Adenoviral Mediated Gene Delivery to Human Umbilical Cord Mesenchymal Stromal Cells for Inner Ear Hair Cell Differentiation

By

Keerthana Devarajan

B.E, Biotechnology, Visvesvaraya Technological University, Belgaum, India, 2008

Submitted to the graduate degree program in Bioengineering and the Graduate Faculty of the University of Kansas School of Engineering in partial fulfillment of the requirements for the degree of Master of Science

Committee members

Dr. Michael Detamore, Committee Chair

Dr. Hinrich Staecker

Dr. Laird Forrest

May 17th, 2011

Date Defended

The Thesis Committee for Keerthana Devarajan certifies
that this is the approved version of the following thesis:

Adenoviral Mediated Gene Delivery to Human Umbilical Cord Mesenchymal Stromal Cells for Inner Ear Hair Cell Differentiation

Committee members

Dr. Michael Detamore, Committee Chair

Dr. Hinrich Staecker

Dr. Laird Forrest

May 17th, 2011

Date Approved

ABSTRACT

Hearing is one of our main sensory systems and having a hearing disorder, although not life threatening, can have a disturbing impact in an individual's quality of life. Approximately 49 million Americans suffer from some form of hearing loss. Sensory neural hearing loss (SNHL) is the most common form, which results from degeneration of inner ear sensory hair cells and auditory neurons in the cochlea. In recent years, there has been an increasing interest in gene delivery to mesenchymal stem cells. Gene delivery approaches to stem cells can provide an opportunity to engineer a variety of specialized cell types. The objective of this thesis was to evaluate the potential of human umbilical cord mesenchymal stromal cells (hUCMSCs) as a possible source for regenerating inner ear hair cells. This thesis was successful in developing an adenoviral mediated gene delivery approach to deliver the *Math1* gene to hUCMSCs. The expression of *Math1* induced the differentiation of hUCMSCs into cells that resembled inner ear hair cells morphologically and immunocytochemically, evidenced by the expression of hair cell-specific and glial cell markers. The results obtained in this thesis demonstrated for the first time that hUCMSCs can differentiate into hair cell-like cells, thus introducing a potential tissue engineering and cell transplantation approach for the treatment of hearing loss.

ACKNOWLEDGMENTS

I would like to acknowledge Drs. Michael Detamore, Hinrich Staecker and Laird Forrest for serving on my thesis committee and providing valuable guidance and insightful suggestions in the preparation of this Master's thesis. I would also like to acknowledge Dr. Yumei Xie, Dr. Shuang Cai and Taryn Babgy for training me on the viral gene delivery technique and for answering my smallest doubts with patience. I thank Dr. Forrest for the extensive training in constructing recombinant vectors and giving me the opportunity to work at his laboratory, Dr. Peter Smith for giving me access to real time PCR equipment, Aritra Bhattacherjee for his constant guidance and help in qRT-PCR technique, from designing primers to analyzing gene expression.

A special thanks to Jennifer Brantley for her valuable guidance in immunocytochemistry and mouse procedures and for making every single day at lab memorable with entertaining conversations. I would like to thank AJ Mellot for being a truly wonderful friend with whom I have had the most enjoyable experiences, be it celebrating success in gene delivery research or hanging out. I also thank Peggy Keefe for being the most supportive and caring laboratory manager, talking to who kept my spirits high while I dealt with homesickness and other issues. I acknowledge my fellow graduate students and post docs for their support in facing challenging research problems. I would like to thank all the undergraduates for assisting with continual lab maintenance. Special thanks to our most talented post doc, Neethu, who always provided insightful suggestions and answers to numerous questions.

I will forever be indebted to my advisor, Dr. Michael Detamore, for being an exceptional mentor, for believing in me, always encouraging and motivating while I dealt with numerous failed experiments and for providing continuous support and guidance on personal and professional matters.

I am extremely grateful to my co-advisor, Dr. Hinrich Staecker for being so kind and supportive, and for all the insightful thoughts and suggestions without which this thesis would not be possible. His energy and enthusiasm be it the lab or the OR is simply awesome and I am glad to have worked with such a great person.

Finally, I want to thank my parents, Devarajan and Manjula, my sister, Sushmitha, my best friends, Punit and Vikas, and my extended family and friends back home for their unconditional love and support in everything that I wanted to do and for believing in me.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF TABLES

CHAPTER 2

CHAPTER 1: Introduction

The overall objective of this thesis was to demonstrate the feasibility of transducing human umbilical cord mesenchymal stromal cells (hUCMSCs) using adenoviral vectors, and to differentiate them *in vitro* into a hair cell-like cell through adenoviral mediated gene delivery of the *Math1* gene. To achieve this objective, two phases were recognized - feasibility phase and differentiation phase. In the feasibility phase, the objective was to transduce hUCMSCs using different adenoviral serotypes (Ad5 and Ad28) and to compare transduction efficiencies between these different serotypes. The next phase was the differentiation phase, which was to induce inner ear hair cell differentiation in hUCMSCs and to characterize the properties of the differentiated cells. The feasibility phase provided information about the ability of hUCMSCs to express genes delivered through an adenoviral vector. In the differentiation phase, the cells were first tested for their ability to respond to the transcription factor *Math1* with vectors driven by the human cytomegalovirus (hCMV) promoter and the glial fibrillary acidic protein (GFAP) promoter. The cells were then differentiated *in vitro* using the vector with an hCMV promoter. Differentiation of hUCMSCs was characterized according to morphology and immunocytochemistry. Gene expression was quantified via quantitative RT-PCR. Additionally, the differentiated cells were tested for their capacity to repair damaged sensory epithelium in murine macular organ cultures *in vitro*. To achieve the overall objective, three specific aims were designed:

1) To investigate the feasibility of transducing hUCMSCs using adenoviral serotypes 5 and 28. This aim was achieved by infecting hUCMSCs with Ad5 or Ad28 carrying the green fluorescent protein (GFP) gene. The cells were transduced with the vector at varying multiplicities of infection (MOI). It was hypothesized that *the highest MOI would give the highest transduction efficiency due to the availability of a higher number of viral particles infecting per cell.* The transduction efficiencies between the two serotypes at varying MOIs were compared by imaging the intensity of fluorescence and the number of cells that fluoresced at days 3, 5 and 10 following transduction.
2) To assess and compare the ability of hUCMSCs to respond to the *Math1* transcription factor using different promoter types. This aim consisted of transducing hUCMSCs with adenoviral vectors driven by the hCMV promoter and the GFAP promoter at the MOI with the highest transduction efficiency in Specific Aim 1. It was hypothesized that *hUCMSCs would show better Math1 expression when transduced with the hCMV promoter driven Ad5 vector than with the GFAP promoter driven Ad5 and Ad28 vectors.*
3) To induce hair cell differentiation in hUCMSCs *in vitro* using the Ad5 vector with the hCMV promoter and the *Math1* gene, and assess the ability of the differentiated cells to repair damaged epithelium in murine macular organ cultures. This aim consisted of two phases, which were performed in parallel. The first phase consisted of transducing hUCMSCs using the vector with the highest *Math1* gene expression from Specific Aim 2. In the second phase, the

differentiated cells were co-cultured with neomycin-treated murine macular organs. It was hypothesized that *when transduced, hUCMSCs would differentiate into cells that morphologically resemble hair cells and express hair cell and glial cell markers. Moreover, it was hypothesized that the differentiated hair cell-like cells would repair damaged sensory epithelium in murine macular organs in vitro.*

The organization of the remaining chapters is as follows:

Chapter 2 serves to provide background information in which the literature pertinent to subsequent chapters is reviewed. Also provided in Chapter 2 is a discussion of hair cell regeneration, inner ear gene therapy, and use of stem cell-based therapies in cochlear repair, highlighting the function of the *Math1* gene and its homologues in hair cell differentiation, and the importance of curing sensory neural hearing loss (SNHL).

After the background information is established in Chapter 2, Chapters 3 – 6 address the experiments performed to satisfy the aforementioned Specific Aims. Chapter 3 serves to address Specific Aim 1, providing a comparison of transduction efficiencies in hUCMSCs between the Ad5 and Ad28 vectors.

Specific Aim 2 is addressed in Chapter 4. In Chapter 4, the ability of hUCMSCs to respond to the *Math1* transcription factor using adenoviral vectors driven by hCMV promoter and GFAP promoter was assessed. The cells were infected at an MOI of 100. *Math1* gene expression was analyzed at day 10 post transduction by immunostaining the cells with myosin VIIa antibody.

Chapter 5 focuses on Specific Aim 3, which involved *in vitro* inner ear hair cell differentiation of hUCMSCs and assessment of the ability of the differentiated hair cell-like cells to repair neomycin-treated murine macular organ cultures. The transduced cells were characterized morphologically and immunostained with hair cell and neuronal markers. Differentiated cells at day 10 were co-cultured with neomycin-treated murine macular organs for 10 days and immunostained with Myosin VIIa to analyze their ability to repair damaged sensory epithelium.

Chapter 6 contains the conclusion. Findings from all experiments are summarized in a global context and future research directions are discussed.

CHAPTER 2: A Review of Gene Delivery and Stem Cell Based Therapies for Regenerating Inner Ear Hair Cells

ABSTRACT

Approximately 49 million Americans suffer from some form of hearing loss and almost 47% of the elderly (75 years or older) have sensory neural hearing loss (SNHL). Cochlear implants and hearing aids are the only treatments available today. Although not life threatening, SNHL isolates an individual from social interactions and affects the quality of life. In contrast to these existing treatments, hair cell restorations via gene delivery and stem cell-based therapies hold a great potential to cure deafness. In this review article, we evaluate some of the advantages and disadvantages of the different viral vectors employed in inner ear gene therapy and the insights gained from the use of embryonic, adult and induced pluripotent stem cells in generating inner ear hair cells.

INTRODUCTION

Hearing loss has become one of the most common disabilities in the United States and can affect almost every age group. The number of people with hearing loss worldwide has been steadily increasing over recent years, reaching almost 49 million people in the US alone (1). According to the National Institute on Deafness and Other Communication Disorders (NIDCD) 2010 statistics, approximately 17% of the American adult population experiences hearing loss and 3 out of every 1000 children are born deaf. The prevalence of hearing loss increases with age, as about 47% of

adults over 75 years old have hearing impairment (NIDCD). Although hearing loss may not be life threatening, it can greatly influence the patient's quality of life, social interactions, and have a significant financial impact on society. (1, 2).

Hearing loss can be conductive, mixed, central or sensorineural. Conductive loss usually occurs due to a block or damage in the outer or middle ear and is most often cured surgically or medically (3). In mixed hearing loss, the problem is both in the outer or middle and the inner ear. Central hearing loss results from damage to the auditory nerves or nuclei of the central nervous system. Sensorineural hearing loss (SNHL) involves damage to the cochlea (inner ear sensory hair cells) or the Eighth nerve. It is irreversible and in most cases a hearing aid is required. SNHL and vestibular dysfunctions account for about 90% of all hearing loss, and they are not curable. Common causes for SNHL are aging, ototoxic drugs, noise induced trauma, inner ear concussion, and immune disorders (4-6). Although only a small percentage of the cases can be treated medically and surgically, advances in molecular and stem cell therapies may provide tools to treat the irreparable damage of hair cells caused by SNHL (3).

The ear is of tremendous importance in sensing the world around us. Aside from being the prime organ for the perception of sound, it also plays a crucial role in balancing the body. The inner ear is a complex structure and has been referred to as a labyrinth (7). Acoustic energy, in the form of sound waves, is channeled into the ear canal where it strikes the tympanic membrane. As the energy hits the stapes located at the oval window, a pressure wave sets the cochlear fluid into motion. This results in

the depolarization and release of a neurotransmitter from the base of the hair cell. Primary auditory afferents then conduct the signal to the brainstem.

Hair cells are sensory receptors located in the inner ear. They appear to be “hair-like” because of the numerous stereocilia that extend from their surfaces. Hair cells are responsible for converting sound into electrical signals that are sent to the brain via the auditory nerve for processing. In the human cochlea, hair cell death can occur due to a variety of causes, such as age related deafness (presbycusis), a high dosage of ototoxic drugs (e.g., gentamycin, cisplatin, aminoglycosides), genetic disorders, infectious diseases, or high levels of noise exposure (7-9). Several molecular pathways and mechanisms are involved in hair cell death. Patients exposed to high doses of aminoglycosides as a treatment regimen for bacterial infections often experience hair cell death. The aminoglycosides are known to activate the intracellular caspase-9 signaling pathway and trigger mitochondria to release cytochrome c into the cytoplasm, which consequently induces apoptosis in hair cells. Noise induced cellular stress activates the JNK signaling pathway and causes neuronal cell death via necrosis. Necrotic hair cell death is less common than apoptotic cell death and is mainly induced by trauma or disease (7, 9, 10).

Over the past 30 years, several attempts have been made in the attempt to understand the mechanics of the hair cells, molecules, genes and pathways involved in hair cell formation and death (11). The process of hair cell regeneration was considered impossible to occur in higher vertebrates until two groups (Dr. Rubel and Dr. Cotanche) serendipitously discovered the amazing phenomenon in birds in the

late 1980s. Ever since, scientists have been conducting experiments in an attempt to understand the mechanics and pathways of avian and mammalian (rat, mouse, guinea pig) hair cell regeneration as they have characteristics similar to the human ear (8, 10, 12-19). An indication of the advancement in the field of hair cell regeneration has been reported in recent years, and we refer readers to numerous outstanding reviews (3, 20-27). This review article will focus on recent therapeutic approaches using stem cell biology and gene therapy to regenerate mammalian inner ear hair cells.

HAIR CELLS, SUPPORTING CELLS AND THEIR FUNCTION

Hair cells

Hair cells are the mechanoreceptors in the inner ear that detect sound, head movements and orientation in space (9, 28). They are present in the sensory epithelium of both the auditory (inner and outer hair cells) and vestibular systems (Type 1 and Type 2 hair cells). All hair cells are surrounded by supporting cells and have physiological and morphological differences. Figure 1 shows the classification of inner ear sensory epithelium, and Table 1 gives the differences between an inner and outer hair cells.

Hair cell structure

Hair cells are flask-shaped cells with extended processes at their apical ends called stereocilia. The stereocilia are made of actin filaments and actin bundling proteins - fimbrin and espin (29). The vestibular system hair cells have one true cilium called the kinocilium that marks the polarity of the cell (Fig. 2). The

kinocilium of hair cells in the auditory system degenerate and hence are not effectively polarized. Hair cells form synaptic connections from neurons at the basal end and terminate in the cochlear or vestibular nuclei of the brainstem. The term "mechanoreceptors" comes from their participation in transforming mechanical energy to electrical energy. In the auditory system, the mechanical energy occurs in the form of a wave, whereas in the vestibular system, the mechanical energy is a result of displacement of hair cells due to the force of gravity or inertia.

Transduction in Hair Cells

The stereocilia are connected together by a filamentous tip link. The tips are connected to cation transduction channels that are involved in calcium and potassium ion exchange. Each hair cell has about 100 transduction channels; the hair cell's mechanical movement and ion exchange control the cell's membrane potential and provide the driving force for auditory or vestibular nerve excitation. Myosin motor proteins are activated by the calcium ions and play an important role in triggering the hair cell's adaptation to mechanical stimuli from stereocilia deflections (11).

Supporting Cells

Supporting cells are the nonsensory cells of the sensory epithelium and do not take part in sound transduction. They are located at the base of the hair cells and surround them, preventing contact between individual cells. The mammalian auditory system has two main types of supporting cells: the Deiter's cells support the outer hair cells at the base, and the pillar cells help in forming the reticular lamina, which

isolates the stereocilia from their cell bodies. A few other supporting cells types include interphalangeal cells, Hesen's cells and Claudius cells (30). Supporting cells of the vestibular system have yet to be studied in detail to understand their physiological and morphological differences (22, 28). Supporting cells are known to play an important role in avian hair cell regeneration by two different mechanisms: mitotic regeneration and transdifferentiation (31). The former occurs when the supporting cell divides mitotically, stimulating one of them to differentiate into a hair cell, whereas the latter occurs when a supporting cell changes its gene expression and becomes a hair cell directly without dividing.

Summary

Hair cells reside in the auditory and vestibular systems and are responsible for mechanoelectrical transduction of sound. Ototoxic drugs or noise induced stress can damage hair cells, thus compromising inner ear function. Supporting cells located at the base of the hair cells have the capability to regenerate hair cells in the vestibular system by mitotic division or transdifferentiation, though supporting cell proliferation may not always occur to replace lost hair cells.

ESSENTIAL GENES IN HAIR CELL DIFFERENTIATION

It is important to identify and characterize genes that govern the ontogeny and differentiation of the cochlear epithelium; identifying such genes can lead to the design of several therapeutic approaches for sensory epithelial cell development and hair cell differentiation. A critical gene responsible for inner ear development is the *Atonal* gene - a protein belonging to the basic helix-loop-helix (bHLH) family of transcription factors that activates the E-box dependent transcription. *Math1*, also known as *Atoh1*, is the mouse homolog of the *Drosophila melanogaster Atonal* gene. The *Math1* gene is essential for the differentiation of sensory hair cells from previously established sensory primordium and is limited to only a subpopulation of the nonsensory supporting cells (32). Studies with embryonic *Math1*-null mice reported a failure to produce hair cells, proving *Math1* as a positive regulator in directing hair cell differentiation (33). Gene delivery studies in guinea pigs, mice, and rats reported an over expression of *Math1* in non-sensory cells, resulting in the production of ectopic immature hair cells outside the sensory epithelium via the transdifferentiation mechanism (14, 32, 34-38). The nonsensory *Math1* expressing cells attracted auditory nerve fibers and developed into mature hair cells (35, 36).

The other homologues of the *Atonal* gene are *Cath1* (chicken atonal homolog), *Xath1* (Xenopus atonal homolog) and *Hath1* (human atonal homolog), although *Math1* is the most extensively studied and used transcription factor (39, 40). Studies with adenoviral expression of *Hath1* in rats showed hair cell production without supporting cell proliferation (41).

Additional genes involved in the control of hair cell fate include *Hes1*, *Hes5*, *BETA2/NeuroD, Jagged1* and Notch Signaling (16, 17). *Hes1* and *Hes5* have been shown to influence cell fate through negative regulation of *Math1* (42, 43). Certain cell cycle kinases also influence inner ear development by regulating cell cycle and inhibiting hair cell differentiation (Refer Table 2.2). *BETA2/NeuroD1* gene has been shown to regulate the formation of sensory and neuronal ganglions in both cochlear and vestibular systems (44). Table 2.2 gives a list of the different genes involved in hair cell differentiation. Figure 2.3 represents a schematic on how different genes interact and contribute to positive and negative regulation of *Math1* transcription factor. These pathways can be induced or inhibited via standard or molecular therapy and additionally can be used to control the differentiation of stem cells.

GENE THERAPY AND STEM CELL-BASED APPROACHES FOR TREATMENT OF SENSORY NEURAL HEARING LOSS

Current therapies for treating hearing loss involve the use of either hearing aids or cochlear implants. Cochlear implants are only available to patients with severe hair cell damage and profound loss of hearing ability. However, the implants are not absolutely efficient in restoring hearing; their performance varies from patient to patient and requires training to adapt to the device. With advances in regenerative medicine using stem cells and gene therapy, several new strategies have emerged with the hope of permanently curing deafness. Some of these strategies are discussed in the following paragraphs.

Gene therapy in the inner ear

As discussed earlier, the *Math1* gene is an essential gene in generating hair cells. Multiple approaches to deliver the *Math1* gene into the inner ear have been evaluated. Most of the approaches involve the injection of viral or non-viral vectors into the inner ear canal to trigger endogenous cells in the organ of corti to differentiate. There are a number of other popular routes of vector administration for inner ear gene therapy that are well explained in the literature (45-48).

An ideal vector is one that would ensure patient safety and effective transformation of undifferentiated cells. The commonly used vectors are derived from adenovirus, adeno-associated virus (AAV), herpes virus and lentivirus. Adenoviral mediated *Math1* and *Hath1* gene delivery has shown promising results *in vivo* for regenerating inner ear hair cells in mammals (14, 34, 35, 37, 49). Adenoviral vectors offer high transfection efficiency and have been extensively investigated in clinical trials for ocular disease and cystic fibrosis (50, 51). The prior experience with adenovectors in the clinic can make them more desirable for commercial and clinical applications. The efficacy of adenoviral vectors can be improved by modifying vector elements, such as using tissue specific promoters or deleting DNA sequences to eliminate production of harmful viral proteins. Adenoviral mediated vestibular hair cell regeneration has been reported to be more efficient with the glial fibrillary acidic protein (GFAP) promoter than the human cytomegalovirus (hCMV) and chicken β actin (cBA) promoters. Additionally, adenovectors with deleted E4 regions resulted in increased tolerability of the cells towards the vector (37, 38, 52). In addition to

delivering the *Math1* gene, adenoviral vectors are used to deliver growth factors such as transforming growth factor-β1 (TGF-β1), glial cell-derived neurotrophic factor (GDNF), and B-cell lymphoma 2 (Bcl-2) to protect hair cells from sound trauma and ototoxic drugs (53, 54). Despite the success of adenoviruses in the effective delivery of *Math1*, short term gene expression and strong immune responses often result, but can be overcome by adeno-associated viruses. Studies show the capacity of adeno-associated viruses to deliver genes to inner ear blood vessels and certain auditory nerve fibers with negligible toxicity when compared to adenoviruses (48, 55). *Herpes simplex* viral vectors (HSV) derived from *Herpes simplex type I* can infect and replicate in non-dividing cells. Studies in cochlear gene therapy using HSV reported dispersed gene expression in the cochlea, limited to auditory and vestibular spiral ganglion neurons (56). HSV mediated gene delivery requires the use of high viral stock volumes due to the difficulty of producing the vectors in high titers. HSV are reported to evoke strong inflammatory responses in guinea pigs (57). Lentivirus is the best available viral vector in terms of transduction efficiency and transgene expression because of its ability to infect both proliferating and non-proliferating cells, including stem cells that are difficult to transduce. Research in lentiviral-mediated gene delivery in the guinea pig cochlea showed gene expression limited to the perilymphatic space in the cochlea. High gene expression was observed in ganglion neurons, glial cells, and supporting cells; however, the vector failed to infect sensory cells. Although lentiviral vectors offer long term gene expression in a variety

of cell types, they are known to have a high risk of evoking a strong immune response and generating a replication-competent virus (58).

Currently, no ideal vector exists for use in inner ear gene delivery. However, adenovector based gene therapy is currently the most widely used form of gene therapy in the inner ear. Newer recombinant forms of adenoviruses carrying the *Math1* gene have shown promising results in generating hair cells in both auditory and vestibular systems. Among all known viral vectors, adenovirus is widely researched in delivering specific genes (*Math1*, GDNF, and Bcl-2) for regenerating and protecting hair cells. Table 2 lists the advantages and disadvantages of using different viral vectors in inner ear gene therapy.

The development and use of different viral vectors in inner ear gene therapy allows us to evaluate the effects of introducing specific genes and therapeutic molecules that can regenerate hair cells and prevent hair cell damage.

Stem cell-based therapy for inner ear hair cell regeneration

In the last decade, there has been a considerable amount of attention directed toward stem cell-based therapies for treating diseases like Alzheimer's, Parkinson's and cardiovascular diseases. The success of stem cells in treating these diseases opened opportunities for researchers to explore the use of stem cells in treating hearing disabilities. Stem cell therapy is based on the concept that, upon transplantation, the undifferentiated stem cell has the capacity to respond and react to surrounding cell signals and differentiate into the appropriate cell type associated

with the signal. Stem cells are a useful way of exploring the molecular pathways that underlie hair cell genesis. Some of the stem cell-based therapeutic strategies that have employed stem cells in the effort to cure hearing loss are listed below.

Embryonic Stem Cells

ESCs are pluripotent and capable of giving rise to cells from any of the three germ layers (59). With respect to hair cell regeneration, it was reported that murine embryonic stem cells can generate inner ear progenitors *in vitro* (60). These ESCs were allowed to form embryoid bodies and were cultured in the presence of epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and basic fibroblast growth factor (bFGF). The newly generated progenitor cells were reported to express markers characteristic of hair cell differentiation and hair cell specific markers. Additionally, the progenitor sensory cells had the capacity to integrate into sensory epithelial layers when injected into the developing inner ear of a chicken and to express hair bundle markers *in vivo*. Another study demonstrated the use of murine ESCs from transgenic *Math1*/nGFP mice (61). The ESCs were differentiated using a step-by-step method toward the ectodermal lineage using otic-inducing growth factors. The generated otic progenitor cells had the capacity to develop into mechanosensitive sensory hair cells *in vitro* and demonstrated immature hair cell transduction currents (61). ESCs have been reported to produce sensory auditory neurons and neural progenitors with the potential to restore auditory function by generating nerve connections to hair cells (62-64). Embryoid bodies from murine ESCs co-cultured with hair cell explants showed neuron-like cells and positive

staining for neurofilament(63). Most of the embryonic stem cell studies have used murine stem cells; however, there have been attempts to differentiate human embryonic stem cells (hESCs) in the presence of growth factors like neurotrophin-3 (NT-3), bFGF, BDNF, EGF and bone morphogenic protein 4 (BMP4). The differentiated cells expressed inner ear and synaptic markers (65-67). hESCs have also been cultured to generate otic progenitors that were capable of differentiating into auditory sensory neurons (66).

Although these studies have demonstrated successful *in vitro* and *in vivo* generation of replacement hair cells and auditory neurons from ESCs, further investigations are crucial in developing treatment strategies for hearing loss because of the controversial and ethical issues linked to ESCs.

Adult Stem Cells

Promising results have been shown with bone marrow mesenchymal stem cells (BMSCs) in the field of hair cell regeneration. Mesenchymal stem cells derived from rat bone marrow have been reported to differentiate into inner ear progenitors and express sensory cell markers including myosin VIIa, espin, Brn3c, p27kip and *jagged2 in vitro* (68). Additionally, the differentiated cells displayed morphological characteristics of hair cell stereociliary bundles. Studies have shown that BMSCs stimulated in the presence of growth factors were able to form neuronal progenitors, and after being transfected with the *Math1* gene, were able to differentiate into inner ear sensory-like cells (68). Adult stem cells also have the potential to deliver gene and therapeutic molecules to other parts of the inner ear. For example, Connexin 26, a

protein present in cochlear gap junctions and supporting cells was expressed when bone marrow stromal cells were transplanted into the perilymphatic space of the mouse cochlea. (69). Bone marrow mesenchymal stem cells also have the potential to differentiate into auditory neurons *in vitro* and *in vivo* (70, 71), demonstrating that a wide variety of inner ear cell types can be generated from stem cells.

Adult stem cells isolated from mouse macular organs have been shown to differentiate into hair cells when cultured with EFG and IGF-1 (72). Adult stem cells isolated from olfactory neuroepithelium expressed hair cell markers and resembled hair cells phenotypically when co-cultured with cochlear cell supernatant (73).

Transplantation studies in mice with bone marrow-derived hematopoietic stem cells (BMHSCs) suggested the possibility of differentiation of BMHSCs into mesenchymal cells and fibrocytes in the adult inner ear (74). The results with BMHSCs show their potential to attenuate cochlear injury by replacing mesenchymal cells and fibrocytes in the inner ear (74).

Adult stem cells from many tissues are now being used to investigate cures for various diseases. They are less controversial when compared to embryonic stem cells. These cells can enter clinical trials involving autologous transplantation therapies and be used in bioengineered products. In treating inner ear disorders, bone marrow-derived stem cells have shown the most favorable results (69, 70, 74, 75).

Induced Pluripotent Stem Cells

The discovery of generating induced pluripotent stem cells (iPSCs) gave a new dimension to stem cell research. iPSCs are produced from adult stem cells, which are reprogrammed to express certain genes and maintain characteristic properties of embryonic stem cells. iPSCs opened up the possibility of generating and using patient-specific stem cells without immune rejection *in vivo* and, unlike hESCs, there are no controversial issues associated with their use. The most important factors in maintaining the pluripotency of ESC lines are *Oct4, c-Myc, Klf4* and *Sox2*. Any adult stem cells forced to express the above four genes under ESC culture conditions can be reprogrammed into ESC-like cells (76). Recently, it was demonstrated that human neural stem cells can be directly reprogrammed to iPSCs by just expressing *Oct4* (77). Like ESCs, most studies with iPSCs in hair cell regeneration are also of murine origin. A recent study showed generation of iPSCs from murine embryonic fibroblasts (61). These fibroblasts were transduced with retroviruses to express *Oct4*, *c-Myc*, *Klf4* and *Sox2*. The generated iPSCs were cultured in a medium containing otic-inducing FGF-3 and FGF-10 to produce otic progenitor cells. The generated otic progenitors differentiated into hair cell-like cells expressing hair cell markers. When co-cultured with fibroblast-like cells from embryonic chicken utricles, the differentiated cells developed hair bundle-like protrusions, responded to mechanical stimulation, and displayed transduction currents (61). Another study explored the use of iPSCs for restoring auditory ganglion neurons. *In vitro* neuronal differentiation of iPSCs was induced by exposing them to stromal cell-derived inducing activity

(SDIA). SDIA is a neural inducing activity demonstrated in stromal cells when they simultaneously produce inducing and inhibitory factors and was developed by Kawasaki *et al.* (78). The differentiated cells were transplanted into the cochleas of mice. iPS cell-derived neurons projecting toward cochlear hair cells were observed 1 week after transplantation (79). Although iPS cell techniques are novel, researchers have started to explore their potential in treating SNHL.

Stem cell therapy holds great promise for curing SNHL; however, there has been little progress to that end. Some of the barriers that exist with the use of stem cells are the formation of tumors and graft-versus-host diseases. Owing to the spiral structure of the cochlea, it is difficult to direct the stem cells into the desired location inside the cochlea. Additionally, the inner ear fluids are rich in potassium ions, providing a challenging environment for the stem cells to differentiate (24). Further investigation with the use of different stem cell types and advancements in the existing approaches are necessary before they can enter the clinical and begin treating hearing disorders.

Summary of Stem Cell-Based Therapies

ESCs have high survival rates and migration capacity when implanted into the cochlea (80, 81). They migrated onto auditory neurons and exhibited neuronal differentiation. However, ESCs exhibited low integration into endogenous tissue and failed to differentiate completely at the implantation site (82, 83). There is also the risk of tumor formation and the risk of transmitting infections with ESCs because they use animal products during the culturing process. ESCs used in inner ear

treatment require the use of immunosuppressive therapy or cloning to avoid graft-vs-host diseases. On the other hand, adult stem cells isolated from bone marrow can easily bypass the immune barriers and overcome the problem of immune rejection. BMSCs also have a high survival rate and can migrate into multiple regions in the cochlea and brain (70, 84-86). Although they can be stimulated to differentiate into a number of cell lineages, they differentiate more toward the mesoderm lineage and can be used to replace degenerated cochlear fibrocytes (87). iPSCs do not have any ethical concerns with their use and are more patient specific, thus eliminating the risk of immune rejection. Their undifferentiated state allows them to migrate to regions surrounding the cochlea. However, one of the major concerns with iPSCs is the time required to produce the individual cell lines (88). They also pose the risk of passing on the DNA from the genetically altered cell to future generations.

Some of the barriers that exist with the use of stem cells are the formation of tumors and graft-versus-host diseases. Another concern is the integration of stem cells in the inner ear. Owing to the spiral structure of the cochlea, it is challenging to direct the stem cells into the desired location and evaluate the integration of differentiated stem cells inside the cochlea. Further investigation with the use of different stem cell types and advancements in the existing approaches are necessary before they can enter the clinic and begin treating hearing disorders.

Nevertheless, these concerns bring opportunities for bioengineers and clinicians to engineer cells and vectors carrying a combination of genes and therapeutics and develop methods and devices to deliver therapeutics at appropriate

locations. Recent advances in stem cell technology and gene based therapies provide the framework required for the development of potential treatment options.

DISCUSSION

The success of current approaches unveils the possibility of using different viral vectors, genes, growth factors and stem cell types in regenerating inner ear hair cells. Nevertheless, more research is still required to overcome challenges involved before these approaches will be ready for clinical and commercial use. Although several studies have reported the generation of hair cell-like cells *in vitro* and *in vivo* (in animals), the outcome and characteristic properties of these cells must be further explored. A cell that simply resembles a hair cell morphologically and expresses hair cell markers would be immature without being able to respond to mechanical stimuli and transfer signals to the auditory neurons. A detailed investigation of their ultra structure and ability to connect to auditory neurons and produce transduction currents is essential. In terms of functional outcomes, crucial to any tissue engineering strategy, the mechanoelectrical transduction and maturity of transplanted hair cell-like cells can be evaluated by auditory brainstem response (ABR) and otoacoustic emissions tests (OAE) in animal studies.

Audiologists and scientists have come a long way toward reaching the goal for hair cell regeneration in mammals by developing inner ear gene therapy strategies in animal models. However, an improvement in the existing gene delivery techniques is required to suit clinical applications.

Some of the drawbacks associated with inner ear gene therapy are addressed below. For example, gene delivery studies have shown that over-expression of the *Math1* gene led to the formation of ectopic hair cells (14, 36). Hair cells are extremely rare and the cochlea contains only about 14,000 hair cells that detect and amplify sound (67), therefore an overabundance of hair cells in the cochlea can also lead to deafness. There are several pathways that control the expression of *Math1* and drive a cell toward differentiating into a hair cell. *Math1* inhibitors and down regulators can be used in addition to targeting the inner ear with only the *Math1* gene. This approach can be used to produce an appropriate number of hair cells. The activation of a notch receptor can up regulate *Hes* and *Hey* genes that are potent inhibitors of *Math1* (16, 17, 43). Inclusion of notch receptor inhibitors in gene therapy can help in boosting *Math1* gene expression. However, a balance of expression and inhibition between the *Math1* gene and *Math1* inhibitor is required for normal inner ear development and hearing.

Besides developing strategies to control the expression of the *Math1* gene, the side effects caused by viral routes also need to be considered. Although viral methods have shown tremendous success in the animal model, there is always the risk of DNA mutations and cancer associated with viruses. Non-viral gene delivery techniques have recently gained attention due to the minimal risks associated in terms of clinical safety and reliability. Another drawback of *Math1* gene therapy is that although it can be a potential cure for hearing loss caused by sound trauma or ototoxic damage, it may not cure hearing loss caused by genetic defects. Stem cell-based therapies *in vivo*

have reported migration of these cells into different areas, and thus an ideal route for delivering cells to the right location in the cochlea is crucial to restoring hearing loss. The large majority of studies have used only animal models and animal stem cells. There is no doubt that these studies have played a major role in gathering valuable information about hair cell regeneration; however, the variation among species requires the need to explore the use of human stem cell lines like bone marrow derived mesenchymal and hematopoietic stem cells, umbilical cord blood and umbilical mesenchymal stromal stem cells, and induced pluripotent stem cells. Research in human stem cells lines will have more clinical relevance.

Gene delivery and stem cells are a potential cure to hearing loss however, there are limitations that must be overcome. Gene therapies must consider using combinations of essential genes and cell cycle inhibitors to control the production of hair cells, and detailed quantification of hair cell functionality is necessary. Gene delivery vehicles with minimal risk of mutagenesis and immune response must also be developed. Finally, an ideal route to implant stem cells inside the cochlea would be essential for successful innervations of hair cells, thereby allowing better, if not pristine transmission of sound with the assistance of a cochlear implant.

CHAPTER 3: Comparison of Transduction Efficiency in Human Umbilical Cord Mesenchymal Stromal Cells between Adenovirus Serotypes 5 and 28

ABSTRACT

Adenoviruses are among the first known vectors that are capable of infecting both proliferating and non-proliferating cells. They are one of the most effective expression systems and occur in several different serotypes. However, the entry of an adenovirus largely depends on the binding of virus fiber protein and the host cell surface receptor. Ad5 vectors rely on coxsackie adenovirus receptor (CAR) and other integrin related receptors to integrate into the cell's genome. Human umbilical cord mesenchymal stromal cells (hUCMSCs) are derived from Wharton's jelly between the blood vessels of the umbilical cord. Since hUCMSCs do not express CAR, protamine sulfate (a polycationic peptide) was used to increase viral adsorption. In this study, we investigated the feasibility of transducing hUCMSCs using Ad5 (Adf11D) and Ad28 (Ad28t.eGFP) green fluorescent protein (GFP) vectors using protamine sulfate as a cross-linking agent and compared the transduction efficiencies between the two serotypes. The results suggest that both Ad5 and Ad 28 can effectively transduce hUCMSCs, however Ad28 achieved higher gene expression and transduction efficiency.

INTRODUCTION

Gene delivery is emerging as a promising approach in the medical and pharmaceutical field. Gene therapy is defined as the transfer of new genetic material or therapeutic molecules into a cell to alter its function at the cellular or molecular level for therapeutic applications (89). The future of gene therapy largely depends on designing vectors that are capable of effectively delivering therapeutic molecules and genes to target cells with minimal cytotoxic effects (90). Recombinant adenoviral vectors were first used to deliver genes in the treatment of cystic fibrosis (91). They are now used in several *in vitro* and *in vivo* experiments due to their remarkable potential of infecting both dormant and dividing cells (92, 93).

The human adenoviruses are among the largest double stranded and most complex non-enveloped viruses. These viruses have 11 different structural proteins and occur in more than 50 serotypes (91, 94). Owing to their complex structure, their cell entry pathway involves several complex steps. The initial step is the binding between the fiber knob domain and the host cell receptor (CAR or CD46). This is followed by the secondary internalization between the RGD penton base and the αυ integrins. The secondary interaction enables entry via clathrin mediated endocytosis. Once the virion enters the cells, it begins to dissemble and is transported to the nuclear pore complex by microtubule trafficking. At the nuclear pore, the viral DNA enters the host cell nucleus (90, 94). From these steps, it is evident that the binding of the virus to the host cell receptor is the most crucial step in gene transfer and adenoviral mediated gene transfer is limited in most cell types due to the absence of

adenoviral receptors. Many cell types, including hematopoietic stem cells and bone marrow mesenchymal stem cells, lack the adenoviral receptors and are not easily infected by Ad5 vectors (95, 96). Native Ad5 based vectors are wild type vectors and have successfully transduced cells expressing CAR and other cell surface integrin receptors (90, 97, 98). A strategy to effectively infect cells without CAR is to use fiber modified vectors (99-102) or to transduce cells with the virus at a high multiplicity of infection (MOI). However, high MOI levels produce high inflammatory responses in host cells, which is undesirable in clinical use.

hUCMSCs are isolated from Wharton's jelly of umbilical cords and have some properties in common with bone marrow mesenchymal stem cells (BMSCs) (103). Umbilical cords represent an abundant and inexpensive cell source. hUCMSCs can differentiate into a number of cell types and offer significant potential in gene delivery techniques (104-107). Although some studies have investigated the transduction of BMSCs (108, 109), there have been few reports on the transduction of hUCMSCs. Qian *et al.* (107) reported lentiviral mediated gene delivery in hUCMSCs and Rachakatla *et al.* (106) reported adenoviral transduction of hUCMSCs using a recombinant fiber-modified adenovector.

In this study, hUCMSCs were transduced using two different adenoviral serotype vectors - Ad5 and Ad28 - at varying multiplicities of infections (MOI) in the presence of protamine sulfate as a cross linking agent. Protamine sulfate is a polycation that increases transduction efficiency by enhancing viral uptake (110, 111). We compared the transduction efficiencies in hUCMSCs by fluorescence

imaging of green fluorescent protein (GFP) at days 3, 5 and 10. We determined which vector and which MOI transduced the maximum number of cells with a high intensity of fluorescence and maximum gene expression.

MATERIALS AND METHODS

Isolation and expansion of hUCMSCs

hUCMSCs were isolated from human umbilical cords according to protocols approved by the University of Kansas Human Subjects Committee (KU-Lawrence IRB approval # 15402, KU Medical Center IRB approval # 10951). The cords were obtained from the hospital and harvested within 24 hours after delivery. The cords were rinsed in phosphate buffered saline (PBS) and cut into small pieces of 2 to 3 cm. The vascular tissue was then removed, and the cords were minced and incubated in 0.2% type II collagenase (298 U/mg; Worthington Biochemical; Lakewood, NJ) in low glucose Dulbecco's modified Eagle's medium (DMEM) for 4 hours at 37°C on a shaker. The digested homogenous solution was diluted in sterile PBS at a 1:4 ratio and centrifuged for 5 minutes at 1100 rpm. The supernatant was discarded and the cells frozen for future use. Frozen hUCMSCs were thawed, plated at a density of 7,000 cells/cm^2, and cultured in low glucose DMEM with 10% fetal bovine serum (FBS-MSC quantified) and 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Cells were fed every 2 days and maintained in a cell culture incubator at 37°C. At 80 - 90% confluency, the cells were trypsinized and passaged**.** hUCMSCs were isolated from 4 different human umbilical cords (n = 4)

and expanded to passage 4 (P4) for the experiments. All experiments were performed in quadruplicate for each cord.

Adenoviral vectors and vector production

Ad5 and Ad28 serotypes were used in this study. The Ad5 (Adf11D) vector backbone had E1, E3, and E4 regions deleted. The production system for the vector provided robust replication of the adenovector and purified stocks at 5×10^{11} and 2×10^{12} total particle units/ml (pu/ml), with a total particle to particle ratio ranging from 3 to 10 pu/fluorescent focus units. The Ad28 (Ad28t.eGFP) vector backbone had E1 region deleted with the transgene expression cassette inserted at the E1 region. They were produced using 293-ORF cells. The production system for the vector provided robust replication of the adenovector and purified stock at 3×10^{12} fluorescent focus units/ml. The total particle units were determined by a spectrophotometric assay that has been standardized and qualified to reliably and robustly quantify the total particles within a single lot of adenovector. The adenovector lots were purified, aliquoted and stored at -80°C. Individual aliquots were used for each experiment to prevent loss of activity associated with freeze-thaw cycles. Both vectors had their transgene (GFP) expression driven by the hCMV promoter, and the expression cassette contained an open reading frame and an SV40 polyadenylation site and transcriptional stop site at the 3' end of the open reading frame (GenVec Inc., Gaithersburg, Md., USA) (54, 112).

Adenoviral transduction of hUCMSCs

hUCMSCs were trypsinized at P4 and the cells were plated in 48-wells at 1,000 cells/well (n=4) and 500 µl hUCMSC medium (low glucose DMEM, 10% FBS, 1% penicillin/streptomycin) was added per well. Cells were allowed to attach for 24h before the virus was added directly to the medium at 10, 50, and 100 multiplicities of infection (MOI) in the presence of 8 µg/ml of protamine sulfate (MP Biomedicals, Irvine, CA) (107, 113). Controls had all the above components but the vector. The plate was gently rocked after adding the vector and incubated at 37°C. The medium was changed after 24h and every 48h for the remaining culture period. The cells were cultured for 10 days and transient gene expression was imaged using a fluorescent microscope at days 3, 5 and 10.

Fluorescent imaging of transduced cells

Transient gene expression at days 3 and 5 was imaged. At day 10, the cells were fixed for 10 minutes in 4% paraformaldehyde in PBS, followed by washing the cells three times in PBS. The cells were then stained using ProLong Gold antifade with DAPI (4', 6-diamidino-2-phenylindole) (Invitrogen Life Technologies, Carlsbad, CA) and imaged. DAPI strongly binds to A-T rich regions in the DNA and stains the cell nuclei. Microscopic images of eGFP-positive cells were acquired using an inverted fluorescent microscope (Nikon Eclipse E2000-U, Melville, NY), QImaging Retiga 2000R camera and QCapture software.

RESULTS

Effects of transducing hUCMSCs at different MOIs with Ad5 and Ad28 serotypes

By visual examination, it was observed that the intensity of fluorescence and the number of cells transduced with both vectors gradually increased from MOI 10 – 100. No fluorescence was observed in the controls. To investigate the transduction efficiencies and the transient GFP gene expression between Ad5 and Ad28 in hUCMSCs, the transgene gene expression was imaged at days 3, 5 and 10 (Figures 3.1 – 3.2). With both vectors, the maximum number of GFP expressing cells was observed at MOI 100. The cells had maximum fluorescence intensity and transduction efficiency at day 5, with gene expression fading off by day 10. However, when the gene expression between both vectors was compared, Ad28 had a higher number of GFP expressing cells and higher fluorescence intensity at all MOIs on all days when compared to Ad5. The results suggest that the Ad28 vector gave better transduction efficiency when compared to the Ad5 vector.

DISCUSSION

Adenovectors are the best known vectors for transducing both dividing and non dividing cell types. It has great potential in clinical applications. One of the major drawbacks of an adenoviral vector reported in several studies is its poor ability to infect cells lacking adenoviral receptors. Adenoviruses depend on cell surfaces for internalization and endocytosis (97, 114). hUCMSCs also lack the coxsackie adenoviral receptor (106). There are several approaches to improve adenoviral transduction efficiency in human stem cells and overcome gene transfer issues associated with CAR dependent entry of the adenovirus. Use of lipofectamine has been reported to improve the transduction efficiency of human hematopoietic stem cells (115). Lactoferrin can be used as a bridge to enhance binding between the host cell and the virus to increase transduction efficiency (116). Increasing the viral concentration can increase efficiency, however, this can be toxic to the cells and cause an inflammatory response (117, 118). Viral adsorption and uptake can be enhanced using polycations like polybrene, protamine sulfate, and polylysine (119). The positively charged polycationic molecules mask the negative charges on the host cell surface and increase the availability of cell surface receptors. The viral vectors can be complexed with the polycations and enter the cells via endocytosis (110, 120).

In this study, we characterized the feasibility of transducing hUCMSCs using Ad5 and Ad28 vectors and compared the transduction efficiencies between the two serotypes. Overall, Ad28 was observed to transduce hUCMSCs better than Ad5 in terms of both the intensity of fluorescence and the number of cells transduced. Both

vectors showed transgene expression at all MOIs, with MOI 100 giving the best gene expression. The cells were imaged at days 3, 5 and 10 to evaluate the transient gene expression in both vectors. Gene expression reached a maximum level on day 5 and gradually declined by day 10.

The use of protamine sulfate as a cross linking agent may have enhanced the uptake of adenoviral particles and contributed to improving the transduction efficiency in both the Ad5 and Ad28 vectors (111, 121). Lentiviral transduction of hUCMSCs has used polybrene as a cross linking agent to enhance efficiency (107). However, preliminary studies of adenoviral transduction in hUCMSCs with polybrene indicated cytotoxicity and cell death. Protamine sulfate is another polycation reported to be an excellent alternative to polybrene (113). Studies with protamine sulfate have demonstrated essentially the same infection efficiency as polybrene with low cytotoxicity on a range of cell types (113) and therefore was used as an alternative to polybrene.

CONCLUSIONS

In this study, the feasibility of transducing hUCMSCs using an adenoviral vector in the presence of protamine sulfate was demonstrated, and a multiplicity of infection of 100 was found to produce the best level of gene delivery in both Ad5 and Ad28 vectors, with Ad28 significantly outperforming Ad5.

CHAPTER 4: Ability of Human Umbilical Cord Mesenchymal Stromal Cells to Respond to *Math1* Transcription Factor via Adenoviral Mediated Gene Transfer Using Different Promoter Types

ABSTRACT

Gene delivery to human umbilical cord mesenchymal stromal cells (hUCMSCs) has vast potential in gene therapy-based treatment strategies, and adenoviruses are currently used in several clinical trials. Adenoviruses have been widely used in targeting several inner ear cell types to deliver the *Math1* gene. *Math1* is a critical gene responsible for the differentiation of sensory hair cells. In this study, we investigated the ability of hUCMSCs to respond to the *Math1* transcription factor driven by the hCMV and GFAP promoters. We demonstrated a relatively higher number of myosin VIIa positive and hair cell-like cells in hUCMSCs infected with a vector driven by the hCMV promoter. The potential of hUCMSCs to express *Math1* suggests the possibility of inducing hair cell differentiation *in vitro*.

INTRODUCTION

Human umbilical cord mesenchymal stromal cells (hUCMSCs) are believed to be multipotent stem cells, and have shown promising results in gene delivery and tissue engineering applications (106, 122). These cells can be an excellent source for cell transplantation therapies and regenerative medicine because of their low immune rejection and non tumorigenic properties (123). However, to make any stem cell useful in clinical applications, it must be differentiated into a specific cell type. Differentiation can be achieved by delivering growth factors in several ways. One

approach is to use a viral vector to deliver a specific gene of interest. In this study, we tested the ability of hUCMSCs to respond to the *Math1* gene. *Math1* is a protein belonging to the basic helix-loop helix family of transcription factors. It is expressed in inner ear hair cells and neural cells in the hindbrain, spinal cord and germinal layer of the cerebellum (33). Studies have reported that *Math1*-null mice failed to produce inner ear hair cells and overexpression of *Math1* led to the production of numerous ectopic hair cells in mice (33, 36). The *Math1* gene therefore plays a key role in the formation of inner ear hair cells.

In the past, several viral vectors have been used to deliver the *Math1* gene in the inner ear to regenerate hair cells, however, the most promising results were obtained with adenoviral vectors at high transduction efficiencies and with low cytotoxic effects (35, 49, 54). Studies have shown transgene expression for up to 3 weeks in the inner ear with adenoviral vectors (49). Another essential property of vectors that plays an important role in transgene expression is the promoter used in constructing these vectors (52). In this study, we assessed the ability of hUCMSCs to express *Math1* driven by two different promoter types – human cytomegalovirus (hCMV) and glial fibrillary acidic protein (GFAP) promoters. Our further experiments to induce hair cell differentiation in hUCMSCs via adenoviral mediated gene delivery used results obtained from this study and transduced cells using AdMath1.11D.

MATERIALS AND METHODS

Isolation and expansion of hUCMSCs

hUCMSCs were isolated from human umbilical cords according to protocols approved by the University of Kansas Human Subjects Committee (KU-Lawrence IRB approval # 15402, KU Medical Center IRB approval # 10951). The cords were obtained from the hospital and harvested within 24 hours after delivery. The cords were rinsed in phosphate buffered saline (PBS) and cut into small pieces of 2 to 3 cm. The vascular tissue was then removed, and the cords were minced and incubated in 0.2% type II collagenase (298 U/mg; Worthington Biochemical; Lakewood, NJ) in low glucose Dulbecco's modified Eagle's medium (DMEM) for 4 hours at 37°C on a shaker. The digested homogenous solution was diluted in sterile PBS at a 1:4 ratio and centrifuged for 5 minutes at 1100 rpm. The supernatant was discarded and the cells frozen for future use. Frozen hUCMSCs were thawed, plated at a density of 7,000 cells/cm^2, and cultured in low glucose DMEM with 10% fetal bovine serum (FBS-MSC quantified) and 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Cells were fed every 2 days and maintained in a cell culture incubator at 37°C. At 80 - 90% confluency, the cells were trypsinized and passaged**.** hUCMSCs were isolated from 4 different human umbilical cords (n = 4) and expanded to passage 4 (P4) for the experiments. All experiments were performed in quadruplicate for each cord.

Adenoviral vectors and vector production

The Ad5 vectors (AdMath1.11D and Ad5.GFAP.Math1) had E1, E3 and E4 regions deleted with an expression cassette of *Math1* driven by the human cytomegalovirus (hCMV) and human glial fibrillary acidic protein (GFAP) promoters respectively. The production system for the vector provided robust replication of the adenovector and purified stocks at 5×10^{11} and 2×10^{12} total particle units/ml (pu/ml), with a total particle to particle ratio ranging from 3 to 10 pu/fluorescent focus units. The Ad28 (Ad28.GFAP.Atoh1) vector backbone had E1 region deleted with an expression cassette of *Atoh1* driven by the GFAP promoter. The Ad28.GFAP.Atoh1 vector particles were produced using 293-ORF cells. The production system for the vector provided robust replication of the adenovector and purified stocks at 5×10^{11} and 2×10^{12} total pu/ml, with a total particle to particle ratio ranging from 3 to 10 pu/fluorescent focus units. The total particle units were determined by a spectrophotometric assay that has been standardized and qualified to reliably and robustly quantify the total particles within a single lot of adenovector. The adenovector lots were purified, aliquoted and stored at -80°C. Individual aliquots were used for each experiment to prevent loss of activity associated with freeze-thaw cycles. The expression cassette contained an open reading frame and an SV40 polyadenylation site and transcriptional stop site at the 3' end of the open reading frame (GenVec Inc., Gaithersburg, Md., USA) as previously described (38, 112).

Adenoviral transduction of hUCMSCs

hUCMSCs were trypsinized at P4 and the cells were plated in 8 well Millicell EZ Slide (Millipore, Billerica, MA) at 1,000 cells/well (n=4) and 500 µl hUCMSCs medium (low glucose DMEM, 10% FBS, 1% penicillin/streptomycin) was added per well. Cells were allowed to attach for 24 hours before the virus was added directly to the medium at a multiplicity of infection (MOI) of 100 in the presence of 8 µg/ml of protamine sulfate (MP Biomedicals, Irvine, CA) (107, 113). Controls did not have any vector added. The chamber slide was gently rocked after adding the vector and incubated at 37°C. The medium was changed after 24h and every 48h for the remaining culture period. The cells were cultured for 10 days and immunostained for myosin VIIa.

Immunocytochemistry

Cells were fixed for 10 minutes with 4% paraformaldehyde in PBS. This was followed by washing three times in PBS. Immunostaining was initiated by blocking the slides for 1 hour with 5% FBS, followed by four 0.2% Tween washes. The cells were then permeabilized with 0.2% Triton X-100 for 10 minutes. Myosin VIIa positive expression was characterized using polyclonal antibody to myosin VIIa. Fixed and permeabilized cells were incubated at 4°C overnight with the primary antibody (1:100). The primary antibody used was a rabbit polyclonal antibody to Myosin VIIa (Proteus Biosciences, Ramona, CA). Slides were then washed four times with 0.2% Tween and incubated with secondary antibody (1:400) for 30 minutes at

room temperature. The secondary antibody with Alexa visualizing fluorescent signals used was Alexa-555-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). All incubations were in a humidified chamber. Incubation was followed by four washes with 0.2% Tween. The slides were mounted in ProLong Gold antifade with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen Life Technologies, Carlsbad, CA) and cover slipped. DAPI strongly binds to A-T rich regions in the DNA and stains the cell nuclei blue. Microscopic images of myosin VIIa-positive cells counterstained with DAPI were acquired using an upright fluorescent microscope (Nikon Eclipse E800 and Q Image Aqua camera) and QCapture software. Images were photomerged and analyzed with Adobe Photoshop CS5 extended (Adobe Systems, San Jose, CA).

RESULTS

Myosin positive cells from human umbilical cord mesenchymal stromal cells

To examine the potential of hUCMSCs to respond to *Math1*, hUCMSCs were transduced with the AdMath1.11D vector driven by the hCMV promoter and the Ad5GFAP.*Math1* and the Ad28.GFAP.Atoh1 vectors driven by the GFAP promoter at a multiplicity of infection of 100.

We detected the expression of the myosin VIIa marker in hUCMSCs transduced with all three vectors. Only a small percentage of cells expressed myosin VIIa. However, the AdMath1.1D vector driven by the hCMV promoter had a relatively higher number of myosin VIIa positive cells when compared to Ad5.GFAP.Math1 and Ad28.GFAP.Atoh1 vectors that were driven by the GFAP

promoter. The positive cells in Ad*Math1*1D transduced cells also demonstrated a degree of anatomical resemblance to hair cells (Figures 4.1 – 4.3).

DISCUSSION

This study tested the potential of hUCMSCs to respond to the *Math1* transcription factor driven by two different promoter types – hCMV and GFAP. In preliminary studies, we demonstrated the expression of the *Math1* gene by immunostaining with a polyclonal antibody to *Atoh1* (Aviva Systems Biology, San Diego, CA). Here, we showed that hUCMSCs are capable of expressing the *Math1* gene by immunostaining with rabbit polyclonal to myosin VIIa antibody. Since the *Math1* gene is essential for hair cell formation, hUCMSCs expressing *Math1* should stain positive for myosin VIIa (33). In this study, AdMath1.11D demonstrated a higher number of myosin VIIa positive cells when compared to the Ad5.GFAP.Math1 and Ad28.GFAP.Atoh1 vectors. Controls also expressed a certain level of myosin VIIa positive cells, however, the expression was minimal when compared to the vector treated cells and there were no cells morphologically resembling hair cells. Previous studies using adenoviruses or adeno-associated viruses for inner ear gene delivery in the cochlea using the CMV and GFAP promoters demonstrated that the expression driven by the GFAP promoter was limited only to vestibular supporting cells, demonstrating the specificity of this promoter type (52, 124). These previous findings explain the low gene expression in hUCMSCs with the transgene cassette driven by the GFAP promoter.

CONCLUSIONS

Previously, the *Math1* gene has been shown to express in rat and mouse bone marrow mesenchymal stem cells when transfected using Lipofectamine (68, 125). However, there have been no reports on viral gene delivery of the *Math1* gene in mesenchymal stem cells. In this study, the ability of hUCMSCs to respond to the *Math1* transcription factor using an adenoviral vector was demonstrated by the presence of myosin VIIa positive cells. We demonstrated for the first time that mesenchymal stem cells can respond to the *Math1* transcription factor delivered using a viral vector. The AdMath1.11D vector was shown to produce a relatively higher number of myosin VIIa positive cells when compared to the Ad5.GFAP.Math1 and Ad28.GFAP.Atoh1 vectors.

CHAPTER 5: Adenoviral Mediated Gene Delivery to Human Umbilical Cord Mesenchymal Stromal Cells for Inner Ear Hair Cell Differentiation

ABSTRACT

Stem cells in the inner ear lack the capacity to spontaneously divide and replace lost or damaged hair cells. Human umbilical cord mesenchymal stromal cells (hUCMSCs) have been reported to differentiate into several cell lineages including bone, cartilage, and neurons and therefore may have the potential to differentiate into inner ear sensory hair cells. In this study, we investigated whether hUCMSCs could differentiate into inner ear hair cells via adenoviral mediated gene delivery of the *Math1* gene. To induce differentiation, hUCMSCs were transduced with the AdMath1.11D vector in the presence of protamine sulfate as a cross-linking agent. Differentiated cells were characterized based on their morphology and immunocytochemistry. Additionally, gene expression was quantified via quantitative RT-PCR. Moreover, the *in vitro* capability of the differentiated hair cell-like cells to repair damaged epithelial cells was demonstrated by co-culturing with neomycin-treated murine macular organs. The results demonstrated for the first time that hUCMSCs can differentiate into hair cell-like cells, thus providing a new potential tissue engineering approach for treating hearing loss.

INTRODUCTION

Sensory neural hearing loss (SNHL) and balance disorders are the most common hearing disabilities and affect approximately 49 million people in the United States. Loss of both auditory and vestibular hair cells is a cause of deafness. A cell source that has the potential to regenerate inner ear hair cells would have tremendous potential in clinical applications. Cells from the Wharton's jelly of umbilical cords are believed to be multipotent mesenchymal stem cells that can differentiate into cell types from all three germ layers (122). Studies have documented *in vitro* differentiation of these human umbilical cord mesenchymal stromal cells (hUCMSCs) into osteocytes, chondrocytes, hepatocytes, adipocytes, neural cells, and pancreatic cells (126-129). hUCMSCs can be a valuable tool for gene delivery and tissue engineering applications. Differentiation of hUCMSCs into nerve tissue raises the possibility of differentiating them into sensory hair cells. Recent work has shown that inner ear progenitor cells can be generated from bone marrow mesenchymal stem cells by using a combination of growth factors and expression of the *Math1* transcription factor (68). Several studies in inner ear gene therapy have reported successful delivery of the *Math1* gene using adenoviral vectors (14, 35, 36, 38, 49). Adenoviruses offer high transfection efficiencies and have substantial clinical experience, which makes them suitable for clinical use. We therefore evaluated the potential of hUCMSCs to express the *Math1* gene and differentiate into hair cells.

In this study, using an adenoviral gene delivery approach to express the *Math1* transcription factor in hUCMSCs, we induced hair cell differentiation. We then

examined the potential of differentiated cells to repair damaged sensory epithelium by co-culturing with murine macular organs.

MATERIALS AND METHODS

Isolation and expansion of hUCMSCs

hUCMSCs were isolated from human umbilical cords according to protocols approved by the University of Kansas Human Subjects Committee (KU-Lawrence IRB approval # 15402, KU Medical Center IRB approval # 10951). The cords were obtained from the hospital and harvested within 24 hours after delivery. The cords were rinsed in phosphate buffered saline (PBS) and cut into small pieces of 2 to 3 cm. The vascular tissue was then removed, and the cords were minced and incubated in 0.2% type II collagenase (298 U/mg; Worthington Biochemical; Lakewood, NJ) in low glucose Dulbecco's modified Eagle's medium (DMEM) for 4 hours at 37°C on a shaker. The digested homogenous solution was diluted in sterile PBS at a 1:4 ratio and centrifuged for 5 minutes at 1100 rpm. The supernatant was discarded and the cells frozen for future use. Frozen hUCMSCs were thawed, plated at a density of 7,000 cells/cm^2, and cultured in low glucose DMEM with 10% fetal bovine serum (FBS-MSC quantified) and 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Cells were fed every 2 days and maintained in a cell culture incubator at 37°C. At 80 - 90% confluency, the cells were trypsinized and passaged**.** hUCMSCs were isolated from 4 different human umbilical cords (n = 4)

and expanded to passage 4 (P4) for the experiments. All experiments were performed in quadruplicate for each cord.

Adenoviral vectors and vector production

The Ad5 (AdMath1.11D) vector backbone had E1, E3 and E4 regions deleted with an expression cassette of *Math1* driven by human cytomegalovirus (hCMV) promoter. The production system for the vector provided robust replication of the adenovector and purified stocks at 5 X 10^{11} and 2 X 10^{12} total particle units/ml (pu/ml), with a total particle to particle ratio ranging from 3 to 10 pu/fluorescent focus units. Total particle units were determined by spectrophotometric assay that has been standardized and qualified to reliably and robustly quantify the total particles within a single lot of adenovector. The adenovector lots were purified, aliquoted, and stored at -80°C. Individual aliquots were used for each experiment to prevent loss of activity associated with freeze-thaw cycles. The expression cassette contained an open reading frame and an SV40 polyadenylation site and transcriptional stop site at 3' end of the open reading frame (GenVec Inc., Gaithersburg, Md., USA) as previously described (38, 112).

Adenoviral transduction of hUCMSCs

hUCMSCs were trypsinized at P4 and the cells were plated in an 8 well Millicell EZ Slide (Millipore, Billerica, MA) at 1,000 cells/well (n=4) and 500 µl hUCMSCs medium (low glucose DMEM, 10% FBS, 1% penicillin/streptomycin)

was added per well. For RT-PCR, cells were plated in 12 well plates at 5,000 cells/well (n = 4) and 1000 µl of hUCMSCs medium was added. Cells were allowed to attach for 24 hours before the virus was added directly to the medium at a multiplicity of infection (MOI) of 100 in the presence of 8 µg/ml of protamine sulfate (MP Biomedicals, Irvine, CA) (107, 113). Controls had every component added but the vector. The chamber slide/well plate was gently rocked after adding the vector and incubated at 37°C. The medium was changed after 24h and every 48h for the remaining culture period. The cells were cultured *in vitro* for 10 days and immunostained for hair cell specific and neuronal markers.

Immunocytochemistry

Cells were fixed for 10 minutes with 4% paraformaldehyde in PBS. This was followed by washing three times in PBS. Immunostaining was initiated by blocking the slides for 1 hour with 5% FBS followed by four 0.2% Tween washes. The cells were then permeabilized with 0.2% Triton X-100 for 10 minutes. Hair cell differentiation in culture was characterized by immunostaining with the hair cell specific marker myosin VIIa and the glial cell marker GFAP, and the neuronal marker neurofilament. Double antibody staining was performed by adding two primary antibodies together - rabbit polyclonal to myosin VIIa (1:100) (Proteus Biosciences, Ramona, CA) and chicken polyclonal to GFAP (1:100) (Abcam, Cambridge, MA). Mouse monoclonal to neurofilament (Invitrogen, Carlsbad, CA) was stained separately at a 1:100 dilution. The cells were incubated overnight at 4°C. Incubation

was followed by four washes with 0.2% Tween and secondary antibody incubation for 30 minutes at room temperature. The secondary antibodies with Alexa visualizing fluorescent signals (1:400) used were Alexa-555-conjugated goat anti-rabbit IgG, Alexa-488-conjugated goat anti-chicken IgG and Alexa-555-conjugated goat anti-mouse IgG (all from Molecular Probes, Eugene, OR). All incubations were done in a humidified chamber. Incubation was followed by four washes with 0.2% Tween. The slides were mounted in ProLong Gold antifade with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen Life Technologies, Carlsbad, CA) and cover slipped. Microscopic images of cells counterstained with DAPI were acquired using an upright fluorescent microscope (Nikon Eclipse E800 and Q Image Aqua camera) and QCapture software. Images were photomerged and analyzed with Adobe Photoshop CS5 extended (Adobe Systems, San Jose, CA).

FM1-43 staining and fixation

FM1-43 FX (Invitrogen, Carlsbad, CA) is a lipophilic dye that has been shown to enter hair cells through transduction channels and is used to detect active transducer channels in the differentiated hair cell-like cells (130, 131). 5 µg/ml of FM1-43 FX staining solution was prepared in ice cold Hanks' Balanced Salt solution (HBSS) (Sigma-Aldrich, St. Louis, MO). The cells were quickly stained for 1 min in the staining solution. The cells were then fixed in ice cold 4% paraformaldehyde in HBSS for 10 minutes on ice. This was followed by three rinses in HBSS. The slides were mounted in ProLong Gold antifade with DAPI (Invitrogen Life Technologies,

Carlsbad, CA) and cover slipped. Microscopic images of cells counterstained with DAPI were acquired using an upright fluorescent microscope (Nikon Eclipse E800 and Q Image Aqua camera) and QCapture software. Images were photomerged and analyzed with Adobe Photoshop CS5 extended (Adobe Systems, San Jose, CA).

Quantitative RT-PCR

Total RNA from the controls and samples was extracted at day 10 post transduction using RNAeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Isolated RNA was converted to cDNA using a TaqMan High Capacity kit (Applied Biosystems, Foster City, CA) in a BioRad i-cycler thermal cycler (BioRad, Hercules, CA). Real time PCR was performed using SYBR green PCR Master Mix (BioRad, Hercules, CA) and an i-cycler (BioRad, Hercules, CA). PCR analysis was performed using cDNA as a template using custom designed primers for the following genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), myosin VIIa, nestin, glial fibrillary acidic protein (GFAP), sex determining region Y-box 2 (Sox2) and paired box gene 2 (Pax2). Primers were designed using Beacon Designer 7.9. Table 5.1 gives the list of primer pairs used and their cDNA product lengths. Custom primers were ordered from Integrated DNA Technologies (Coralville, IA). A $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative level of expression for each target gene. Gene expression was normalized to controls and GAPDH.

LIVE/DEAD Assay for cell viability

Cell viability at day 3 post transduction was assessed using a LIVE/DEAD assay kit for cell viability (molecular Probes, Eugene, OR). hUCMSCs at P4 from each of the four cords were plated in a 24 well plate at 5,000 cells/well (n =3). Cells were incubated with LIVE/DEAD reagent (dye concentration 2mM Calcein AM and 4 mM Ethidium Bromide) for 10 minutes at 37°C in the dark. Cells were imaged using an inverted fluorescent microscope (Nikon, Melville, NY) and QCapture software.

Co-culture of AdMath1.11D transduced hUCMSCs with murine macular organs

Macular organs from mice were harvested as previously described (38). Adult C57B16 mice (12 months old, male and female) were sacrificed with intraperitoneal beuthanasia and decapitated under approved IACUC protocol (# 2008-1746). The otic capsule was exposed, and the macular organs were identified by finding the otolithic membranes. Using no. 5 watchmaker forceps, the saccule and utricle were removed. The organs were cultured on a Millicell membrane (Milli-pore Corp., Bedford, MA) suspended in 2,000 µl of Dulbecco modified Eagle medium supplemented with N1 (Sigma, St.Louis, MO) + 100U/ml penicillin and 5.5 µl /mL of 30% glucose. After 24 hours *in vitro* (37°C, 5% carbon dioxide), cultures were treated with 10^{-3} mol/L neomycin for 48 hours. Experimental cultures (n = 4 per experimental group) were treated with previously transduced hUCMSCs. *Math1* gene expressing cells and GFP expressing cells were obtained from AdMath1.11D and Ad28.eGFP transduced

hUCMSCs cultures respectively. The cells were trypsinized at day 10 post transduction and suspended in organ culture medium at 50,000 cells/mL. 250 μl of this suspension was added to each macular organ (63). Negative control cultures were placed in a medium not treated with neomycin and no cells were added. A second negative control culture was placed in complete medium without neomycin and no cells were added. Positive control cultures were treated with GFP expressing hUCMSCs. Organ cultures were incubated in a CO_2 incubator at 37°C for 10 days with medium changes every 3 days.

Immunostaining

At day 10, explants were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes. This was followed by three PBS washes. Immunostaining was initiated by blocking and permeabilizing explants in 10% FBS and 0.3% Triton X-100 for 30 minutes. This was followed by incubation with primary antibody (1:500) at 4°C overnight. The primary antibody used was rabbit polyclonal to myosin VIIa (Proteus Biosciences, Ramona, CA). The explants were washed thrice with PBS and incubated in secondary antibody (1:400) for 30 minutes. The secondary antibody with Alexa visualizing fluorescent signals used was Alexa-555-conjugated goat anti rabbit IgG (all from Molecular Probes, Eugene, OR). All incubations were done in a humidified chamber. This was followed by three PBS washes again. The explants were mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) using ProLong Gold antifade with DAPI (Invitrogen Life Technologies,

Carlsbad, CA) and cover slipped. Microscopic images of explants were acquired using an upright fluorescent microscope (Nikon Eclipse E800 and Q Image Aqua camera) and QCapture software. Images were analyzed with Adobe Photoshop CS5 extended (Adobe Systems, San Jose, CA).

RESULTS

Characterization of hair cell like differentiation of hUCMSCs *in vitro*

Differentiated cells (n = 4) from hUCMSC cultures were characterized based on morphology and immunocytochemistry. Spindle-shaped fibroblast-like cells (characteristic morphology of hUCMSCs) were observed in the negative controls; however, the transduced cell populations showed a small percentage of pear-shaped cells with extended protrusions resembling the ciliary bundles in hair cells. These cells had centrally located nuclei. The ciliary protrusions and cytoplasm of the pear-shaped cells were positive for myosin VIIa and GFAP antibodies (Figure 5.1). In addition, FM1-43 fluorescent labeling was observed in a small percentage of transduced cells (Figure 5.2). The transduced cells did not stain positive for the neurofilament antibody (Figure 5.3).

Quantification of gene expression

Untreated hUCMSCs were used as controls. All relative gene expression values for each target gene were normalized to controls and GAPDH. Expression of myosin VIIa was detected in the transduced cell population at day 10, although

controls also showed low levels of myosin VIIa expression. Transduced hUCMSCs demonstrated a small change in gene expression when compared to the controls. Nestin expression was detected in the transduced cells; however, nestin expressed high standard deviations when compared to other genes. Sox2 and Pax2 expressions were detected at PCR amplification cycles close to 35. There was no significant difference in the gene expression levels of myosin VIIa, nestin, Pax2 and Sox2 between the control and transduced groups. GFAP expression failed to show up on real time PCR analysis (Figure 5.4).

Transduced cell viability

Cell viability was determined with Calcein AM (green) and Ethidium Bromide (red) fluorescent markers. Green color indicated live cells, while the red color indicated dead cells. hUCMSCs transduced with the AdMath1.11D vector at a multiplicity of infection of 100 demonstrated high cell viabilities at 3 days post transduction, indicating low cytotoxic effects of adenoviral mediated gene delivery in hUCMSCs (Figure 5.5).

Co-culture of AdMath1.11D transduced cells and macular organ explants

We investigated the potential of AdMath1.11D transduced hUCMSCs to repair damaged sensory epithelium in macular organ explants. After 10 days of co-culture with macular organs, hair cell-like cells were found in one of the four explants (Figure 5.6). Negative controls without neomycin showed the presence of hair cells in

the sensory epithelium. Negative controls with neomycin treatment did not show any myosin VIIa positive hair cells due to the absence of hair cells. In addition, the positive controls failed to show any migration of GFP expressing hUCMSCs (Figure 5.6).

DISCUSSION

hUCMSCs are believed to be multipotent stem cells and have been reported to differentiate into multiple cell lineages belonging to all three germ layers. hUCMSCs have been shown to give rise to cells from chondrogenic, osteogenic, myogenic, adipogenic, neurogenic, hepatogenic, and pancreatic lineages (126-129, 132). In this study, we demonstrated the possibility of extending the range of lineages for hUCMSCs to include inner ear sensory hair cells. Studies of *Math1* expression in inner ear progenitor cells have indicated that this transcription factor plays an important role in the differentiation of hair cells (33). Studies with rat bone marrow mesenchymal stem cells have demonstrated their potential to express *Math1* and differentiate into inner ear progenitor cells in the presence of otic-inducing growth factors (68).

Our results show that hUCMSCs infected with an adenovector can express the *Math1* transcription factor driven by the human cytomegalovirus (hCMV) promoter, and differentiate into hair cell-like cells. Studies with mesenchymal stem cells have indicated that a stem cell that has differentiated partially or completely into an inner ear progenitor cell can express early otic, hair cell, and neuronal markers (125).

Immunocytochemical results on the transduced cell population in our experiments demonstrated differentiated cells that expressed the hair cell-specific markers myosin VIIa and FM1-43 and the glial cell marker GFAP. Myosin VIIa is a hair cell-specific marker that stains actin binding domains in myosin motor proteins present in the transduction channels (11). GFAP is an astrocyte marker expressed in sensory epithelial cells in the inner ear (133). Differentiated cells were characterized anatomically and immunocytochemically. Myosin VIIa and GFAP positive cells were pear-shaped and displayed protrusions at one end that resembled ciliary bundles in hair cells. Cell morphology indicated differentiation into hair cell-like cells, and positive expressions of myosin VIIa and GFAP indicated immature features of hair cell precursor cells and neuroepithelial cells (133). FM1-43 positive stains indicated the presence of active transducer channels in the hair cell-like cells. The differentiated cells did not express the neuronal marker neurofilament indicating that the *Math1* gene did not induce any neuronal phenotypes. Only a small percentage of the transduced cell population was observed to demonstrated hair cell morphology and gene expression. Previous research has shown that the notch signaling pathway can inhibit differentiation, resulting in the prevention of a hair cell fate commitment (125). This may be one of the reasons for low transduction efficiencies and partial differentiation of hUCMSCs.

Quantitative gene expression results from RT-PCR demonstrated expression of myosin VIIa, nestin, Pax2, and Sox2. Although the PCR results demonstrated a certain level of gene expression, the reliability of the data in detecting minute levels

of gene expression may be compromised. Only a few cells in the entire transduced population were reported to be differentiated and due to the lack of a selection gene in the vector (AdMath1.11D) backbone, the differentiated cells could not be separated from the rest of the population for gene expression analysis. In our study, RNA was isolated from the entire cell population consisting of a mixture of differentiated and undifferentiated cells. Therefore, the chances of PCR detecting the desired gene are very bleak with the inclusion of the RNA from all of the undifferentiated cells. Immunocytochemistry results were positive for GFAP while RT-PCR failed to detect GFAP. This discrepancy may be because GFAP is reported to express only in cells that are partially or completely differentiated to sensory epithelium or neuroepithelium (133). The gene expression may have been at extremely low levels that were difficult to detect within the first few amplification cycles of RT-PCR. On the other hand, myosin VIIa was detected by RT-PCR. This can be explained from the low levels of myosin VIIa expressed in untreated hUCMSCs, though not sufficient enough to induce hair cell differentiation in hUCMSCs. We can therefore conclude that *Math1* expression is important for inducing development of hair cell characteristics in hUCMSCs.

Immunocytochemistry of transduced hUCMSCs and macular organ co-cultures demonstrated the presence of myosin positive cells, indicating the potential of hUCMSCs to engraft and integrate into the sensory epithelium. Experimental group treated with GFP expressing hUCMSCs failed to show GFP expression in sensory epithelium owing to the short term transient gene expression of GFP.

However, myosin positive hair cell-like cells were observed in only one of the four explant co-cultures. This result may be explained as follows. It is possible that the one explant actually received a sufficient number of *Math1* expressing cells and demonstrated its ability to regenerate hair cells in neomycin-treated macular organs, or the neomycin treatment failed to damage the hair cells in the sensory epithelium for this explant alone. Another possibility to consider is the multipotent properties of hUCMSCs and their capability of differentiating into hair cell-like cells responding to inductive signals from the surrounding cells in the sensory epithelium. Evaluation of the integration of differentiated cells in tissue can be challenging owing to the complex structure of the inner ear. More importantly, these results provide the basic information required for further *in vitro* and cell transplantation studies. Studies have demonstrated the successful co-culture of stem cells with cochlear explants (63, 68). Therefore, co-culturing cochlear explants with cells that are genetically manipulated *in vitro* to express *Math1* using viral vectors can potentially be used to regenerate inner ear hair cells.

The findings in this study indicated that hUCMSCs may have the potential to differentiate into inner ear sensory cells for use as a source of transplantation in curing SNHL and other hearing disorders. Stem cells in the inner ear lack the capacity to differentiate and replace damaged hair cells (27) and therefore a cell source like hUCMSCs with the capacity to express *Math1* can be used in therapeutic applications. Our findings suggest for the first time that hUCMSCs can differentiate into hair cell-like cells via the adenoviral mediated gene delivery of *Math1*, thus

providing a significant finding in the field of tissue engineering and a potential strategy to cure sensory neural hearing loss.

CHAPTER 6: Conclusion

Previous studies that attempted to regenerate inner ear hair cells have successfully differentiated embryonic and mesenchymal stem cells using a combination of several otic-inducing growth factors or by expression of an externally delivered *Math1* gene. The studies in this thesis made the first attempt in using mesenchymal stem cells from human umbilical cord stroma for regenerating hair cells. Our results examined the potential use of human umbilical cord mesenchymal stromal cells (hUCMSCs) in stem cell-based and tissue engineering approaches to restore hearing loss.

Hair cell differentiation in hUCMSCs was induced *in vitro* via adenoviral mediated gene delivery of the *Math1* gene. The differentiated cells resembled hair cells based on morphology and immunocytochemical markers. Differentiated cells expressed hair cell specific markers myosin VIIa and FM1-43 and the glial cell marker GFPA. The findings indicated the development of hair cell-like cells. However, transduction efficiencies were low and only a small percentage of the cells differentiated into hair cell-like cells. This may suggest partial differentiation of stem cells. The notch signaling pathway, *Hes1*, *Hes5,* and *Jagged1* genes are known to be potent down regulators of *Math1* expression. They cause lateral inhibition wherein one cell is singled out from a group of cells for a given fate. Notch signaling inhibits *Math1* gene expression and hair cell differentiation, preventing a cell from committing to the hair cell fate. Future work can investigate the application of notch

inhibitors like γ-secretase inhibitors at varying concentrations to prevent the lateral inhibition caused by notch signaling.

Future studies with the use of hUCMSCs for hair cell differentiation can focus on delivering adenoviral vectors at high multiplicity of infection and using a combination of growth factors like IGF-1, NT-3, bFGF, EGF, and BDNF to supplement the differentiation process and achieve a higher percentage of differentiated cells. Although, MOIs greater than 100 may cause random integration of virus into host chromosome and generate replication competent viruses and evoke immune responses. Co-culturing partially differentiated cells with inner ear sensory epithelial cells may also induce differentiation in stem cells. Additionally, a vector designed to contain an antibiotic selection marker can be used to isolate the differentiated cells from the transduced population and test their potential to regenerate hair cells *in vivo*. Fluorescence activated cell sorting (FACS) analysis can be considered to isolate differentiated cells from the transduced population. Quantitative RT-PCR on an isolated, differentiated cell population would give a more representative analysis of the target genes expressed than the data obtained in this thesis. Regardless, the success achieved with hUCMSCs in this thesis work has laid a foundation to explore numerous ways of inducing hair cell differentiation. This thesis examined the potential of hUCMSCs as a possible source to engineer inner ear hair cells to restore hearing loss. In the future, cell transplantation treatments for hearing loss may include the delivery of a pure, differentiated hUCMSC population to the inner ear, with or without the augmentation of growth factors.

REFERENCES

1. Cotanche D, Kaiser C. Hair cell fate decisions in cochlear development and regeneration. Hearing research. 2010.

2. Oshima K, Suchert S, Blevins N, Heller S. Curing hearing loss: Patient expectations, health care practitioners, and basic science. Journal of communication disorders. 2010;43(4):311-8.

3. Pauley S, Kopecky B, Beisel K, Soukup G, Fritzsch B. Stem cells and molecular strategies to restore hearing. Panminerva medica. 2008;50(1):41.

4. Suter AH, von Gierke HE. Noise and public policy. Ear and hearing. 1987;8(4):188.

5. Clark WW, Bohne BA. Effects of noise on hearing. JAMA. 1999;281(17):1658.

6. Fligor BJ, Cox LC. Output levels of commercially available portable compact disc players and the potential risk to hearing. Ear and hearing. 2004;25(6):513.

7. Bodmer D. Protection, regeneration and replacement of hair cells in the cochlea: implications for the future treatment of sensorineural hearing loss. Swiss Med Wkly. 2008;138(47-48):708-12.

8. Cheng AG, Cunningham LL, Rubel EW. Mechanisms of hair cell death and protection. Current opinion in otolaryngology & head and neck surgery. 2005;13(6):343.

9. Matsui J, Cotanche D. Sensory hair cell death and regeneration: two halves of the same equation. Current opinion in otolaryngology & head and neck surgery. 2004;12(5):418.

10. Kharlamova A, Aarts N. A Review of Past and Present Hair Cell Regeneration Techniques.

11. Gillespie P, Müller U. Mechanotransduction by hair cells: models, molecules, and mechanisms. Cell. 2009;139(1):33-44.

12. Rubel E, Oesterle E, Weisleder P, editors. Hair cell regeneration in the avian inner ear1991.

13. Weisleder P, Rubel E. Hair cell regeneration in the avian vestibular epithelium. Experimental neurology. 1992;115(1):2-6.

14. Huang Y, Chi F, Han Z, Yang J, Gao W, Li Y. New ectopic vestibular hair cell-like cells induced by Math1 gene transfer in postnatal rats. Brain research. 2009;1276:31-8.

15. Dooling R, Dent M. New studies on hair cell regeneration in birds. Acoustical Science and Technology. 2001;22(2):93-9.

16. Wang G, Chatterjee I, Batts S, Wong H, Gong T, Gong S, et al. Notch signaling and Atoh1 expression during hair cell regeneration in the mouse utricle. Hearing research. 2010.

17. Zine A, Van De Water T, de Ribaupierre F. Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. Development. 2000;127(15):3373.

18. Corwin J. Regeneration in the auditory system. Experimental neurology. 1992;115(1):7-12.

19. Stone J, Rubel E. Cellular studies of auditory hair cell regeneration in birds. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(22):11714.

20. Kwan T, White P, Segil N. Development and Regeneration of the Inner Ear. Annals of the New York Academy of Sciences. 2009;1170(International Symposium on Olfaction and Taste):28-33.

21. Edge A, Chen Z. Hair cell regeneration. Current Opinion in Neurobiology. 2008;18(4):377-82.

22. Matsui J, Ryals B. Hair cell regeneration: An exciting phenomenon... But will restoring hearing and balance be possible? Journal of Rehabilitation Research and Development. 2005;42(4):187.

23. Bermingham-McDonogh O, Rubel E. Hair cell regeneration: winging our way towards a sound future. Current Opinion in Neurobiology. 2003;13(1):119-26.

24. Brigande J, Heller S. Quo vadis, hair cell regeneration? Nature neuroscience. 2009;12(6):679-85.

25. Collado M, Burns J, Hu Z, Corwin J. Recent advances in hair cell regeneration research. Current opinion in otolaryngology & head and neck surgery. 2008;16(5):465.

26. Beisel K, Hansen L, Soukup G, Fritzsch B. Regenerating cochlear hair cells: quo vadis stem cell. Cell and tissue research. 2008;333(3):373-9.

27. Li H, Corrales C, Edge A, Heller S. Stem cells as therapy for hearing loss. Trends in Molecular Medicine. 2004;10(7):309-15.

28. Matsui JI, Parker MA, Ryals BM, Cotanche DA. Regeneration and replacement in the vertebrate inner ear. Drug Discovery Today. 2005;10(19):1307-12.

29. LeMasurier M, Gillespie P. Hair-cell mechanotransduction and cochlear amplification. Neuron. 2005;48(3):403-15.

30. White P, Doetzlhofer A, Lee Y, Groves A, Segil N. Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. Nature. 2006;441(7096):984-7.

31. Stone J, Oesterle E, Rubel E. Recent insights into regeneration of auditory and vestibular hair cells. Current opinion in neurology. 1998;11(1):17.

32. Chen P, Johnson J, Zoghbi H, Segil H. The role of Math1 in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. DEVELOPMENT-CAMBRIDGE-. 2002;129(10):2495-505.

33. Bermingham N, Hassan B, Price S, Vollrath M, Ben-Arie N, Eatock R, et al. Math1: an essential gene for the generation of inner ear hair cells. Science. 1999;284(5421):1837.

34. Izumikawa M, Minoda R, Kawamoto K, Abrashkin K, Swiderski D, Dolan D, et al. Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nature medicine. 2005;11(3):271-6.

35. Kawamoto K, Ishimoto SI, Minoda R, Brough DE, Raphael Y. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. Journal of Neuroscience. 2003;23(11):4395.

36. Zheng J, Gao W. Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nature neuroscience. 2000;3(6):580-6.

37. Baker K, Brough D, Staecker H. Repair of the vestibular system via adenovector delivery of Atoh1: a potential treatment for balance disorders. 2009.

38. Staecker H, Praetorius M, Baker K, Brough D. Vestibular hair cell regeneration and restoration of balance function induced by Math1 gene transfer. Otology & Neurotology. 2007;28(2):223.

39. Cao S. The Current Status and Prospects of Gene Therapy for the Inner Ear. Human Gene Therapy. (ja).

40. Kim P, Helms AW, Johnson JE, Zimmerman K. XATH-1, a Vertebrate Homolog of Drosophila Atonal, Induces Neuronal Differentiation within Ectodermal Progenitors* 1. Developmental biology. 1997;187(1):1-12.

41. Shou J, Zheng J, Gao W. Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of Hath1. Molecular and Cellular Neuroscience. 2003;23(2):169-79.

42. Zine A, Aubert A, Qiu J, Therianos S, Guillemot F, Kageyama R, et al. Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. The Journal of Neuroscience. 2001;21(13):4712.

43. Zheng J, Shou J, Guillemot F, Kageyama R, Gao W. Hes1 is a negative regulator of inner ear hair cell differentiation. Development. 2000;127(21):4551.

44. Liu M, Pereira FA, Price SD, Chu M, Shope C, Himes D, et al. Essential role of BETA2/NeuroD1 in development of the vestibular and auditory systems. Genes & development. 2000;14(22):2839.

45. Okano T, Nakagawa T, Kita T, Endo T, Ito J. Cell–Gene Delivery of Brain-Derived Neurotrophic Factor to the Mouse Inner Ear. Molecular Therapy. 2004;14(6):866-71.

46. Oshima K, Shimamura M, Mizuno S, Tamai K, Doi K, Morishita R, et al. Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats. The FASEB journal. 2004;18(1):212.

47. Rejali D, Lee VA, Abrashkin KA, Humayun N, Swiderski DL, Raphael Y. Cochlear implants and ex vivo BDNF gene therapy protect spiral ganglion neurons. Hearing research. 2007;228(1-2):180-7.

48. Jero J, Mhatre AN, Tseng CJ, Stern RE, Coling DE, Goldstein JA, et al. Cochlear gene delivery through an intact round window membrane in mouse. Human Gene Therapy. 2001;12(5):539-48.

49. Praetorius M, Brough D, Hsu C, Plinkert P, Pfannenstiel S, Staecker H. Adenoviral vectors for improved gene delivery to the inner ear. Hearing research. 2009;248(1-2):31-8.

50. Campochiaro PA, Nguyen QD, Shah SM, Klein ML, Holz E, Frank RN, et al. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. Human Gene Therapy. 2006;17(2):167-76.

51. Alton E, Kitson C. Gene therapy for cystic fibrosis. Expert Opinion on Investigational Drugs. 2000;9(7):1523.

52. Praetorius M, Hsu C, Baker K, Brough D, Plinkert P, Staecker H. Adenovector-mediated hair cell regeneration is affected by promoter type. Acta oto-laryngologica. 2010;130(2):215-22.

53. Kawamoto K, Yagi M, Stöver T, Kanzaki S, Raphael Y. Hearing and hair cells are protected by adenoviral gene therapy with TGF- 1 and GDNF. Molecular Therapy. 2003;7(4):484-92.

54. Pfannenstiel S, Praetorius M, Plinkert P, Brough D, Staecker H. Bcl-2 Gene Therapy Prevents Aminoglycoside-Induced Degeneration of Auditory and Vestibular Hair Cells. Audiology and Neurotology. 2009;14(4):254-66.

55. Luebke AE, Foster PK, Muller CD, Peel AL. Cochlear function and transgene expression in the guinea pig cochlea, using adenovirus-and adeno-associated virus-directed gene transfer. Human Gene Therapy. 2001;12(7):773-81.

56. Staecker H, Li D, O Malley B, Van De Water T. Gene expression in the mammalian cochlea: a study of multiple vector systems. Acta oto-laryngologica. 2001;121(2):157-63.

57. Derby ML, Sena-Esteves M, Breakefield XO, Corey DP. Gene transfer into the mammalian inner ear using HSV-1 and vaccinia virus vectors. Hearing research. 1999;134(1-2):1-8.

58. Han JJ, Mhatre AN, Wareing M, Pettis R, Gao WQ, Zufferey RN, et al. Transgene expression in the guinea pig cochlea mediated by a lentivirus-derived gene transfer vector. Human Gene Therapy. 1999;10(11):1867-73.

59. Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. Stem cells. 2001;19(3):193-204.

60. Li H, Roblin G, Liu H, Heller S. Generation of hair cells by stepwise differentiation of embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(23):13495.

61. Oshima K, Shin K, Diensthuber M, Peng A, Ricci A, Heller S. Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. Cell. 2010;141(4):704-16.

62. Martinez-Monedero R, Edge A. Stem cells for the replacement of inner ear neurons and hair cells. International Journal of Developmental Biology. 2007;51(6/7):655.

63. Coleman B, Fallon J, Pettingill L, de Silva M, Shepherd R. Auditory hair cell explant co-cultures promote the differentiation of stem cells into bipolar neurons. Experimental cell research. 2007;313(2):232-43.

64. Corrales CE, Pan L, Li H, Liberman MC, Heller S, Edge ASB. Engraftment and differentiation of embryonic stem cell–derived neural progenitor cells in the cochlear nerve trunk: Growth of processes into the organ of corti. Journal of neurobiology. 2006;66(13):1489-500.

65. Shi F, Corrales CE, Liberman MC, Edge ASB. BMP4 induction of sensory neurons from human embryonic stem cells and reinnervation of sensory epithelium. European Journal of Neuroscience. 2007;26(11):3016-23.

66. Chen W, Jongkamonwiwat N, Jonhson S. FGF signaling can induce the generation of cochlear progenitors from hESCs with the potential to differentiate into functional hair cell-like cells and neurons UK National Stem Cell Network Oxford. UK National Stem Cell Network. 2009:99.

67. Rivolta MN, Li H, Heller S. Generation of inner ear cell types from embryonic stem cells. METHODS IN MOLECULAR BIOLOGY-CLIFTON THEN TOTOWA-. 2006;330:71.

68. Jeon S, Oshima K, Heller S, Edge A. Bone marrow mesenchymal stem cells are progenitors in vitro for inner ear hair cells. Molecular and Cellular Neuroscience. 2007;34(1):59-68.

69. Sharif S, Nakagawa T, Ohno T, Matsumoto M, Kita T, Riazuddin S, et al. The potential use of bone marrow stromal cells for cochlear cell therapy. Neuroreport. 2007;18(4):351.

70. Matsuoka AJ, Kondo T, Miyamoto RT, Hashino E. In vivo and In vitro Characterization of Bone Marrow Derived Stem Cells in the Cochlea. The Laryngoscope. 2006;116(8):1363-7.

71. Kondo T, Johnson SA, Yoder MC, Romand R, Hashino E. Sonic hedgehog and retinoic acid synergistically promote sensory fate specification from bone marrow-derived pluripotent stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(13):4789.

72. Li H, Liu H, Heller S. Pluripotent stem cells from the adult mouse inner ear. Nature medicine. 2003;9(10):1293-9.

73. Doyle KL, Kazda A, Hort Y, McKay SM, Oleskevich S. Differentiation of adult mouse olfactory precursor cells into hair cells in vitro. Stem cells. 2007;25(3):621-7.

74. Lang H, Ebihara Y, Schmiedt R, Minamiguchi H, Zhou D, Smythe N, et al. Contribution of bone marrow hematopoietic stem cells to adult mouse inner ear: mesenchymal cells and fibrocytes. The Journal of comparative neurology. 2006;496(2):187-201.

75. Tan BTG, Lee MMG, Ruan R. Bone marrow derived cells that home to acoustic deafened cochlea preserved their hematopoietic identity. The Journal of comparative neurology. 2008;509(2):167-79.

76. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663-76.

77. Kim JB, Greber B, Araúzo-Bravo MJ, Meyer J, Park KI, Zaehres H, et al. Direct reprogramming of human neural stem cells by OCT4. Nature. 2009;461(7264):649-3.

78. Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S, et al. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron. 2000;28(1):31-40.

79. Nishimura K, Nakagawa T, Ono K, Ogita H, Sakamoto T, Yamamoto N, et al. Transplantation of mouse induced pluripotent stem cells into the cochlea. Neuroreport. 2009;20(14):1250.

80. Hildebrand MS, Dahl HHM, Hardman J, Coleman B, Shepherd RK, De Silva MG. Survival of partially differentiated mouse embryonic stem cells in the scala media of the guinea pig cochlea. JARO-Journal of the Association for Research in Otolaryngology. 2005;6(4):341-54.

81. Sekiya T, Kojima K, Matsumoto M, Kim TS, Tamura T, Ito J. Cell transplantation to the auditory nerve and cochlear duct. Experimental neurology. 2006;198(1):12-24.

82. Hu Z, Ulfendahl M, Olivius N. Central migration of neuronal tissue and embryonic stem cells following transplantation along the adult auditory nerve. Brain research. 2004;1026(1):68-73.

83. Jongkamonwiwat N, Zine A, Rivolta N. Stem Cell Based Therapy in the Inner Ear: Appropriate Donor Cell Types and Routes for Transplantation. Current drug targets. 2010;11(7):888-97.

84. Crain BJ, Tran SD, Mezey E. Transplanted human bone marrow cells generate new brain cells. Journal of the neurological sciences. 2005;233(1-2):121-3.

85. Naito Y, Nakamura T, Nakagawa T, Iguchi F, Endo T, Fujino K, et al. Transplantation of bone marrow stromal cells into the cochlea of chinchillas. Neuroreport. 2004;15(1):1.

86. Vlastarakos P, Nikolopoulos T, Tavoulari E, Papacharalambous G, Tzagaroulakis A, Dazert S. Sensory cell regeneration and stem cells: what we have already achieved in the management of deafness. Otology & Neurotology. 2008;29(6):758.

87. Kamiya K, Fujinami Y, Hoya N, Okamoto Y, Kouike H, Komatsuzaki R, et al. Mesenchymal stem cell transplantation accelerates hearing recovery through the repair of injured cochlear fibrocytes. American Journal of Pathology. 2007;171(1):214.

88. Lanza R. Stem cell breakthrough: don't forget ethics. Science. 2007;318(5858):1865.

89. Goessler UR, Riedel K, Hörmann K, Riedel F. Perspectives of gene therapy in stem cell tissue engineering. Cells Tissues Organs. 2006;183(4):169-79.

90. Chailertvanitkul V, Pouton CW. Adenovirus: a blueprint for non-viral gene delivery. Current opinion in biotechnology. 2010.

91. Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nature medicine. 2001;7(1):33-40.

92. Strayer DS. Viral gene delivery. Expert Opinion on Investigational Drugs. 1999;8(12):2159-72.

93. Wilson JM. Adenoviruses as gene-delivery vehicles. New England Journal of Medicine. 1996;334(18):1185-7.

94. Nemerow G, Pache L, Reddy V, Stewart P. Insights into adenovirus host cell interactions from structural studies. Virology. 2009;384(2):380-8.

95. Rebel VI, Hartnett S, Denham J, Chan M, Finberg R, Sieff CA. Maturation and Lineage Specific Expression of the Coxsackie and Adenovirus Receptor in Hematopoietic Cells. Stem cells. 2000;18(3):176-82.

96. Wickham T. Targeting adenovirus. Gene therapy. 2000;7(2):110-4.

97. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science. 1997;275(5304):1320.

98. Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(7):3352.

99. Hidaka C, Milano E, Leopold PL, Bergelson JM, Hackett NR, Finberg RW, et al. CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. Journal of Clinical Investigation. 1999;103:579-87.

100. Koizumi N, Mizuguchi H, Hosono T, Ishii-Watabe A, Uchida E, Utoguchi N, et al. Efficient gene transfer by fiber-mutant adenoviral vectors containing RGD peptide. Biochimica et Biophysica Acta (BBA)-General Subjects. 2001;1568(1):13-20.

101. Krasnykh V, Dmitriev I, Mikheeva G, Miller CR, Belousova N, Curiel DT. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. Journal of virology. 1998;72(3):1844.

102. Mizuguchi H, Koizumi N, Hosono T, Utoguchi N, Watanabe Y, Kay MA, et al. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene therapy. 2001;8(9):730-5.

103. Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, et al. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. Stem cells. 2006;24(3):781-92.

104. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem cells. 2004;22(7):1330-7.

105. Can A, Karahuseyinoglu S. Concise Review: Human Umbilical Cord Stroma with Regard to the Source of Fetus Derived Stem Cells. Stem cells. 2007;25(11):2886-95.

106. Rachakatla RS, Marini F, Weiss ML, Tamura M, Troyer D. Development of human umbilical cord matrix stem cell-based gene therapy for experimental lung tumors. Cancer Gene Therapy. 2007;14(10):828-35.

107. Qian H, HANG X, Xu W, Zhu W, Cao H, Chen Y, et al. Lentivirus-modified human umbilical cord mesenchymal stem cells maintain their pluripotency. Biotechnology and applied biochemistry. 2010;55:53-62.

108. Conget PA, Minguell JJ. Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. Experimental hematology. 2000;28(4):382-90.

109. Meyerrose TE, Roberts M, Ohlemiller KK, Vogler CA, Wirthlin L, Nolta JA, et al. Lentiviral Transduced Human Mesenchymal Stem Cells Persistently Express Therapeutic Levels of Enzyme in a Xenotransplantation Model of Human Disease. Stem cells. 2008;26(7):1713-22.

110. Lin T, Gu J, Zhang L, Davis J, Huang X, Cabbini G, et al. Enhancing adenovirus-mediated gene transfer in vitro and in vivo by addition of protamine and hydrocortisone. The Journal of Gene Medicine. 2003;5(10):868-75.

111. Lanuti M, Kouri C, Force S, Chang M, Amin K, Xu K, et al. Use of protamine to augment adenovirus-mediated cancer gene therapy. Gene therapy. 1999;6(9):1600.

112. Schlecker C, Praetorius M, Brough D, Presler R, Hsu C, Plinkert P, et al. Selective Atonal gene delivery improves balance function in a mouse model of vestibular disease. Gene therapy. 2011.

113. Cornetta K, Anderson WF. Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene-transfer: implications for human gene therapy. Journal of virological methods. 1989;23(2):187-94.

114. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins [alpha] v [beta] 3 and [alpha] v [beta] 5 promote adenovirus internalization but not virus attachment. Cell. 1993;73(2):309-19.

115. Byk T, Haddada H, Vainchenker W, Louache F. Lipofectamine and related cationic lipids strongly improve adenoviral infection efficiency of primitive human hematopoietic cells. Human Gene Therapy. 1998;9(17):2493-502.

116. Adams WC, Bond E, Havenga MJE, Holterman L, Goudsmit J, Karlsson-Hedestam GB, et al. Adenovirus serotype 5 infects human dendritic cells via a CAR-independent receptor pathway mediated by lactoferrin and DC-SIGN. Journal of General Virology. 2009:vir. 0.008342-0v1.

117. Gonzalez R, Vereecque R, Wickham TJ, Facon T, Hetuin D, Kovesdi I, et al. Transduction of bone marrow cells by the AdZ. F (pK7) modified adenovirus demonstrates preferential gene transfer in myeloma cells. Human Gene Therapy. 1999;10(16):2709-17.

118. Smith JS, Keller JR, Lohrey NC, McCauslin CS, Ortiz M, Cowan K, et al. Redirected infection of directly biotinylated recombinant adenovirus vectors through

cell surface receptors and antigens. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(16):8855.

119. Clark PR, Stopeck AT, Brailey JL, Wang Q, McArthur J, Finer MH, et al. Polycations and cationic lipids enhance adenovirus transduction and transgene expression in tumor cells. Cancer Gene Therapy. 1999;6(5):437.

120. Jacobsen F, Hirsch T, Mittler D, Schulte M, Lehnhardt M, Druecke D, et al. Polybrene improves transfection efficacy of recombinant replication deficient adenovirus in cutaneous cells and burned skin. The Journal of Gene Medicine. 2006;8(2):138-46.

121. Yang Y, Hsieh Y. Protamine sulfate enhances the transduction efficiency of recombinant adeno-associated virus-mediated gene delivery. Pharmaceutical research. 2001;18(7):922-7.

122. Wang L, Ott L, Seshareddy K, Weiss ML, Detamore MS. Musculoskeletal tissue engineering with human umbilical cord mesenchymal stromal cells. Regenerative Medicine. 2011;6(1):95-109.

123. Fong CY, Richards M, Manasi N, Biswas A, Bongso A. Comparative growth behaviour and characterization of stem cells from human Wharton's jelly. Reproductive biomedicine online. 2007;15(6):708-18.

124. Stone IM, Lurie DI, Kelley MW, Poulsen DJ. Adeno-associated virus-mediated gene transfer to hair cells and support cells of the murine cochlea. Molecular Therapy. 2005;11(6):843-8.

125. Edge AB, MA, US), inventor MASSACHUSETTS EYE & EAR INFIRMARY (Boston, MA, US), assignee. GENERATION OF INNER EAR CELLS. United States. 2009.

126. Campard D, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. Gastroenterology. 2008;134(3):833-48.

127. Chao KC, Chao KF, Fu YS, Liu SH. Islet-like clusters derived from mesenchymal stem cells in Wharton's Jelly of the human umbilical cord for transplantation to control type 1 diabetes. PLoS One. 2008;3(1):e1451.

128. Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DÖ, et al. Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. Stem cells. 2007;25(2):319-31.

129. Mitchell K, Weiss M, Mitchell B, Martin P, Davis D, Morales L, et al. Matrix cells from Wharton's jelly form neurons and glia. Stem cells. 2003;21(1):50-60.

130. Gale J, Marcotti W, Kennedy H, Kros C, Richardson G. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. The Journal of Neuroscience. 2001;21(18):7013.

131. Taura A, Kikkawa YS, Nakagawa T, Ito J. Hydrogen protects vestibular hair cells from free radicals. Acta oto-laryngologica. 2010;130(S563):95-100.

132. Kadner A, Hoerstrup SP, Tracy J, Breymann C, Maurus CF, Melnitchouk S, et al. Human umbilical cord cells: a new cell source for cardiovascular tissue engineering. The Annals of thoracic surgery. 2002;74(4):1422-8.

133. Rivolta MN, Holley MC. Cell lines in inner ear research. Journal of neurobiology. 2002;53(2):306-18.

134. Martin F, Clark J. Introduction to Audiology (with CD-ROM): Allyn & Bacon, Inc. Needham Heights, MA, USA; 2005.

135. Newby H, Popelka G. Audiology, Englewood Cliffs. NJ: Prentice-Hall; 1992.

136. Sataloff J. SERIES TITLE: YEAR: 1966 PUB TYPE: Book. 1966.

137. Willott JF. Aging and the auditory system: Anatomy, physiology and psychophysics: Whurr Pub Ltd; 1991.

138. Kiernan AE, Pelling AL, Leung KKH, Tang ASP, Bell DM, Tease C, et al. Sox2 is required for sensory organ development in the mammalian inner ear. Nature. 2005;434(7036):1031-5.

139. Oesterle EC, Campbell S, Taylor RR, Forge A, Hume CR. Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. JARO-Journal of the Association for Research in Otolaryngology. 2008;9(1):65-89.

140. Kiernan AE, Cordes R, Kopan R, Gossler A, Gridley T. The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. Development. 2005;132(19):4353.

141. Kiernan AE, Xu J, Gridley T. The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. PLoS genetics. 2006;2(1):e4.

142. Sage C, Huang M, Vollrath MA, Brown MC, Hinds PW, Corey DP, et al., editors. Essential role of retinoblastoma protein in mammalian hair cell development and hearing2006: National Acad Sciences.

143. Sage C, Huang M, Karimi K, Gutierrez G, Vollrath MA, Zhang DS, et al. Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. Science. 2005;307(5712):1114.

144. Bodson M, Breuskin I, Lefebvre P, Malgrange B. Hair cell progenitors: identification and regulatory genes. Acta oto-laryngologica. 2010;130(3):312-7.

145. Chen P, Segil N. p27 (Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. Development. 1999;126(8):1581.

146. Chen P, Zindy F, Abdala C, Liu F, Li X, Roussel MF, et al. Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. Nature cell biology. 2003;5(5):422-6.

147. Löwenheim H, Furness DN, Kil J, Zinn C, Gültig K, Fero ML, et al. Gene disruption of p27Kip1 allows cell proliferation in the postnatal and adult organ of Corti. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(7):4084.

148. Holt J, Johns D, Wang S, Chen Z, Dunn R, Marban E, et al. Functional expression of exogenous proteins in mammalian sensory hair cells infected with adenoviral vectors. Journal of neurophysiology. 1999;81(4):1881.

149. Weiss MA, Frisancho JC, Roessler BJ, Raphael Y. Viral-mediated gene transfer in the cochlea. International journal of developmental neuroscience. 1997;15(4-5):577-83.

150. Peter AELCR, Poulsenb GVDDJ. Adenoviral and AAV-Mediated Gene Transfer to the Inner Ear: Role of Serotype, Promoter, and Viral Load on In vivo and In vitro Infection Efficiencies. Gene therapy of cochlear deafness: present concepts and future aspects. 2009;66:87-98.

151. Li Duan M, Bordet T, Mezzina M, Kahn A, Ulfendahl M. Adenoviral and adeno-associated viral vector mediated gene transfer in the guinea pig cochlea. Neuroreport. 2002;13(10):1295.

152. Lalwani A, Walsh B, Reilly P, Muzyczka N, Mhatre A. Development of in vivo gene therapy for hearing disorders: introduction of adeno-associated virus into the cochlea of the guinea pig. Gene therapy. 1996;3(7):588.

153. Kho ST, Pettis RM, Mhatre AN, Lalwani AK. Safety of adeno-associated virus as cochlear gene transfer vector: analysis of distant spread beyond injected cochleae. Molecular Therapy. 2000;2(4):368-73.

154. Glorioso J, DeLuca N, Fink D. Development and application of herpes simplex virus vectors for human gene therapy. Annual Reviews in Microbiology. 1995;49(1):675-710.

APPENDIX A: FIGURES

- CHAPTER 1: No figures.
- CHAPTER 2: Figures 2.1 - 2.3
- CHAPTER 3: Figures 3.1 - 3.2
- CHAPTER 4: Figures 4.1 - 4.3
- CHAPTER 5: Figures 5.1 - 5.6
- CHAPTER 6: No figures.

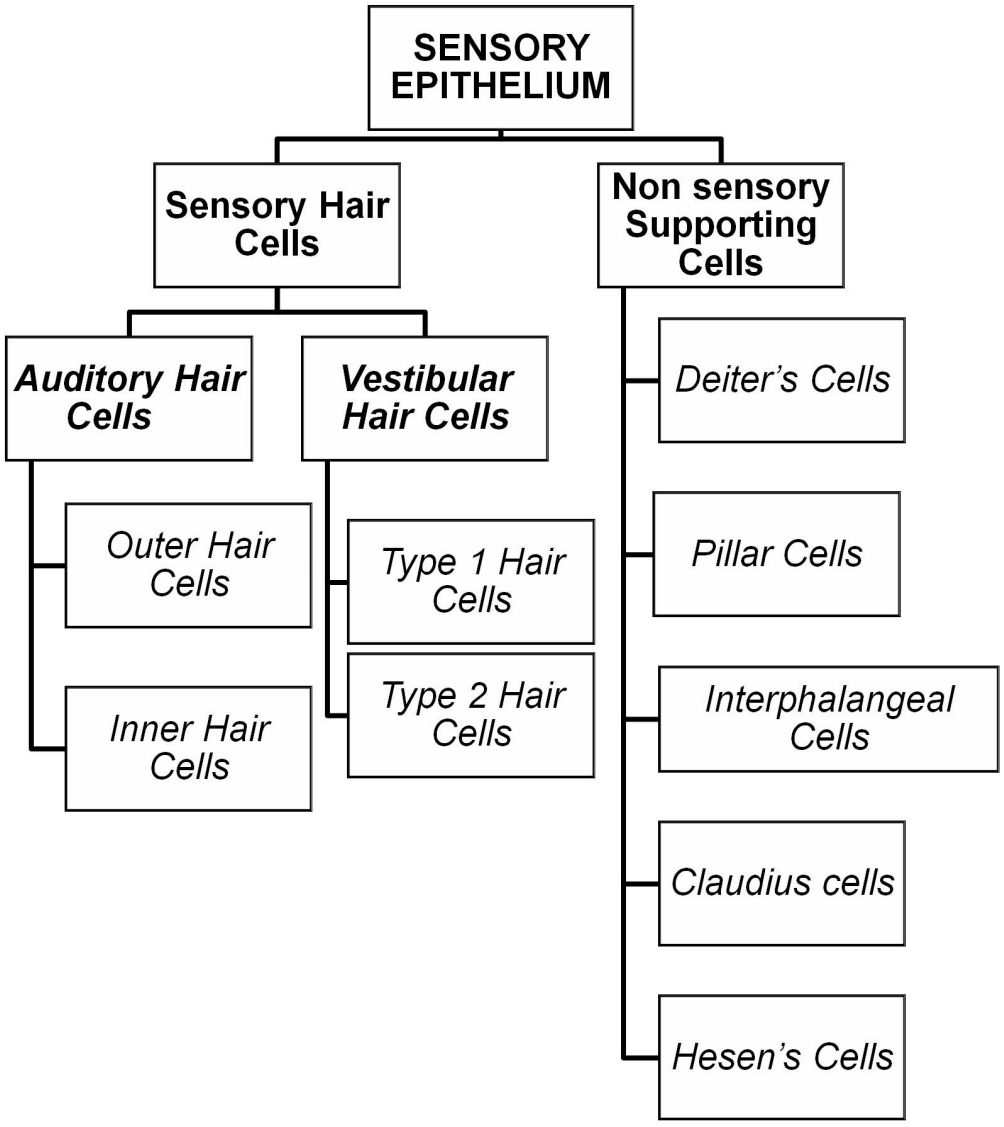

Figure 2.1: Classification of inner ear sensory epithelium.

Sensory epithelium in the inner ear is composed of both sensory and non sensory cell types that have morphological and physiological differences.

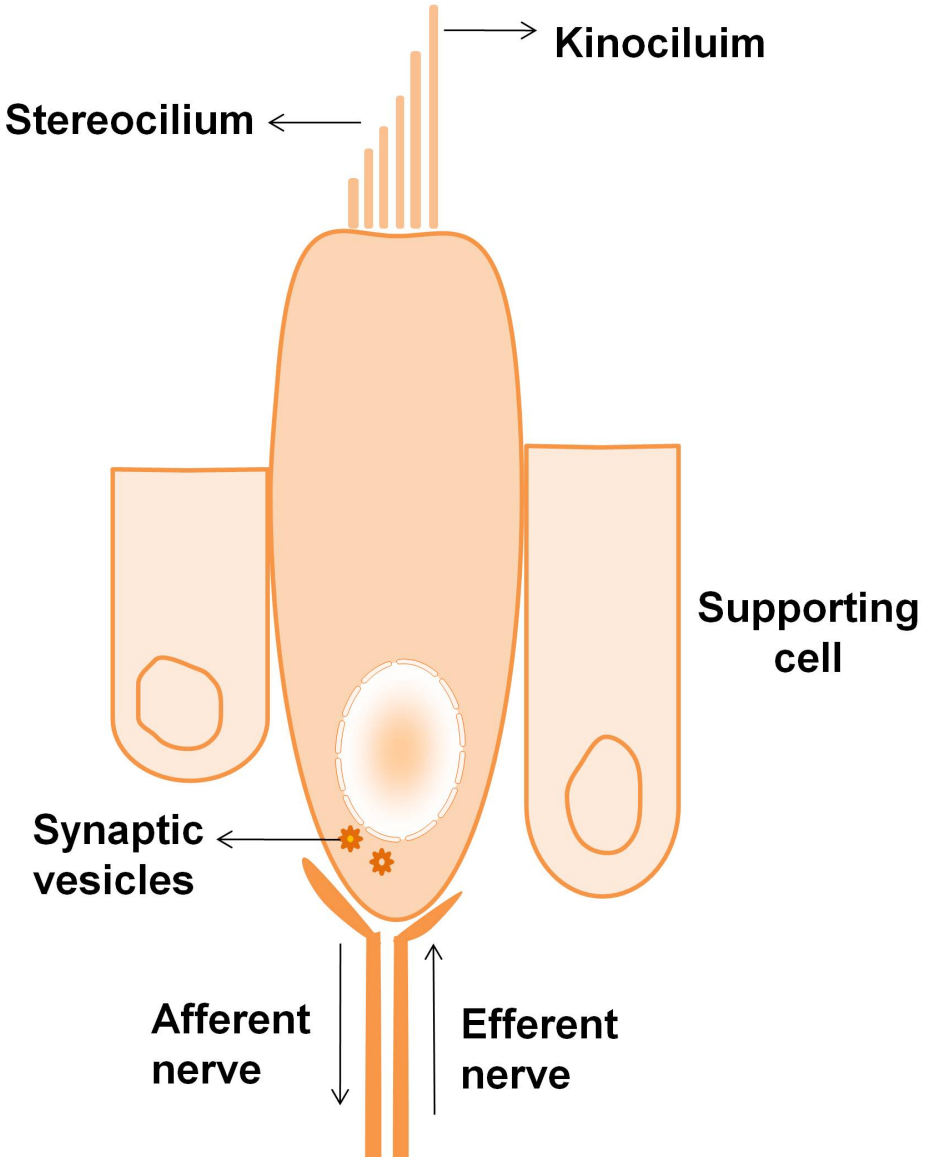

Figure 2.2: Structure of a typical hair cell.

Schematic representation of a hair cell, showing the flask-shaped hair cell supported by supporting cells at the base. The stereocilia at the top are connected by filamentous tip links that participate in transduction and ion exchange.

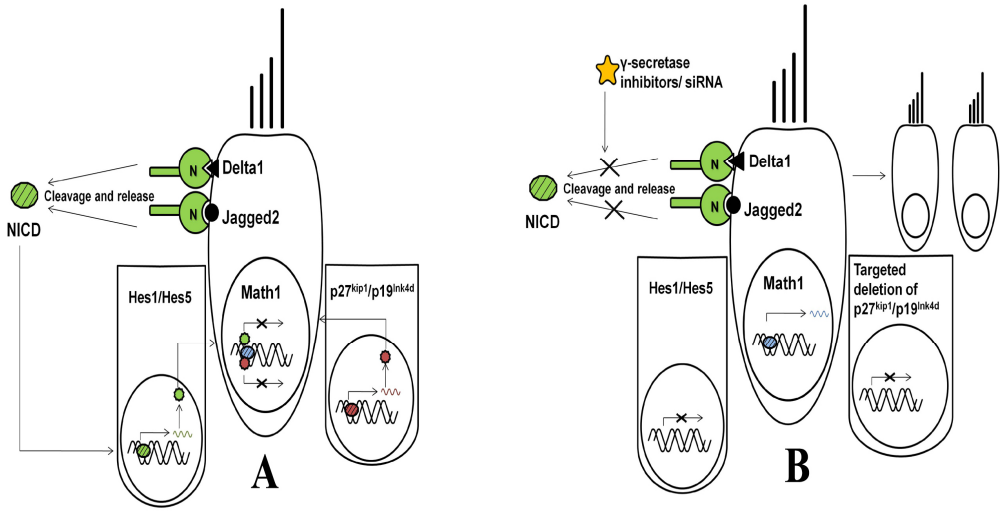

Figure 2.3: Schematic on the interaction of different genes and their contribution to positive and negative regulation of *Math1* transcription factor.

(A) Hair cells express hair cell-specific *Math1* transcription factor and notch ligands – Delta1 and Jagged2. Notch receptor (N) binds to the ligands, cleaves with the help of γ-secretase and releases Notch Intracellular Domain (NICD). NICD enter the nucleus of supporting cells and activated *Hes1/Hes5* transcription factors. *Hes1/Hes5* proteins inhibit *Math1* gene expression. Alternatively, expression of $p27^{kip1}$ and $p19^{Ink4d}$ in early progenitor supporting cells repress *Math1* expression and maintain supporting cell fate. (B) In the presence of γ-secretase inhibitors and targeted deletion of $p27^{kip1}$ and $p19^{Ink4d}$ genes, ectopic expression of *Math1* occurs producing supernumerary hair cells.

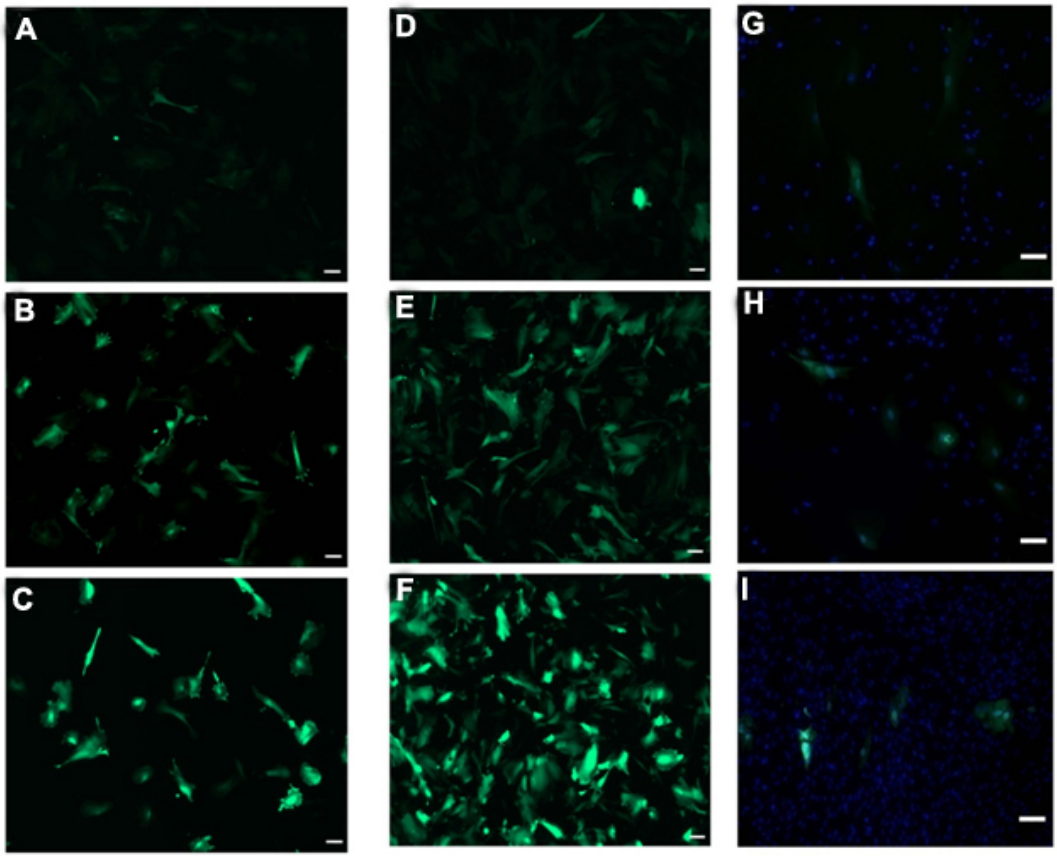

Figure 3.1: Adenoviral mediated expression of GFP in hUCMSCs transduced with Ad28.eGFP vector at 3, 5 and 10 days post transduction.

The figure shows the effect of MOI on the expression level of introduced GFP. Vertical panels (A-C), (D-F) and (G-I) represent transient gene expression at days 3, 5 and 10 respectively. Horizontal panels (A, D, G), (B, E, H) and (C, F, I) represent the effect of MOIs 10, 50 and 100 respectively on the expression level of adenovirally introduced GFP. Note that maximum gene expression was at day 5 and at MOI 100. The figures represent a random microscopic field for one cord. The results were consistent among cords. Scale bar = 100 µm.

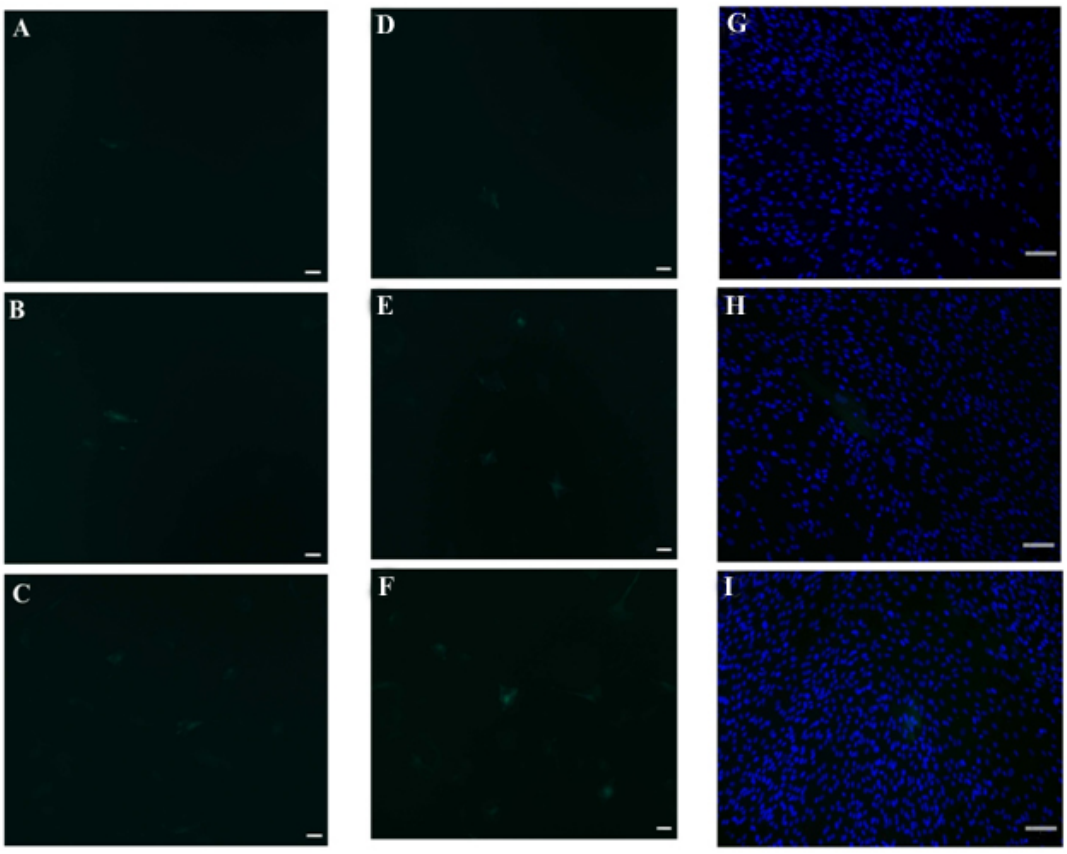

Figure 3.2: Adenoviral mediated expression of GFP in hUCMSCs transduced with Adf11D.eGFP vector at 3, 5 and 10 days post transduction.

The figure shows the effect of MOI on the expression level of introduced GFP. Vertical panels (A-C), (D-F) and (G-I) represent transient gene expression at days 3, 5 and 10 respectively. Horizontal panels (A, D, G), (B, E, H) and (C, F, I) represent the effect of MOIs 10, 50 and 100 respectively on the expression level of adenovirally introduced GFP. Note that maximum gene expression was at day 5 and at MOI 100. The figures represent a random microscopic field for one cord. The results were consistent among cords. Scale bar = 100 µm.

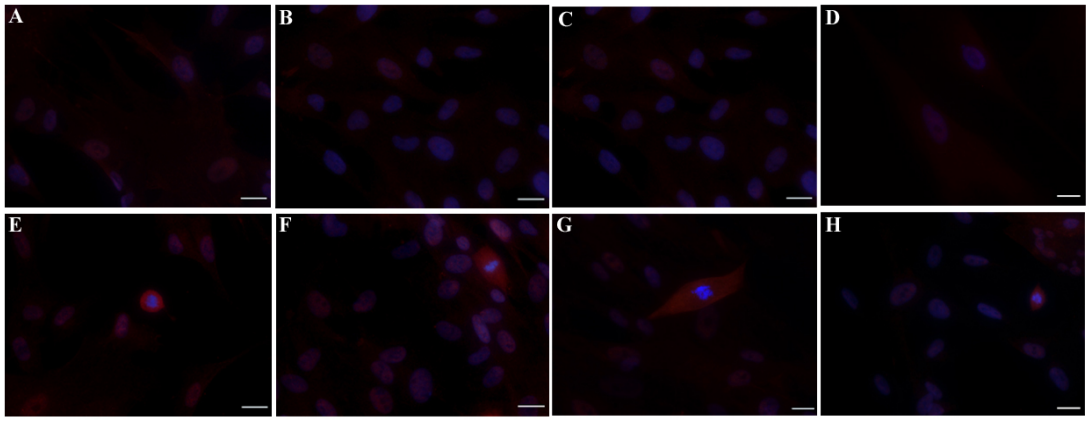

Figure 4.1: Characterization of *Math1* gene expression by immunostaining with myosin VIIa in hUCMSCs transduced with the AdMath1.11D vector

(A-D) controls from each cord that did not have any vector treatment. (E-H) myosin VIIa positive cells from each cord. Vertical panels represent a randomly selected microscopic field for each cord. Cells were infected at MOI 100 and immunostained 10 days post transduction. The position of nuclei is represented by DAPI staining (blue). Immunostaining indicated myosin VIIa positive cells (red) and hair cell-like cells. Scale bar = 20 µm.

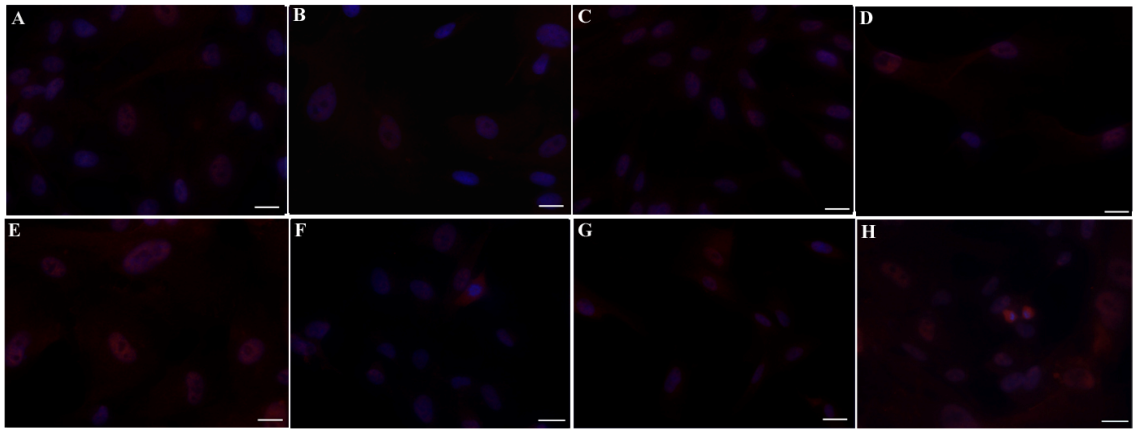

Figure 4.2: Characterization of *Math1* gene expression by immunostaining with myosin VIIa in hUCMSCs transduced with Ad28.GFAP.Atoh1 vector.

(A-D) represent controls from each cord that did not have any vector treatment. (E-H) represent myosin VIIa positive cells from each cord. Vertical panels represent a randomly selected microscopic field for each cord. Cells were infected at MOI 100 and immunostained 10 days post transduction. The position of nuclei is represented by DAPI staining (blue). Immunostaining demonstrated low levels of myosin VIIa expression (red). Scale bar = 20 µm.

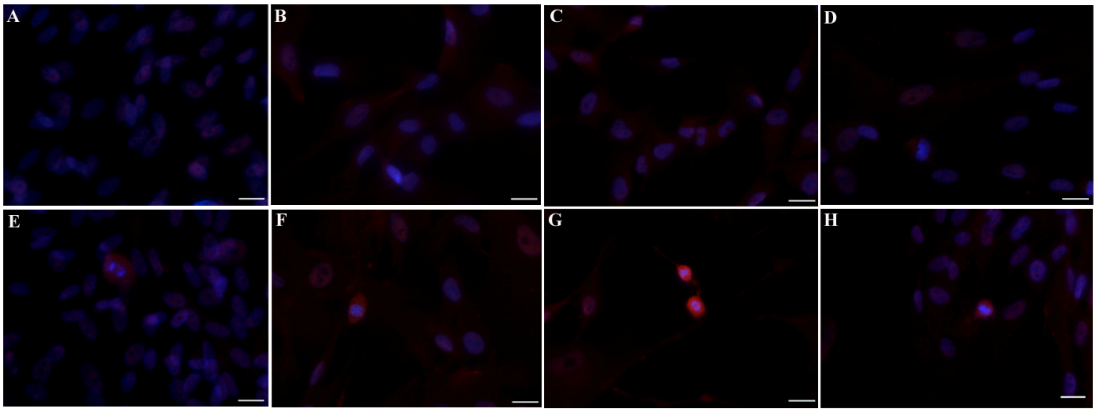

Figure 4.3: Characterization of *Math1* gene expression by immunostaining with myosin VIIa in hUCMSCs transduced with Ad5.GFAP.Math1 vector.

(A-D) represent controls from each cord that did not have any vector treatment. (E-H) represent myosin VIIa positive cells from each cord. Vertical panels represent a randomly selected microscopic field for each cord. Cells were infected at MOI 100 and immunostained 10 days post transduction. The position of nuclei is represented by DAPI staining (blue). Immunostaining demonstrated low levels of myosin VIIa expression (red). Scale bar = 20 µm.

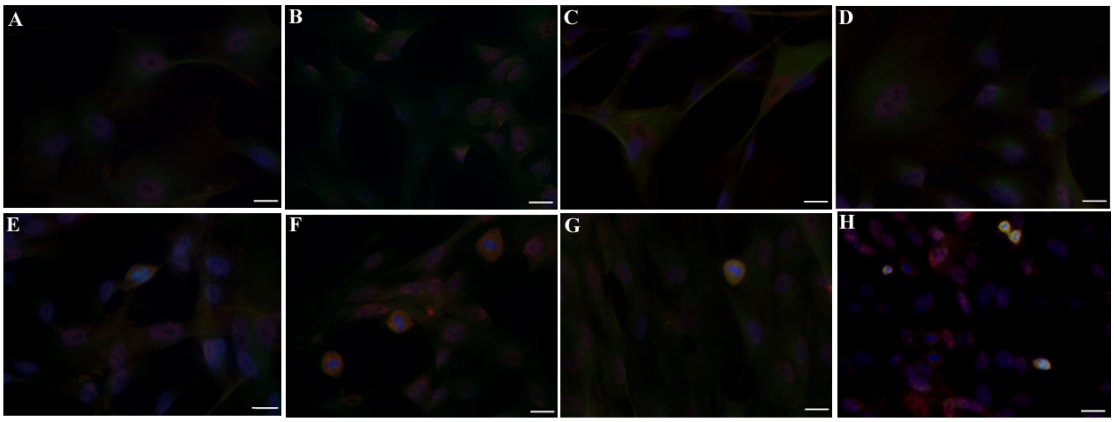

Figure 5.1: Characterization of hair cell-like cells in hUCMSC culture transduced with AdMath1.11D vector using specific hair cell and glial cell markers.

(A-D) controls from each cord that did not have any vector treatment. (E-H) myosin VIIa and GFAP positive cells from each cord. Vertical panels represent a randomly selected microscopic field for each cord. Cells were infected at MOI 100 and immunostained 10 days post transduction. The position of nuclei is represented by DAPI staining (blue). Immunostaining demonstrated myosin VIIa and GFAP positive pear-shaped cells with an extended protrusion at one end, similar in appearance to inner ear hair cells. Yellow color indicates expression of myosin VIIa and GFAP in differentiated cells. Scale bar = 20 µm.

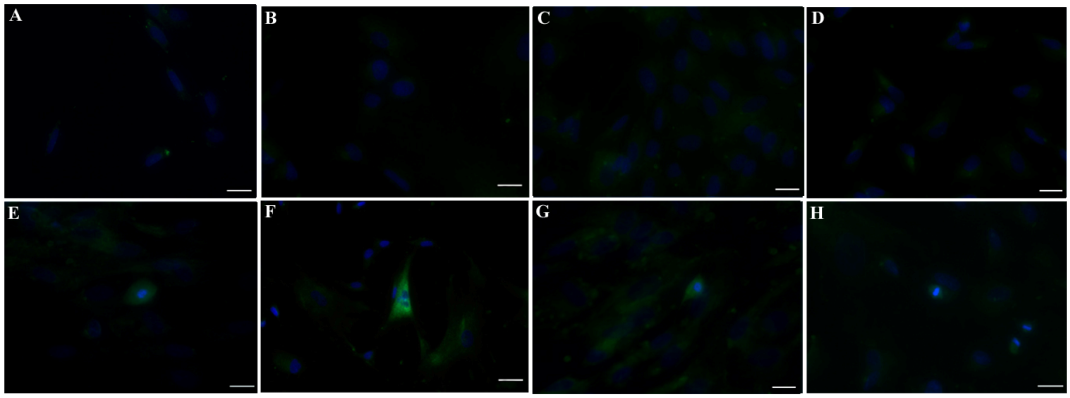

Figure 5.2: Characterization of hair cell-like cells in hUCMSC culture transduced with AdMath1.11D vector using specific hair cell marker – FM1-43.

(A-D) controls from each cord that did not have any vector treatment. (E-H) FM1-43 positive cells from each cord. Vertical panels represent a randomly selected microscopic field for each cord. Cells were infected at MOI 100 and immunostained 10 days post transduction. The position of nuclei is represented by DAPI staining (blue). FM1-43 labels stereocilia transduction channels in hair cells (green) indicating transduction activity in differentiated hair cell-like cells. Scale bar = 20 µm.

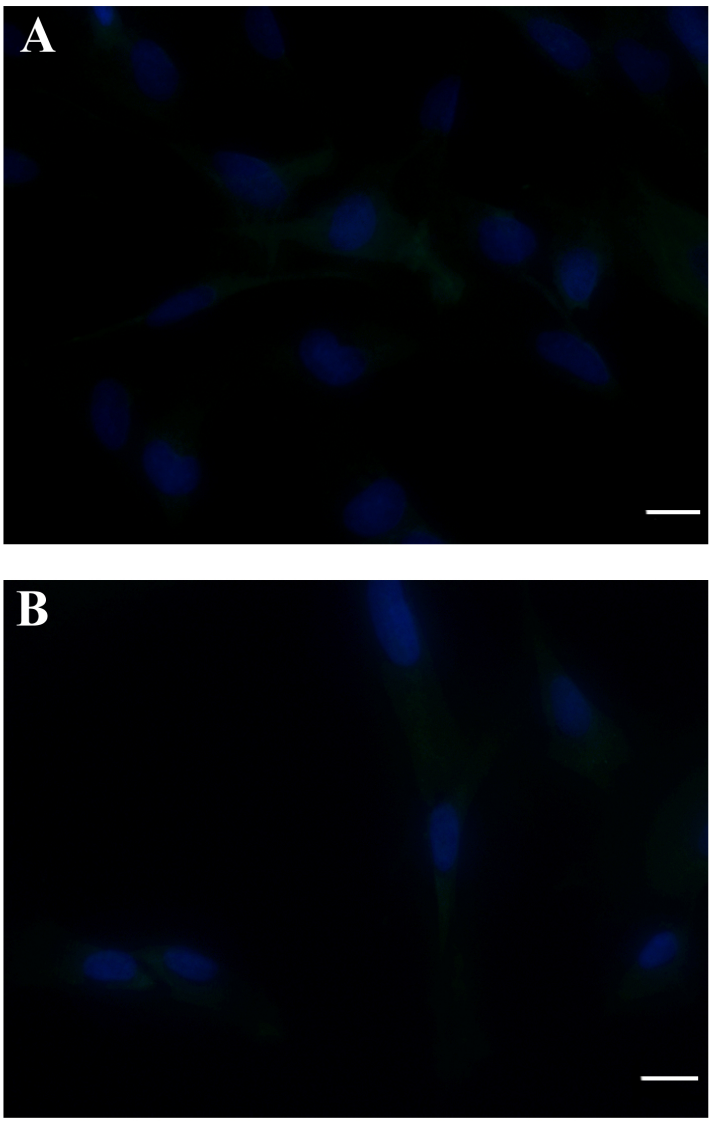

Figure 5.3: Characterization of neuronal expression by immunostaining with neurofilament in hUCMSCs transduced with AdMath1.11D vector.

(A) control that did not have any vector treatment. (B) transduced cells that did not show neurofilament positive cells, indicating an absence of neural differentiation in *Math1* transduced cells. Cells were infected at MOI 100 and immunostained 10 days post transduction. The position of nuclei is represented by DAPI staining (blue). The figures represent a random microscopic field for one cord. The results were consistent among cords. Scale bar = 20 µm.

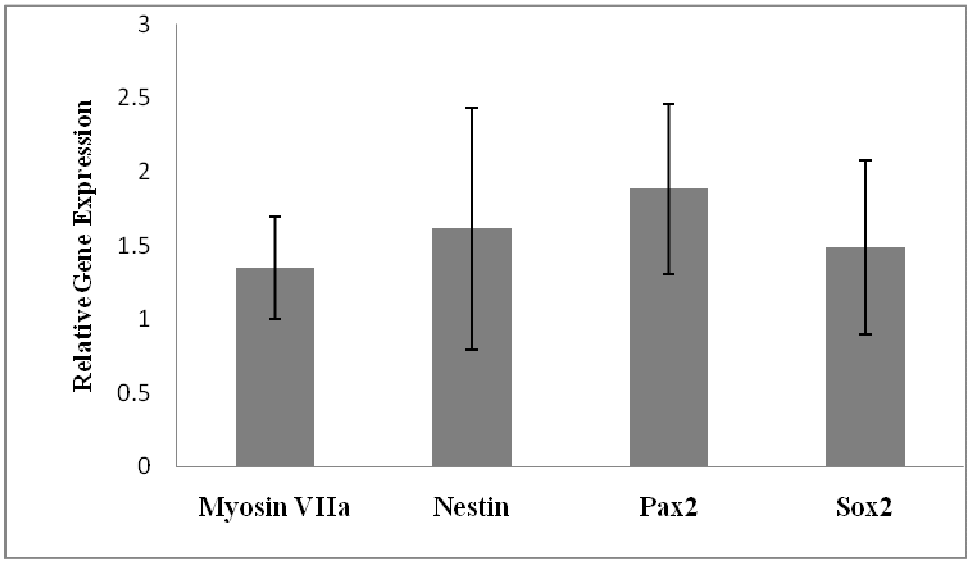

Figure 5.4: Gene expression analysis of myosin VIIa, nestin, GFAP, Pax2 and Sox2

All relative gene expression values for each target gene at day 10 post transduction were normalized to controls and GAPDH. Values are reported as mean ± standard deviation, n = 3. A low level of myosin expression was reported in transduced cells. There were no statistically significant differences in the gene expression levels of myosin VIIa, nestin, Pax2 and Sox2 between control and transduced group.

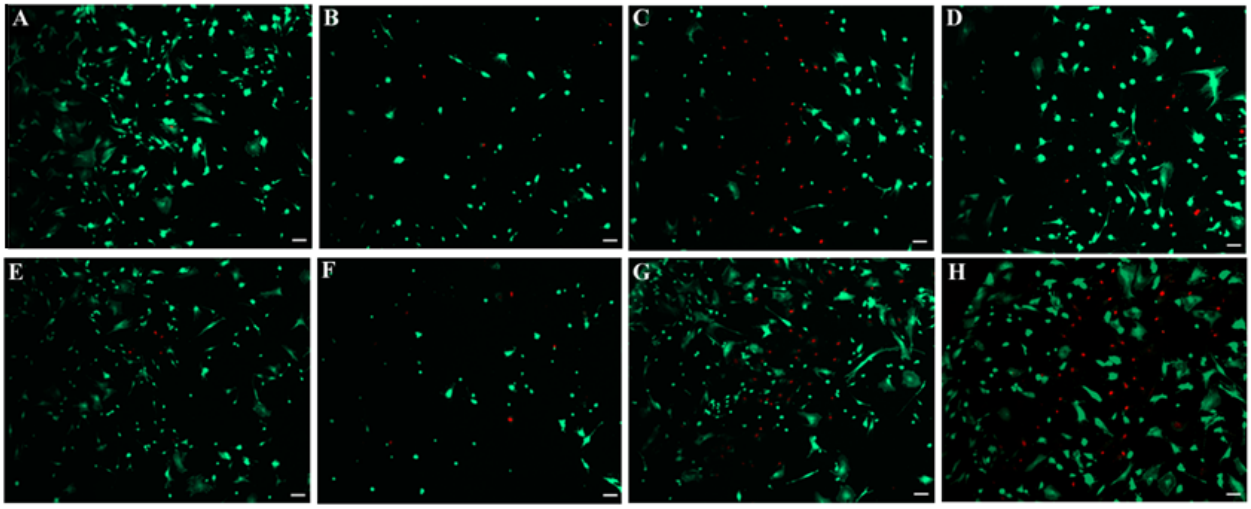

Figure 5.5: LIVE/DEAD images for AdMath1.11D transduced hUCMSCs.

(A-D) represent controls from each cord that did not have any vector treatment. (E-H) represent hUCMSCs from each cord transduced with AdMath1.11D vector at MOI 100 at day 3 post transduction. Live cells are stained green, while dead cells are stained red. The results demonstrated high cell viability in transduced cells indicating low cytotoxic effects of AdMath1.11D. Scale bar = 100 µm.

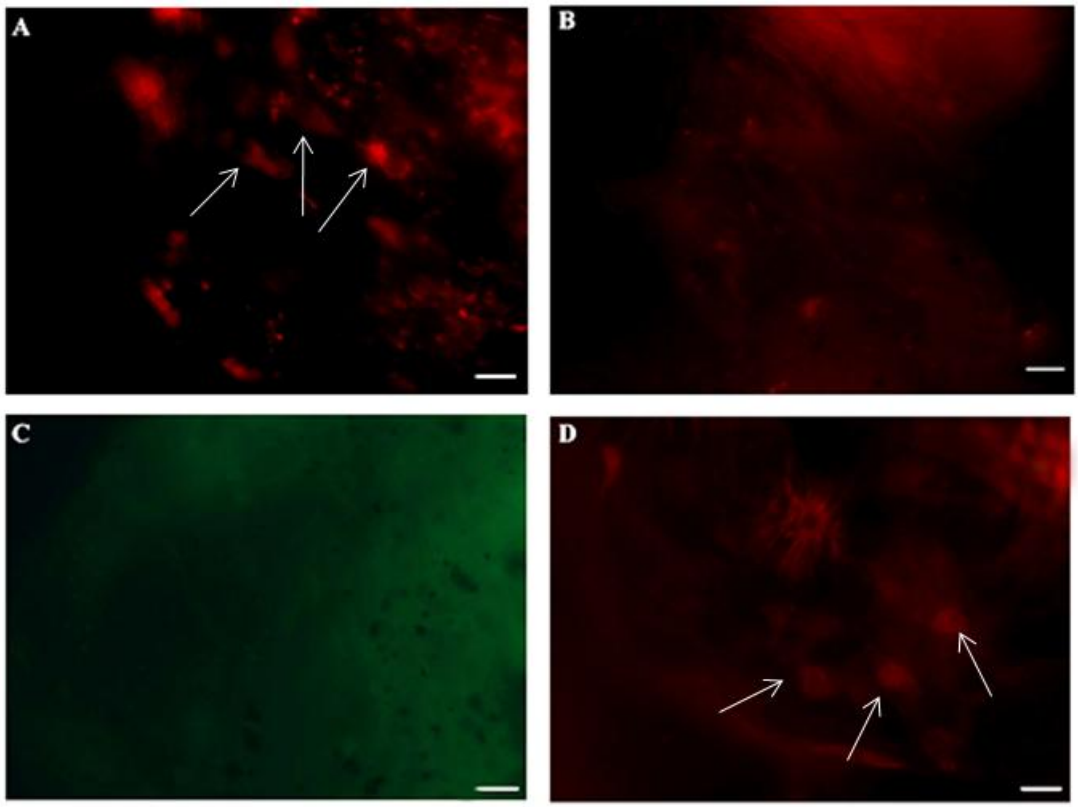

Figure 5.6: Co-culture of transduced hUCMSCs and macular organs.

(A), (B) Negative controls without and with neomycin treatment respectively. Both the negative controls were not treated with cells. (C) Positive control that was treated with neomycin and GFP expressing hUCMSCs. (D) Experimental group that was treated with neomycin and differentiated hUCMSCs. Cultures were stained for myosin VIIa at day 10. The results demonstrated myosin VIIa positive cells (red) in experimental group. Arrows point to myosin VIIa positive cells. All figures represent one explant from each group. Scale bar = 20 µm.

APPENDIX B: TABLES

- CHAPTER 1: No tables
- CHAPTER 2: Table 2.1 – 2.3
- CHAPTER 3: No tables
- CHAPTER 4: No tables
- CHAPTER 5: Table 5.1
- CHAPTER 6: No tables

Table 2.1: Differences between inner and outer hair cells

FACTOR	INNER HAIR CELLS	OUTER HAIR CELLS	Ref.
Arrangement	Arranged in a single row	Arranged in three parallel rows	(134-136)
Shape	Round and small	Long and slim	(10)
Function	Transduce mechanical energy to neural signals	Appear to impact the sensitivity of the cochlea	(10)
Effects	Sensory Neural Hearing loss	Alter properties of cochlear input to the brain	(137)
Approximate number	3,000 to 3,500	9,000 to 12,000	(135, 136)

Table 2.2: Summary of different genes used in inner ear gene therapy

Gene	Role	Reference
Math1	Also known as *Atoh1*. Primary gene responsible for hair cell differentiation. Other homologues include *Hath1*, *Cath1* and *Xath1*.	(33, 35, 39-41)
Hes1 and *Hes5*	Mammalian homologues of Hairy and Enhancer-of-split gene. Expressed in supporting cells and known to be negative regulators of *Math1*. However, a balance between *Hes1/Hes5* is required to control the production of supernumerary hair cells and normal development of inner ear.	(42, 43)
Sox2	Responsible for development of inner ear sensory epithelium and is expressed in supporting cells and inner ear progenitors. Acts upstream of *Math1* and maintains mitotic and transdifferentiation functions of supporting cells.	(138-140)
Jagged2	Member of the notch signaling pathway. Expressed in supporting cells of auditory and vestibular system. Required for the normal development of inner ear sensory organs.	(17, 140, 141)
BETA2/NeuroD	Expressed in neurons and neural precursor cells. Promotes the formation of ganglion neurons in the cochlea. Absence of *BETA2/NeuroD* can compromise hair cell function.	(44)

pRb	Required for cell-cycle exit of embryonic mammalian hair cells but not for their early differentiation. Deletion of pRb from progenitor cells leads to proliferation of hair cells and supporting cells.	(142-144)
$p27^{kip1}$ and $p19^{Ink4d}$	Cyclin-dependent kinase inhibitor. Expressed in sensory progenitors during the early embryonic development of the cochlea. Regulates cell cycle and inhibits hair cell differentiation.	(145-147)

Table 2.3: Summary of different viral vectors used in inner ear gene therapy

Vector	Advantages	Disadvantages	Ref.
Adeno virus	1) Transfect a wide variety of cell types in the inner ear including spiral ganglions, outer hair cells, spiral ligament, stria vascularis, and mesenchymal cells in both auditory and vestibular systems. 2) Can produce high titer values that allow injection of small dose volumes for gene therapy. 3) Transgene expression up to 3 weeks can be achieved. The short duration of gene expression is ideal for hair cell regeneration because prolonged *Math1* expression can produce too many ectopic hair cells and compromise hearing. 4) Effective in delivering *Math1* gene to regenerate hair cells and Bcl-2 to protect hair cells from damage. 5) Allows insertion of large DNA segments. Recombinant forms can take up to 30kb foreign DNA. 6) Infects dividing and non-dividing cells with very high transduction efficiencies, both *in vitro* and *in vivo*. 7) Widely researched in clinical studies in both animals and humans, giving a better ability to tackle clinical complications that can arise.	1) Evokes a strong host immune response. 2) Does not offer long term gene expression. 3) Entry of the viral vectors is largely dependent on a host receptor called the coxsackie virus receptor (CAR).	(35, 37, 54-56, 148, 149)

Adeno-associated Virus	1) Effectively transfect most inner ear cell types *in vivo*. Studies show transgene expression in cochlear blood vessels, nerve fibers and spiral limbus cells. 2) Effective in targeting stria vascularis and delivering tropic factors like NT-3, BDNF, VEGF and FGF. 3) Transgene expression can occur up to 24 weeks *in vivo*. 4) Non toxic to inner ear cells and evokes low immune response. 5) Lacks pathogenicity and has never been associated with any known human disease making them suitable for clinical applications.	1) Only effective with *in vivo* inner ear gene therapy. 2) Successful transgene expression *in vivo* depends on route of vector administration, limited to only direct injection of vector. 3) Previous studies have shown possible dissemination of vector from target tissue. 4) Offers only a limited payload capacity owing to its small size. 5) Risk of insertional mutagenesis. 6) No substantial clinical experience. 7) Vector entry in to host largely depends on heparin sulfate receptor.	(48, 150-153)
Herpes Simplex Virus	1) Effectively known to target non dividing cells, specific to nerve cells, spiral ganglion, vestibular ganglion and mesenchymal cells in mice and guinea pigs. 2) Newer recombinant vectors offer stable and long term gene expression of up to 8 weeks. 3) Can take large DNA fragments.	1) Evokes a strong immune response. 2) Transfection is limited only to non dividing neuronal cells. 3) Large size of the virus makes it difficult to	(56, 57, 154)

		manipulate. 4) The virus does not integrate into the host genome; hence gene expression can be unstable. 5) Difficult to produce high titer values and requires injection of high vector volumes. 6) No substantial clinical experience.	
Lenti virus	1) Transfect both dividing and non-dividing cells, including stem cells that are very difficult to transfect. 2) Effectively transduce spiral ganglion neurons and supporting cell *in vitro*. 3) Studies indicate transgene expression in perilymphatic space for up to 2 weeks.	1) Limited to use in production of genes only in the perilymph. 2) Limited dissemination of vector and not suitable for sensory cell transduction. 3) Failure to transduce cells *in vivo*. 4) Can randomly integrate into host chromosome and capable of generating a replication competent virus. 5) No clinical experience and safety concern arise from human immune deficiency virus origin.	(58, 107)

Table 5.1: Primer pairs and cDNA product length

Marker	Forward primer sequence 5' – 3'	Reverse primer sequence 5' – 3'	cDNA length
Myosin VIIa	GAGAGAGGGAGAGACAAG	CTCTTCCTCTGTCTGGCT	100 bp
Nestin	GAAGATACGGTGGAGAAG	CTTCCCAGTGAAGCCATC	120 bp
GFAP	AACTCCAACTAACAAGAA	CATAACAACAGGAATCAG	151 bp
Pax2	CTACACGCCCATTAAAGC	TACAGAGAAGCCAACA	152 bp
Sox2	ATGGTTGTCTATTAACTTGT	TCTCTCCTCTTCTTTCTC	128 bp
GAPDH	TAACTCTGGTAAAGTGAAT	ACTTGATTTAGGGAT	191 bp