Mouse Embryonic Stem Cells Differentiated into Neuron-like cells or Schwann Cell-like Cells for the Development of Strategies to Ameliorate Deafness

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
(Biomedical Engineering)
in The University of Michigan
2012

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To my husband Ram, my daughters Surabi and Sahana and my parents who encouraged me in pursuing my dream
ACKNOWLEDGEMENTS

First of all, I would like to thank my mentor, Kate Barald, for all of the guidance she provided me during all these years in her laboratory. She greatly supported me in developing my skills and endurance in performing research, science problem solving and writing scientific papers. She also supported me in the ups and downs in my personal life. Her mentorship has been invaluable to me both personally and scientifically.

Thanks to my committee members for their valuable input into my dissertation research. Professors Karl Grosh, Jan Stegeman, El-Sayed Mohamed, and Shuichi Takayama also deserve thanks for all their helpful comments and suggestions on the thesis itself.

Thanks so much to all of the Barald lab members, both past and present, for their friendship and assistance. Special thanks to Beth Smiley for teaching me PCR skills, Therese Roth for showing me the techniques involved in ES cell culture and for being my chief collaborator for many years, Fumi Ebisu for teaching me the necessary skills to isolate statoacoustic ganglia and spiral ganglia and organ of Corti explants and ways to culture them and Andy Chervenak for helping me stage mouse pups and obtain embryos at appropriate stages. I also specially thank Dr. Joong Park who helped me with the design and use of the microfluidic device, which he developed and Josh White for making microfluidic devices whenever I needed them and helping me resolve device setup problems. I thank Chris Edward for helping me to do the live cell imaging in the Department of Cell and Developmental Biology’s Microscopy and Imaging Laboratory. I would like to thank Bobak Mosadegh for many hours of scientific discussion and for teaching me so much.

Thanks also to my professorial collaborators, Dr. Shuichi Takayama for supporting and guiding the microfluidic research for these studies. I also specially thank Dr. Rich Hume for helping us conduct electrophysiological studies on ES-
derived differentiated neuron like cells and teaching me electrophysiological techniques and Dr. Bryan Pfingst for providing us with the cochlear implant electrodes that were used in this research.

I would like to thank the NIH-sponsored MBSTP and TEAM for supporting me for three years of T32 support (2 from MBSTP and 1 from TEAM) and I would like to thank the administrative staffs who are involved in both programs, particularly Lorie Kochanek (MBSTP) and Pat Schultz (TEAM).

Thanks to Maria Steele for coordinating and guiding me through the maze of fulfilling all the requirements to successfully complete the BME curriculum and defend this thesis.

I would also like to express my gratitude to everyone who helped me pursue my dream that I have not personally named. I have been touched and impressed by so many people, and they have all enriched my life in ways too numerous to mention.
TABLE OF CONTENTS

DEDICATION ........................................................................................................................................... ii

ACKNOWLEDGEMENTS ................................................................................................................... iii

LIST OF FIGURES .................................................................................................................................... viii

LIST OF TABLES ...................................................................................................................................... x

LIST OF ABBREVIATIONS .................................................................................................................. xi

ABSTRACT ................................................................................................................................................ xiii

CHAPTER I ................................................................................................................................................. 1

INTRODUCTION

1.1 Hearing loss ........................................................................................................................................ 1

Fate of SGN in hearing loss .......................................................................................................................... 4

Influence of Trophic, Neurotransmitter and Electrical Activity on SGN survival ....................... 5

MIF acts as a neurotrophin at the earliest stages of inner ear development ......................... 8

Role of DHA in neuronal maturation: neurite outgrowth, fasciculation, synaptogenesis .......................... 11

1.2 Different therapy strategies in promoting SGN survival, regeneration and regrowth. 12

Promoting SGN survival .......................................................................................................................... 14

Replacing the mature SGN – Cell based therapy: .............................................................................. 17

Transplantation of neurons from other ganglia into the cochlea ..................................................... 18

Possible use of endogenous sources of stem cells ........................................................................... 20
Induced stem cells derived from exogenous sources: ES cell, Neural stem cells and Immortalized inner ear cell lines .........................................................20
Regrowth of remaining SGN peripheral processes...........................................22

1.3 Combinational therapy - SGN survival and regrowth of SGN to improve .........22
Cellular interface with Cochlear electrode ..........................................................23

1.4 Microfluidic technology in Cell-Cell interaction study ...................................24

1.5 Our Research ..................................................................................................27
1.6 References .....................................................................................................32

CHAPTER II ........................................................................................................42
DIFFERENTIATION OF A COMMON POPULATION OF MOUSE EMBRYONIC
STEM CELLS INTO SPIRAL GANGLION NEURON-LIKE CELLS AND SCHWANN
CELL-LIKE CELLS IN A MICROFLUIDIC DEVICE: STUDIES OF CELL-CELL
INTERACTIONS THAT MIGHT AID APPROACHES TO REGENERATIVE
MEDICINE

2.1 ABSTRACT ....................................................................................................42
2.2 INTRODUCTION .............................................................................................43
2.3 MATERIALS AND METHODS ........................................................................45
2.4 RESULTS .........................................................................................................49
2.5 CONCLUSION .................................................................................................60
2.6 REFERENCES ..................................................................................................68
CHAPTER III ..........................................................................................................................71

DEVELOPMENT OF TWO STEM CELL-BASED THERAPIES TO AMELIORATE
HEARING IMPAIRMENT OR POTENTIATION OF A COCHLEAR IMPLANT

3.1 ABSTRACT .........................................................................................................................71

3.2 INTRODUCTION ..............................................................................................................72

3.3 MATERIALS AND METHODS .........................................................................................76

3.4 RESULTS .........................................................................................................................80

3.5 CONCLUSION ..................................................................................................................87

3.6 REFERENCES ..................................................................................................................92

CHAPTER IV

CONCLUSION .........................................................................................................................108
LIST OF FIGURES

CHAPTER I

Figure 1.1: Section through the cochlea of the mammalian (human) inner ear ...............2
Figure 1.2: Tonotopic map ................................................. 3
Figure 1.3: Healthy Organ of Corti ........................................ 5
Figure 1.4: Sources of trophic support for spiral ganglion neurons ....................... 7
Figure 1.5: “Cartoon from Chapter 3: A cochlear implant wire (in grey above left) has been “coated” with a hydrogel encapsulating MIF-producing mESC-derived SC-like cells (seen as nucleated individual cells in this cartoon): ........................................ 10
Figure 1.6: Cochlear prostheses (electrode in left figure) implanted in the Scala tympani of the inner ear with no or few surviving hair cells and very few SGNs. ....................... 14
Figure 1.7: Cartoon of a Cochlear implant coated with neurotrophin producing Schwann Cells to attract SGN and restore SGN function ........................................ 24

CHAPTER II

Figure 2.1: The mES cells differentiated into SGN-like cells and Schwann cells in the microfluidic device ................................................................. 50
Figure 2.2: Observations of SGN-like cells and Schwann Cell-like cell interactions ...... 52
Figure 2.3: Neurite measurements of the SGN-like cells differentiated in the microfluidic device. ................................................................. 53
Figure 2.4: Neurite measurements using MetaMorph® software on the differentiated SGN-like cells. ................................................................. 55
Figure 2.5: RTqPCR shows OCT4 expression in undifferentiated mES cells and SGN-like neurons differentiated from mES cells ........................................ 57
Supplemental Figure 2.1: mES cell neuronal differentiation conditions with and without DHA

Supplemental Figure 2.2: mES cells differentiated using different growth factors (NGF, CNTF) and the Cytokine (MIF) with and without DHA

Supplemental Figure 2.3: Control experiment - neuronal differentiation media in both the inlet.

Supplemental Figure 2.4: The close up view of the directional outgrowth in the channel

CHAPTER III

Figure 3.1: Ion channel protein expression

Figure 3.2: Patch clamp studies performed on the neuron-like cells

Figure 3.3: CI coated hydrogels with encapsulated SCC-like cells

Figure 3.4: Neuron-like cells derived from mES cells exposed to MIF as a differentiation factor and DHA as a maturation factor

Figure 3.5: Migration of embryonic mouse (Eday12.5) SAG neurites after 2 weeks in culture.

Figure 3.6: SGN isolated from 6 day BalbC mouse pups interacting with variously prepared CI
LIST OF TABLES

CHAPTER II

Table 2.1: Differentiation conditions .................................................................65
Table 2.2: Gene markers ...................................................................................66
Table 2.3: Shear stress measurement ...............................................................67
LIST OF ABBREVIATIONS

SGN – Spiral ganglion neuron
mES – mouse Embryonic stem cells
SAG – Statoacoustic ganglion
HC – Hair cell
SC – Schwann cell derived from mES cells
SuC – Supporting cells
SW10 – Schwann cell line
O of C – Organ of Corti
CI – Cochlear Implant
CNTF – Ciliary neurotrophic factor
NGF – Nerve growth factor
MIF – Macrophage migration inhibitory factor
DHA – Docosahexaenoic acid
F12 – Neuronal Basal media
Oct-4 – Undifferentiated stem cell marker
Ngn1 – Neurogenin1
NeuroD – Neurogenic differentiation transcription factor
Nfil – Neurofilament marker protein
TrkB – Tyrosine kinase receptor B
TrkC – Tyrosine kinase receptor C
Prph – Peripherin
Gaba-A-R – \(\gamma\)-aminobutyric acid ionotropic receptor

VgluT1 – Vesicular glutamate transporter 1

Sp19 – Sodium ion channel

Kv3.1 – Potassium ion channel

WT – Wild type

KO – Knock out

Tuj1 – Tubulin 1 Neurofilament marker

(SC)- CI – Schwann cell coated CI
ABSTRACT

An injury to mechanosensory cells in the inner ear or to the neurons that innervate them can cause sensorineural hearing loss (SNHL). The mechanosensory cells in the cochlea, called auditory Hair Cells (HCs), play an important role in hearing. If these cells degenerate due to acoustic trauma, ototoxic drug exposure, cochlear infection, genetic abnormalities or age, hearing function can be lost or severely compromised. Loss of auditory system neurons, particularly the loss of spiral ganglion neurons can also lead to hearing loss. Regeneration of auditory system inner ear sensory cells does not occur in mammals, including humans, after loss due to injury, drug exposure or noise exposure, in contrast to avians, fish and frogs where such cells are replaced. Partial or complete auditory HC loss not only affects hearing but also affects the survival of the spiral ganglion neurons (SGN) that innervate them, further aggravating hearing loss, which leads to limited therapeutic options for patients. Although in humans, neuron loss is usually slower and less severe than HC loss, the loss of one cell population also affects the other. Despite recent advances in hearing restoration using cochlear implants (CI), also called cochlear prostheses, efficacy of such therapy still remains challenging. Success of the CI is limited by the number of healthy spiral ganglion neurons present in the inner ear at the time of implant and the number of such neurons that make functional contact with the implant. Recently there have been several studies investigating the survival, restoration and regeneration of spiral ganglion neurons, but neuronal survival and CI success depend on the presence of many crucial signal-processing pathways both during and after hearing loss. Regenerative therapy for hearing loss based on cell implants—particularly if stem cell based—is promising because one might implant exogenous or endogenous cells that are already fully or partially differentiated into spiral ganglion-like cells into the inner ear. We have successfully differentiated mouse embryonic stem cells (ES) into spiral ganglion neuron-like cells in culture using the inner
ear’s first neurotrophin, macrophage migration inhibitory factor (MIF) and docosahexaenoic acid (DHA) as a neuronal maturation factor. Therefore, an embryonic stem cell-based approach holds some promise to restore hearing function if such spiral ganglion-like cells could a) be implanted in a deaf inner ear and b) make functional connections with a cochlear implant. Our results show that the ESC-derived SGN like cells showed similar characteristic properties using combination of MIF and DHA as differentiation factor to those of the primary SGN they resemble. These neuron-like cells demonstrated directional outgrowth towards a wild-type mouse target Organ of Corti in the culture. Another way to enhance CI/SGN contacts would be to enhance directed neural cell migration/directional neurite outgrowth of the remaining SGN processes to the CI in response to soluble molecular cues, such as those normally produced by the target tissue. We have explored introducing such cell based cues through the use of hydrogel encapsulated embryonic stem cell derived Schwann cells that are used to coat a CI. These ESC-derived Schwann Cells make MIF, to which adult SGN respond because they have receptors for the neurotrophic cytokine. Development of Schwann cell coated CI with neuronal guidance properties might be used to compensate for the loss of HC to provide sustained trophic support to the SGN for survival. This study also included observing cell – cell interactions between ESC derived neurons and ESC-derived Schwann Cells in vitro. We therefore mimicked the microenvironment of the inner ear and established the cell-cell interactions by using a microfluidic device. The cell interaction studies show that ESC-derived Schwann cells can provide molecular cues for the directional outgrowth of neurites from ESC derived neuron-like cells, statoacoustic ganglia and spiral ganglia excised from embryonic and postnatal mice, respectively. Collectively, these biological findings and use of microfluidics technology have led to advances in the understanding of directional outgrowth responses of both ganglionic neurons and of embryonic stem cell derived neuron-like cells. This progress towards development of novel methods of using both stem cell-derived SGN and stem cell derived- Schwann cells could eventually be used in therapeutic applications to attempt to restore hearing loss in human patients.
Chapter I: Introduction

1.1 Hearing loss:

Sensory Hair cells (HC) serve as the mechanosensory cells responsible for both auditory system function and vestibular system function in vertebrates. "Making an effort to listen: mechanical amplification in the ear" (Hudspeth, 2008). Mammalian auditory hair cells are located within a structure called the organ of Corti (O of C) that sits on the basilar membrane in the inner ear’s snail shaped cochlea, lying within the temporal bone in the skull (Figure 1). HC derive their eponymous and rather fanciful name from the elaborate and polarized rows of stereocilia (actually long microvilli) that emerge from the apical surface of each HC, called the hair bundle, which are embedded in the gelatinous tectorial membrane (Richardson et al., 1987). The Organ of Corti is located in the Scala media (SM), the middle fluid-filled tube of the three such tubes found within the cochlea. In recent years, much progress has been made in identifying the transcriptional basis of the hair cell phenotype (Barald and Kelley, 2004). During the development of the mammalian ear, differentiation as a hair cell may depend on the presence of a single basic helix–loop–helix (bHLH) transcription factor known as Atoh1 (Warchol, 2007). Mammalian cochlear HCs are of two distinct functional types: the outer and inner hair cells (OHC/IHC). Inner hair cells (IHC) are responsible for converting incoming sound signals that reach the ear drum (tympanic membrane) through the air, but which subsequently reach the IHC through the fluid medium of the SM (endolymph) to outgoing neural signals to the brain. OHC, which have evolved only in mammals, provide a complex amplification function (Ashmore, 2008). Loss of or damage to HCs results in a decrease in hearing sensitivity, which is called sensorineural hearing loss (SNHL).
The precise arrangement of HC on the basilar membrane in the cochlea results in what is called a “tonotopic map”. HC in different regions of the basilar membrane are capable of responding to different frequencies of sound—HC at the base of the cochlea respond to high frequency sounds and those at the apex respond to low frequency sounds. Therefore, tonotopy in the auditory system begins with the cochlea, and is specified from high to low frequency along the basilar membrane in the O of C where the basilar membrane vibrates at different sinusoidal frequencies due to variations in thickness and width along the length of the membrane. The membrane is narrower at the high frequency (base) end of the cochlea and wider at the low frequency (apex) region of the cochlea (Figure 2). The nerves that convey information from the different regions of the
basilar membrane HC are themselves said to encode frequency “tonotopically”. Tonotopy then projects through the vestibule cochlear nerve and associated midbrain structures to (Simon et al., 2009).

Figure 1.2: Tonotopic map: HC at the base of the cochlea respond to high frequency sounds and those at the apex respond to low frequency sounds. Source: http://universe-review.ca/R10-16-ANS.htm.

If mechanosensory cells of the vertebrate inner ear are lost or are functionally impaired due to acoustic trauma, ototoxic drugs, cochlear infection, genetic abnormalities or ageing, this can cause sensorineural hearing loss (SNHL) (Rubel et al., 1991). Regeneration of most vestibular and all auditory inner ear sensory HC does not occur in mammals, including humans (Rubel et al., 1991). This is in distinct contrast to the robust regenerative capacity of such HC in avians and other vertebrates, including frogs and fish (Stone and Cotanche, 2007),(Walshe et al., 2003). Scientists have been focusing on the possible differences between—for example—chickens and mammals at the molecular, cellular and organ level for many years, seeking clues from chickens that might someday enable regeneration of the complex O of C and its hearing function in the mammal. The spiraling Organ of Corti (O of C) in the inner ear, besides the morphologically prominent and obvious HC, also contains the supporting cells (SuC), one of which underlies each HC, and bundles of spiral ganglion neurons that innervate
the HC (Figure 1). Mammalian inner ear development occurs during early embryogenesis between embryonic days 12 (E12) and E16 and mammalian HC generation stops after this critical period (Ruben and Sidman, 1967), (Holmes et al., 2011), (Fritzsch et al., 1997b). The HC and their underlying supporting cells (SuC) of the inner ear provide molecular cues to support the directional outgrowth of and the survival of the bipolar spiral ganglion neurons, which synapse with HCs and conduct the encoded sound from the hair cells to the brain stem (Wei et al., 2007; Wei and Yamoah, 2009). Therefore, partial or complete HC population loss not only affects hearing through loss of mechanosensory function but can also affect the survival of spiral ganglion neurons (SGN) with which they make contact. Loss of the neurons further aggravates hearing loss, which, because neither cell population is spontaneously regenerated in mammals, leads to limited therapeutic options for patients who experience loss of HC, loss of neurons or both.

**Fate of SGN in hearing loss:**

The spiral ganglion in the inner ear is populated by bipolar neurons, which connect the HC with central neurons in auditory brain stem nuclei. Once past embryonic development, adult SGNs depend on the integrity of cells of the O of C, particularly the HC with which SGNs make synaptic contact (Stankovic et al., 2004) for their continued survival. Neurotrophins play crucial roles in the development, synaptic connectivity and long-term maintenance of distinct sets of neurons of the central and peripheral nervous systems (Fritzsch et al., 1997b; Shinohara et al., 2002). When deprived of their peripheral target (the HC), the spiral ganglion neurons (SGNs) progressively degenerate due to the loss of connectivity to both the brain stem and the HC (Figure 3). SGN degenerate following the absence of trophic inputs supplied by HC/SC and even SCwann Cells, including neurotrophins, transcription factors and neurotransmitters released by the HC and supporting cells in the O of C (Shepherd et al., 2008).
Influence of Trophic, Neurotransmitter and Electrical Activity on SGN survival:

Neurotrophic factors are endogenous soluble proteins, which are synthesized by supporting cells (SuC), HC and by neurons themselves (resulting in autocrine control loops), which regulate the survival, growth, morphological plasticity and molecular synthesis pathways, including synthesis of neurotransmitter and other proteins in neurons that maintain both their early path-finding and growth functions as well as the later differentiated functions of neurons (Alexi and Hefti, 1993; Wise et al., 2010b). Neurotrophic factors that are active in the central and peripheral nervous systems include the so-called neurotrophins (NTs), nerve growth factor, ciliary neurotrophic factor, epidermal growth factor (EGF), fibroblast growth factors (FGFs), insulin-like growth factors (IGF), and members of the glial cell line–derived neurotrophic factor (GDNF) families (Deister and Schmidt, 2006). In the developing inner ear, the best characterized
of these factors are the NTs. Two members of this family, neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF), are expressed in the developing cochlear and vestibular sensory epithelia respectively (Fritzsch et al., 1997a). Their cognate tyrosine kinase receptors, which belong to the Trk family, are expressed in the sensory neurons of the inner ear ganglia (Pirvola and Ylikoski, 2003). During fetal and early postnatal development, BDNF and NT3 are synthesized by both the inner and the outer hair cells, while trkB and trkC receptors are expressed in the spiral ganglion neurons (Sobkowicz et al., 2002). However, recent work from our laboratory has demonstrated that the earliest “neurotrophins” in the inner ear are not the already identified classical “neurotrophins” but are instead immune system cytokines, including macrophage migration inhibitory function (Holmes et al., 2011; Shen et al., 2011).

During development of the otocyst the auditory and vestibular neuroblasts are amongs the first cell types that are specified (Hemond and Morest, 1991). Their differentiation depends upon two bHLH transcription factors, neurogenin 1 (ngn1) and NeuroD. In null mouse mutants for ngn1, both auditory and vestibular neurons are absent, demonstrating that this gene is essential for the differentiation of all inner ear sensory neurons (Ma et al., 1998). Ma et al. (1998) suggested that NeuroD and b3-tubulin are part of the same regulatory cascade downstream of ngn1. Numerous molecular markers characteristic of auditory neuroblasts, including the transcription factors GATA3, NeuroD, Brn3a and Islet1 are expressed in SGN cells differentiated from auditory neuroblast cell lines (Nicholl et al., 2005),(Lanier et al., 2007). In particular, the POU-domain transcription factors are widely expressed in the developing ear. Members of the Brn-3 family of POU-domain factors are expressed only in the sensorineural elements of the inner ear, and might therefore be expected to play a unique role in the development of these tissues(Lanier et al., 2007).
Spiral ganglion neurons (SGNs) derive trophic support from multiple sources. Inner Hair cells are the source of synaptic activity and stimulate membrane electrical activity. Neurotrophic factors (NTFs) are produced by HCs, SCwann cells, the cochlear nerve and SGNs, which themselves also provide trophic support for SGN survival (Wei et al., 2007)(Figure3). Neurotransmitter release from HC underlies maintenance of SGN electrical properties and survival (Ehrenberger and Felix, 1991). In addition to NTs and transcription factors, signals generated by synaptic and electrical neural activity likely also regulate SGN survival and neurite growth. Neurotransmitter release from hair cells drives membrane electrical activity in SGN, also supporting their survival (Roehm and Hansen, 2005). SGNs, which are electrically active, are capable of rapidly changing their membrane potential. Membrane electrical activity is required for the relay of sensory information to the central nervous system; it may also play a role in the establishment and maintenance of afferent cochlear innervation. An excitatory amino acid, probably L-glutamate, is the most promising candidate for neurotransmitter function at the inner hair cell afferent fiber synapses in the cochlea (Despres and Romand, 1994). The first electrophysiological evidence comes from (Bobbin, 1979) and (Comis, 1970), who showed that glutamate and aspartate mimic the effect of acoustic stimulation, and thus the action of the endogenous neurotransmitter, by increasing the spontaneous firing of the primary auditory neurons(Bobbin, 1979), together with that of their axons(Comis and Leng, 1979). Electrical stimulation, membrane depolarization, and invocation of activation signaling pathways all promote SGN survival(Shepherd et al., 2008). GABA
trophic effects on neurite growth and synaptogenesis, for example, are well known (Puel, 1995), (Usami et al., 2001).

**MIF acts as a neurotrophin at the earliest stages of inner ear development:**

Macrophage Migration Inhibitory Factor (MIF) is a pluripotent cytokine (a signaling molecule involved in cell-cell communication) that is not only critical for immune system development and function (Grieb et al., 2010) and inflammatory responses but also with cell growth and differentiation as well as wound repair and carcinogenesis (Conroy et al., 2010; Gilliver et al., 2011). MIF is also involved in cell growth and embryonic development mainly in the nervous system (Itoh et al., 2003), (Suzuki et al., 1996) and has been implicated in neurodegenerative disorders including multiple sclerosis (Nishihira and Ogata, 2001). Thus, MIF in its multifarious roles, has become a major target protein in a variety of pathophysiological states; anti-MIF antibodies and antagonists could be applied therapeutically in clinical situations for treatment of various diseases, including MS (Nishihira and Ogata, 2001).

MIF plays a key role not only in peripheral nerve regeneration, but also in the viability of SCwann cells through the suppression of apoptosis-related proteins such as p53 (Nishio et al., 2002). The current studies discussed in this thesis may shed light on novel roles of MIF in the nervous system, and could bolster the possibility of using very low concentrations of MIF in therapeutic applications of this cytokine to prevent or delay nerve damage (Nishio, 2002). Although many other laboratories previously demonstrated the presence of MIF in peripheral nerves, and demonstrated that MIF mRNA expression was up-regulated after axotomy (Yang et al., 2006), the role of MIF in nerve injury and regeneration has not been evaluated in the auditory system (Nishio et al., 2002). Our recent research studies suggest that MIF could act as a neurotrophin in the early development of sensory systems, including the auditory system in mice and zebrafish (Shen et al., 2011), (Holmes et al., 2011), (Bank et al., submitted). Our laboratory showed that the zebrafish mif pathway is involved in the development of both sensory hair cell (HC) and sensory neuronal cell survival in the ear, is critical for HC differentiation, semicircular canal formation, statoacoustic ganglion (SAG) development, and lateral line HC differentiation (Shen et al., 2011), (Holmes et al., 2011). This is
consistent with our finding that MIF is expressed in the developing mammalian and avian auditory systems and promotes mouse and chick statoacoustic ganglion (SAG), the forerunner of the cochleovestibular ganglion, neurite outgrowth and neuronal survival, demonstrating key instructional roles for MIF in vertebrate otic development (Shen et al., 2011). MIF is known to be downstream of retinoic acid (RA) in the induction of a neuronal phenotype from embryonic stem cells (Sarkar and Sharma, 2002) and our studies (Bank et al., submitted) have shown that MIF alone can elicit a neuronal phenotype from mouse ES cells at concentrations in the picomolar range. Work of Suzuki and colleagues (2004) demonstrated that in Xenopus, also MIF is a critical player in neuraxis formation (Suzuki et al., 2004). Work from our laboratory demonstrated that zebrafish mif genes (there are 2 in the zebrafish) also contributed to nervous system and inner ear formation (Shen et al., 2012).

MIF’s documented involvement in sensory system development—including eye development (Zaidi et al., 2011) and auditory system development (Holmes et al., 2011; Shen et al., 2012) leads to our present experiments to test the idea that MIF might also be involved in auditory sensory system—regeneration of either sensory cells or neurons. For this neurotrophic cytokine to play a role in any aspects of regeneration or restoration of hearing function in the auditory system, several conditions must be met. 1. In addition to MIF’s being expressed at the right time and in the right place to affect development, 2. If adult sensory cells and/or neurons are to be able to respond to MIF, MIF receptors must be present on the adult cells. 3. If endogenous MIF expression is confined to embryonic times and doesn’t extend into adulthood, then an exogenous source of MIF needs to be supplied and the correct concentrations determined in order for HC and or SGN to respond to the exogenously introduced cytokine.

The embryonic otocyst is a potent source of MIF in development (Bank et al submitted) and indeed, MIF is the primary bioactive factor in promoting directional SAG neurite outgrowth and SAG neuronal survival as part of the well-documented otocyst-derived factor (ODF) (Bianchi and Cohan, 1991), Bank et al., submitted). Both Otocyst-derived MIF and purified MIF are directional neurite outgrowth factors and survival factors for embryonic statoacoustic ganglion neurons (Bank et al., submitted) and adult SGN (Ebisu et al., submitted). In addition, addition of commercially available mouse or
human MIF to mouse embryonic stem cells produces neuron-like cells with many of the characteristics of SGN (Ramamurthy et al, in preparation). Mouse embryonic stem cells that were induced to become SCwann Cell-like also produce MIF (Ramamurthy et al., in prep.). MIF knockout mice are hearing impaired (Bank et al, submitted).

All of these findings both in our laboratory and in other laboratories have encouraged us to take a two faceted approach to determine if MIF might prove of therapeutic value in the injured auditory system or in an auditory system in which sequellae of genetic disease are becoming apparent (e.g Usher’s Syndrome) (Steel et al., 1997). The experiments in this thesis demonstrate that we have been successful at inducing a SGN-like phenotype from mouse embryonic stem cells using very low concentrations of MIF as a neurotrophin and neuronal development inducing agent (Bank et al., submitted). As a second approach, mESC induced to become SCwann Cell like (Roth et al., 2008; Roth et al., 2007), which produce MIF (All SCwann Cells produce MIF during the normal course of their development; Roth et al. 2008), have been used to “coat” a cochlear implant. MIF-producing mESC-derived SC-like cells have been encapsulated in hydrogels and observing interactions in culture with a) mouse SAG neurons; b) mouse SGN or c) SGN-like “neurons” derived from mouse embryonic stem cells (the same stem cell population used to produce the MIF-producing SC-like cells) (Figure 5). Inducing neuronal maturation of mESC-derived neuron-like cells with DHA.

Figure 1.5: “Cartoon from Chapter 3: A cochlear implant wire (in grey above left) has been “coated” with a hydrogel encapsulating MIF-producing mESC-derived SC-like cells (seen as nucleated individual cells in this cartoon): Interactions in culture with a) mouse SAG neurons; b) mouse SGN or c) SGN-like “neurons” derived from mouse embryonic
stem cells (the same stem cell population used to produce the MIF-producing SC-like cells; “a and b” are illustrated above.

**Role of DHA in neuronal maturation: neurite outgrowth, fasciculation, synaptogenesis:**

Mammalian cells, including neurons, are not capable of synthesizing the fatty acid Docosahexaenoic acid (DHA) de novo, which is critical for maturation of neuronal cells’ membrane and synaptic properties in both the Central and peripheral nervous systems (Heinemann and Bauer, 2006; Walczewska et al., 2011). Evidence that DHA n-3 (fatty acid chain 22:6 n-3) is esterified to phosphoglycerides in the neuronal plasma membrane has come from studies of synaptosomes or synaptosomal plasma membranes from the brains of rats (Glomset, 2006). An adequate level of DHA in cell membranes is important for many functions of neural cells and this is presumably the reason for DHA sequestration in the adult mammalian brain during dietary α-linoleic acid deficiency (Valentine and Valentine, 2004). DHA-containing phospholipids in membranes are flexible and membranes possessing a high content of these phospholipids can be relatively thin, while remaining more permeable to ions and small molecules, have looser lipid packing, and are more "dynamic" than membranes composed of other fatty acid containing phospholipids (Uauy and Dangour, 2006). Furthermore, these membranes create an appropriate environment for the types of integral membrane proteins that are highly condensed in neurons, including receptors, ion channels, enzymes, and peripheral proteins (Walczewska et al., 2011) The most significant effects of DHA are related to its role in neural development and maturation of neurons in sensory systems (Uauy et al., 2000). Dietary DHA can apparently decrease axonal dysfunction, reduce loss of cytoskeletal proteins and reduce demyelination after spinal cord injury (Ward et al., 2010). In previous research work, scientists investigated some of the possible mechanisms underlying the neuroprotective effects of DHA on spinal cord injury pathology (Huang et al., 2007). DHA treatment after injury led to a significant reduction in a number of indices of oxidative damage. This may contribute to the reduction in neurofilament loss, as these proteins are highly sensitive to oxidative stress, which leads to alterations in their structure and the eventual collapse of the cytoskeleton (Gelinas et
Glutamate-induced excitotoxicity is reduced and neuronal survival is increased by supplementation with DHA in vitro (Wang et al., 2003) and in vivo (Hogyes et al., 2003) models of neuronal injury. DHA supplementation in the neuronal culture showed increased population of neurons with longer neuritis (Dagai et al., 2009). We therefore elected to aid “neuronal” maturation of the mESC-derived neuron-like cells by exposing them to DHA (5-10 micro molar). With the combination of MIF as neurotrophic factor and DHA as neuronal maturation factor supplementing in the neuronal base media we propose that the mES cells can differentiate into SGN-like cells.

1.2 Different therapy strategies in promoting SGN survival, regeneration and regrowth.

Many researchers are involved in designing mechanisms to regenerate HCs and/or maintain or regrow the neuronal processes of SGN with the objective of restoring hearing function. Potential strategies to replace lost sensory HCs include: (1) replacement of lost cochlear cells with transplanted stem cells induced to become HC-like that are implanted in the damaged inner ear (Parkins, 1985) and (2) recruitment of inner ear endogenous stem cells or progenitor cells to generate new HCs in the injured inner ear.

Embryonic stem cells and adult stem cells from various tissues can to some limited extent be induced to differentiate into new HC-like cells (Jeon et al., 2007; Martinez-Monedero and Edge, 2007; Rivolta et al., 2006). These “replacement hair cells” might then be able to re-establish correct synaptic connections with existing statoacoustic neurons, resulting in nearly complete functional recovery (Bermingham-McDonogh and Reh, 2011; Rubel, 1985). The chicken is capable of regenerating its otic epithelium of mechanosensory hair cells by a normal transdifferentiation process in which SuC can become HCs (Stone et al., 1998), (Rubel and Stone, 1996). However, these approaches are not yet possible in the adult mammalian O of C in vivo (Breuskin et al., 2008). HC replacement therapy, whether with endogenous inner ear stem cells, transdifferentiated cells or exogenously introduced cells or stem cells, is an enormously complicated process and even if the molecular and cellular cues and processes that allow regeneration in chickens and fish were to be identified, it is not clear how they could be applied to
mammalian systems. The only viable mechanism for restoring hearing to a deaf mammalian ear in which HC loss has become an overwhelming factor is to introduce a cochlear prosthesis or cochlear implant (CI) to replace lost HC. The CI bypasses HC function and directly provides electrical signals to stimulate any remaining SGN. The lost HCs are replaced by implanting cochlear prostheses, which detect sound signals (via microphone) and send electrical input to the brain via any remaining SGNs in close proximity to the CI (Figure 6a and 6b). The limitations in such treatment options for patients include the number of viable SGNs present in the inner ear that are capable of making contact with the CI. However, the success of the cochlear prosthesis depends on the functionality of the remaining excitable auditory nerve fibers, and their loss severely compromises the effectiveness of the implant and the hearing benefits it provides.

Studies show a clear relationship between the total number of viable auditory neurons available for CI stimulation and the performance of subjects (e.g. in speech recognition) receiving cochlear implants. At least 25% of the full complement of SGNs should be available to restore hearing successfully after prostheses implantation (Pettingill et al., 2011). Scientists and researchers have concomitantly invested their time in developing a source of SGN replacements to restore hearing when the HCs can be replaced by CI (Evans et al., 2009; Richardson et al., 2007). The success of CI efficacy depends on the functional integrity of the auditory nerve and its central projection, which, unfortunately, has poor integrity in patients with auditory neuropathy (Matsuoka et al., 2007). The longer the period between a patient’s deafness onset and CI implantation, the worse the prognosis for hearing restoration (Hurley, 1999; Shikowitz, 1991). Promoting SGN survival using a variety of different methods and potential strategies could prolong the survival of remaining SGN, induce regrowth of neurites or regenerate SGN and/or provide endogenous or exogenous molecular cues on the CI to induce the maintenance, neurite regrowth or directional outgrowth of existing SGNs peripheral processes. Guidance of the regrowing axons to appropriate targets (e.g. the CI) in greater numbers would also help restore hearing function.
Figure 1. 6: Cochlear prostheses (electrode in left figure) implanted in the Scala tympani of the inner ear with no or few surviving hair cells and very few SGNs. Source: http://www.iurc.montp.inserm.fr/cric/audition/english/corti/fcorti.htm Modified. Figure 6b: Cochlear prostheses implanted in the inner ear bypassing the HC function. Source: www.cochlea.com.

**Promoting SGN survival:**

Promoting SGN survival and neurite outgrowth towards the CI are the main objectives currently under study for improving the efficacy of the implanted cochlear
prostheses. In recent years researchers have investigated a number of growth factors that have the potential to regulate survival or recovery of auditory neurons (Deister and Schmidt, 2006; Kopke et al., 1996; Miller et al., 1997). Coincidental with a focus on molecules that may help to restore cellular function and/or neurite outgrowth to the CI are efforts to develop novel delivery methods (Hendricks et al., 2008). Researchers have been investigating the use of mini osmotic pumps, viral vectors and stem cells as a means of providing direct application of growth factors or sources of growth factors or neurotrophins (cellular or acellular) to the inner ear (Bianchi and Raz, 2004). Scientists have developed different methods for providing therapeutic agents to treat damaged spiral ganglion neurons, which are discussed in this section.

It has been demonstrated that direct cochlear infusion of neurotrophic factors such as neurotrophin-3, brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor enhances the survival of SGN after inner hair cell loss (Malgrange et al., 1996). Treatment with brain derived neurotrophic factor (BDNF) administered chronically via a mini-osmotic pump into the Scala tympani, resulted in enhanced SGN survival over untreated deafened ears or deafened ears treated with artificial perilymph (Malgrange et al., 1996). These observations suggest that there are survival factors in the inner ear, including those produced by direct activation of auditory nerve fibers, which may serve to maintain the auditory nerve (Ernfors et al., 1996). These factors may be applied following deafness to maintain and enhance neural populations and to increase benefits to the profoundly deaf receiving cochlear implants (Miller et al., 1997). Clinically safe and effective drug delivery methods are required. The finite delivery period of osmotic pumps means that long-term neurotrophic factor treatment would require repeated replacement of the pumps, thereby increasing the risk of infection associated with such surgical procedures. While refillable pump-based systems have been shown to function adequately for up to 8 months in rats (Praetorius et al., 2002), the long-term bioactivity of neurotrophic factors at body temperature has not been confirmed, and the regular refilling of these devices still poses a risk for the introduction of infection directly into the cochlea, which could inadvertently result in conditions that produce deafness. While cerebrospinal fluid shunts and are routinely implanted in the CNS on a chronic basis, infection is a major cause of
morbidity and mortality of these devices, with infection rates in the order of 8–10% for both (Ferguson et al., 2007; Strege et al., 2004). Such outcomes would be totally unacceptable if translated to an intracochlear drug delivery system. Prevention of infection within the inner ear is extremely important in order to minimize further damage to and loss of SGNs, and also to avoid infection spreading via the cochlear aqueduct to the CNS. Gene therapy is an alternative method for increasing neurotrophin expression within the cochlea, and in fact *in situ* transduction of the spiral ganglion with genes encoding BDNF, NT3 or GDNF has been shown to support some degree of SGN survival (Di Domenico et al., 2011; Husseman and Raphael, 2009; Kawamoto et al., 2001).

Neurotrophic factor application and electrical stimulation by cochlear implant (CI) in combination have been shown to enhance neuron survival (Winter et al., 2007). Exogenous replacement of either BDNF/NT3 or both neurotrophins protects SGNs from degeneration after deafness to some extent (Evans et al., 2009; Wise et al., 2010a). Researches previously incorporated NT3 into the conducting polymer polypyrrole (Ppy) synthesized with para-toluene sulfonate (pTS) to investigate whether Ppy/pTS/NT3-coated cochlear implant electrodes could provide both neurotrophic support and electrical stimulation for SGNs (O'Leary et al., 2009). Enhanced and controlled release of NT3 was achieved when Ppy/pTS/NT3-coated electrodes were subjected to electrical stimulation. Release studies demonstrated slow passive diffusion of BDNF from Ppy/pTS/BDNF and Ppy/pTS/NT3, with electrical stimulation significantly enhancing BDNF release over a 7 day period. A 3-day SGN explant assay was used to demonstrate that neurite outgrowth from explants was ~12-fold greater when the coating polymers contained BDNF or NT3, although electrical stimulation did not increase neurite outgrowth further (Richardson et al., 2009; Wise et al., 2011b). Drug-releasing conducting polymer coatings on cochlear implant electrodes present a clinically viable method to promote preservation of SGNs without adversely affecting the function of the cochlear implant (Richardson et al., 2009).

Ex vivo gene transfer, which involves the transduction of a host population of cells *in vitro* followed by transplantation of these cells *in vivo*, is another alternative to neurotrophin therapy within the inner ear. Importantly, previous studies have reported
that cells genetically modified to over-produce neurotrophins can continue to express the transgene for at least 12 months (Grill et al., 1997) (Tuszynski et al., 1998), providing further support for this technique as a potential therapeutic approach for prevention of SGN degeneration in deafness. Providing an exogenous cocktail of neurotrophins by using various methods in combination with electrical stimulation may prevent further HC loss or SGN degeneration, but these applications do not seem to have a long term effect in restoring HC and/or SGN, which are severely damaged in some cases of SNHL. The intracochlear infusion of neurotrophic factors via a mini-osmotic pump has been shown to prevent some deafness-induced spiral ganglion neuron (SGN) degeneration; however, the use of pumps may increase the incidence of infection within the cochlea, making this technique unsuitable for neurotrophin administration in a clinical setting. To improve the long-term delivery of neurotrophin and to avoid infection using pump systems, researchers investigated the combination of cell- and gene-based techniques on SGN survival (Hu et al., 2009). Specifically, SCwann cells were genetically modified to over-express either BDNF or NT3 and the survival effects of these cells on SGNs were determined in vitro (Pettingill et al., 2011). BDNF-SCs supported SGN survival in vitro better than NT3-SCs. While both types of neurotrophin over-expressing SCwann cells significantly enhanced SGN survival in comparison to the control SCwann cells, the BDNF-SCs elicited the most potent survival effects (Pettingill et al., 2008).

Generally, systemic administration of neurotrophins has not been effective due to rapid degradation of these molecules, a need for higher concentrations than can be achieved with such small volumes capable of introduction within the end organ, and the potential for systemic toxicity (Apfel et al., 2000). However, long-term transgene expression would require the use of viral vectors, which also raises concerns regarding clinical applicability. A variety of cell based therapies are potential therapeutic options for maintaining SGN populations or for regenerating or regrowing SGN processes that have better connections between the brain stem and the CI.

**Replacing the mature SGN – Cell based therapy:**

Replacement of degenerated peripheral or central neurons by stem cell–derived neurons requires differentiation of the stem cell to the appropriate phenotype, degree of
neuronal maturation and synaptogenic capacity and directed growth of projections to reestablish functional neural circuits. Neural regeneration from stem cells presents challenges in addition to the initial differentiation SCeme, such as overcoming apoptotic cell death, preventing rejection, avoiding inadvertent tumor formation or cell overgrowth and encouraging new neurons to grow processes from the cell to the host target in as direct a path as possible. While differentiation of stem cells to neurons and survival of these cells after engraftment in a host have been demonstrated, it has been more difficult to demonstrate growth of neuronal processes from these neurons to defined targets (Corrales et al., 2006). Mature neurons are post-mitotic differentiated cells that are unable to reenter the cell cycle and repopulate a depleted SGN if there is any damage or loss of neurons—or perhaps of glial cells. In the case of SNHL, if there are few surviving neurons, three different strategies have been suggested to replace the SGNs in SNHL. The first strategy involves transplanting neuronal tissue using other ganglionic cells than those that normally innervate the cochlea. The cochlea could support such transplanted cells by providing an environment that is suitable for, in one example grafted dorsal root ganglion neuronal tissues (He et al., 2010). A second strategy is the possible use of endogenous stem cells of various kinds that might be present in the inner ear itself that could be induced to differentiate into cells that resemble the missing SGNs as closely as possible (Altschuler et al., 2008; Hu and Ulfendahl, 2006). The third possibility is to induce exogenous stem cells introduced into the inner ear to serve the function of SGNs. Such exogenous sources of cells could include embryonic or adult stem cells or iPS cells or immortalized cell lines, such as our otocyst-derived cell lines (Barald et al, 1997; Bianchi et al., 2005, Thompson et al., 2003; Germiller et al., 2004). The third strategy is the most promising for SGN functional restoration due to the ready availability of such exogenous cell sources, whereas the endogenous stem cell availability is limited both because such endogenous stem cell populations have not been readily identified in the inner ear (Jongkamonwiwat et al., 2010; Li et al., 2004) and because inducing a relatively small population to differentiate appropriately or in isolating the stem cells from fully developed cochlea, growing them and reimplanting them present enormous challenges to both the researcher and the physician.

Transplantation of neurons from other ganglia into the cochlea
If extensive degeneration of SGN has occurred, the population of excitable elements for transmitting auditory information to the central nervous system is insufficient for acceptable implant function. Unfortunately, such degeneration is an end-stage result after long-term hair cell deprivation. Neural grafting of differentiated or embryonic neurons is a potential strategy to help restore auditory function following loss of spiral ganglion cells. The reconstruction of a neural pathway from the cochlea to the brainstem, however, is enormously problematic. Hu et al. examined the survival of fetal dorsal root ganglion (DRG) neurons allografted into the cochlea of adult guinea pigs (Hu et al., 2005a; Regala et al., 2005). Their findings demonstrate that fetal DRG neuronal implants allografted into the adult cochlea can survive albeit in small numbers and the survival of the implanted DRGs increases with supplemental exogenous neurotrophic factors (Hu et al., 2005a). These earlier findings from the Swedish group may help establish whether a biologically active implant could be used to replace the spiral ganglion cells and reconstruct a functional pathway from the hearing organ to the cochlear nuclei in the brainstem following trauma. (Olivius et al., 2003) The replacement or enhancement of the auditory nerve itself would be of immediate clinical benefit and allow the implantation of a patient population now untreatable with cochlear implants (El-Amraoui and Petit, 2010; Hu et al., 2009). The choice of fetal dorsal root ganglia (DRG) as a source of heterologous sensory cell replacement was prompted by the finding that these neurons successfully survived peripheral transplantation, and are able to send axons into the CNS and to make functional synapses with CNS neurons if exogenous neurotrophin supplementation is also given. However, even without exogenous neurotrophins a substantial number of implanted DRG neurons were reported to survive, perhaps supported by growth promoting factors in the surrounding neuronal tissues, and/or by autocrine mechanisms in the DRGs themselves (Regala et al., 2005). The total number of surviving grafted DRG neurons increased significantly when combined with exogenous neurotrophic factors, but the number of implanted animals with surviving DRGs did not increase as a result of neurotrophic factor treatment (Olivius et al., 2004). The transplantation of mouse embryonic DRGs was combined with infusion of exogenous NGF into the adult rat cochlea. The outgrowing DRG axons have been observed to penetrate the osseous modiolus towards the spiral ganglion region. Although
neurite projections were found between the implanted DRG neurons and the host cochlear neurons, any functionality was not documented (Hu et al., 2005b). In order to enhance the survival of DRG neurons following transplantation, NGF was infused into the inner ear for a period of 2 weeks. At 3-week postoperative survival time, the a significantly larger number of implanted DRG neurons survived in the NGF group than in the non-NGF group (Hu et al., 2005b; Martinez-Monedero et al., 2008). This illustrates that exogenously supplemented NGF could enhance DRG neuronal survival following transplantation into the adult inner ear, but functional restoration has still been elusive (Hu et al., 2005b).

Possible use of endogenous sources of stem cells.

Stem cells were isolated by “oto”sphere formation from the utricle of vestibular organ of a mouse and the spheres were differentiated into nestin- and tubIII- positive neurons in a culture system (Martinez-Monedero and Edge, 2007). Glial cell line-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) induced differentiation of the progenitor cells present in the spheres. Neuronal processes fasciculated and migrated in the tissue culture dishes with a speed of 10–30 μm/h, and some cells had up to 6 mm long neurites co expressing TrkB and TrkC receptors (Nakaizumi et al., 2004). These results suggest that the mammalian auditory nerve has the capability for self-renewal and replacement. Transplantation of progenitor cells together with an established and reproducible means of inducing neural differentiation and neurite outgrowth may facilitate strategies for better repair and treatment of auditory neuronal damage (Rask-Andersen et al., 2005).

Induced stem cells derived from exogenous sources: ES cells, neural stem cells and immortalized inner ear cell lines.

Stem cells (adult, embryonic or induced pluripotent stem (iPS) cells, which are somatic cell derivatives) could become a renewable source of replacement cells that could be used to treat neurodegenerative diseases (El Seady et al., 2008) or to replace lost or damaged sensory neurons in the central nervous system (CNS), peripheral nervous system (PNS) or in sensory organs such as the inner ear. Our laboratory derived (Barald et al., 1997; Germiller et al., 2004) immortalized (IMO) cell lines from the inner ear of
the 9.5 day embryonic immortomouse (Germiller et al., 2004). The IMO2B1 cell line has been characterized most extensively (Bianchi et al., 2005; Germiller et al., 2004). IMO2B1-conditioned medium promoted outgrowth of SAG explants of both chick and mouse and also promoted outgrowth and survival of dissociated chick and mouse SAG neurons similar to that observed with chick or mouse otocyst derived factor (ODF). Otocyst-derived ODF is made by incubating 8 otocysts of either 4-5 embryonic day Chick embryos or 10-11 day embryonic mouse embryos in 100 microlitres of serum-free medium (Bianchi et al, 1993, 1998 etc. Bank et al, submitted). It is important to note that the cells of the IMO2B1 line maintain many of the characteristics of developing inner ear sensory epithelia (Baral and Kelley, 2004; Germiller et al., 2004). It is believed that the IMO2B1 cell line represents a multipotential precursor population that can give rise either to HC-like or SC-like cells under the influence of specific culture conditions (Baral and Kelley, 2004). The IMO2B1 cell line therefore appears to be an excellent source of material for identifying the bioactive neurite-outgrowth promoting and neuronal cell survival promoting factor(s) released by cells of the early stage inner ear, the otocyst (Bianchi et al. 2005; Bank et al., submitted). We reported (Bianchi et al, 2005) the initial characterization of the first abundant source of ODF-like bioactivity and the first observation of the chemokine/cytokine MCP-1 as an inner-ear-derived, neurite-promoting molecule. Although Monocyte Chemoattractant protein 1 is one of the cytokines in ODF, it is neither the only nor the most important one. Later more extensive proteomic studies were used to identify MIF in the same IMO-derived ODF source (Bank et al., submitted). The biologically active proteins, including the cytokines/chemkines MCP1, MIF, RANTES and TNF-alpha were found in ODF generated from the IMO cell lines and were shown to be involved in outgrowth and survival of SAG and involved in other aspects of inner ear development.(Bianchi et al., 2005) (Bank et al., submitted). Dr. AltusCur laboratory developed SGN-like cells derived from mES cells resembling the characteristics of SGNs of the auditory nerve using Neurog1, BDNF, and GDNF as differentiation factor and found in the scala tympani and modulus of the cochlea of after the SGN cells were implanted(Reyes et al., 2008).

The major pitfall in the cell-based therapeutic approaches is cell survival after a relatively limited period of implantation in the inner ear. The long term survival rate of
the implanted cells is very limited (Reyes et al., 2008). The reasons for such limited survival of the cells may be due to the fluid microenvironment of the inner ear, which may lack the appropriate molecular cues to support the survival of the implanted cells, which have largely been implanted into the Scala Tympani (Reyes et al., 2008). To overcome these cell survival issues researchers have developed a novel combinational therapy approach to have a chance of better connections between the CI and the implanted cells.

**Regrowth of remaining SGN peripheral processes.**

The functional outcomes of implanting a CI depend on the channel interaction of the CI and the success of functional connection formation between the neurons and the current source from the CI. Strategies to encourage regrowth and axonal guidance of the remaining SGN towards the CI will increase channel interactions and bring the neuronal processes into close proximity with the CI, which should provide a potential advantage in CI performance.

**Combinational therapy - Survival and Regrowth of SGN to improve CI efficacy:**

In the normal nervous system and in sensory systems such as the eye and ear, neurotrophic factors are mostly synthesized by target tissues and are used to maintain the viability of nerve-cell bodies and to encourage directed neurite outgrowth towards target tissue. After nerve injury, neurotrophic factors are synthesized by non-neuronal cells (SCwann cells and fibroblasts) in the nerve trunk, and act to support the outgrowth of axons in regenerative responses. Transplantation of neurotrophin-overexpressing SCwann cells into the cochlea may provide an alternative means of delivering neurotrophic factors to the deaf cochlea for therapeutic purposes (Pettingill et al., 2008). The use of cell based sources of NT for restorative therapies provides a clinically viable NT delivery technique that is less susceptible to adverse side effects than pump based delivery methods (Apfel, 2001). Choroid plexus cells produce and release a cocktail of NTs. These cells were encapsulated in biocompatible alginate and implanted into the neonatal deafened cat cochlea in combination with a cochlear implant electrode array (McCall et al., 2010). This combination therapy approach showed that the SGNs and
their peripheral processes were protected and enhanced by electric stimulation for an extended period of time— at least for 8 months. (Wise et al., 2011a)

**Cellular interface with cochlear electrode**

The importance of the cellular interface with the cochlear protheses emphasized the importance of developing electrodes with inert materials for less impedance on the electrical stimulus (Brackmann, 1976). Several types of inert metal and alloys including platinum and iridium oxide, platinum and iridium alloys have been used in such bionic applications (Parkins, 1985). Metal alloys were used for making electrodes for both CIs and deep brain stimulator electrodes (Lu et al., 2005; Paulat et al., 2011). Replacing the metal components of a CI with biocompatible materials could improve electrode performance with less impedance and provide better cell compatibility (Thompson et al., 2010). Biocompatible conducting electrodes made of biobased conducting materials can also be incorporated with active drug, morphogen, growth factor or cytokine releasing mechanisms or with engineered biological molecules (Richardson et al., 2009) and can be developed with controlled release systems for consistent release of the therapeutic molecules over time. Another exciting feature of organic conducting polymers is the ability to create the electrode under physiological conditions, which permits fabrication in the presence of living cells (Hendricks et al., 2008; Wallace et al., 2009). These approaches enable the integration of an electrode with living cells, and should facilitate improvements in the performance of electrode-based biomedical devices (Wallace et al., 2009). In our study we incorporated mESC-derived SCwann cell like cells into a sodium alginate hydrogel, which we used to coat a platinum iridium electrode of a type that has been widely used in clinical applications (Niparko et al., 1989). The encapsulated ES derived Schwann-like cells provide trophic support (in our case, MIF) and growth of existing SGN has been followed (by cinematography) towards the CI (Figure5). A cell based implantation SCeme is illustrated in the cartoon below (Figure 7).
Figure 1.7: Cartoon of a Cochlear implant coated with neurotrophin producing Schwann Cells to attract SGN and restore SGN function. Source: Modified from Wallace.G.G. (Wallace et al., 2009) In our modification of this technical approach, ES derived SCwann cells were coated on CI, in a sodium alginate hydrogel, which can provide MIF (a product they produce normally both in vitro and in vivo) and potentially other neuron-supporting molecular cues.

1.4 Microfluidic technology in Cell-Cell interaction study

Recent developments in micro and nano fabrication (Microelectromechanical system MEMS) techniques have provided new model systems for cell and developmental biologists to carry out experiments in model systems that mimic features of in vivo cellular microenvironments that cannot be provided in conventional macro scale culture conditions. Microfluidic systems, which can be designed by the investigator through computer technology, provide microenvironments for cell and tissue growth and maturation that are on orders of magnitude smaller dimensional scales than conventional tissue culture, sparing media, expensive reagents (including hormones, growth substances, morphogens, cytokines, siRNA, oligonucleotide morpholinos) and providing a translucent chamber, usually the size of a conventional microscope slide, which can be grown in a tissue culture incubator, but removed at intervals for microscopic observation and other assessment techniques, including electrophysiology (Torisawa et al.2009). Most of these systems employ the principle of differentially moving fluid streams, in which, for example, in a biological application, two parallel streams of fluids move over cells-- or even a single cell-- in such a manner that the differential speed of the moving fluid streams keeps them separate—so that , for example, the chemical composition of
the two streams can be kept separate and non-mixable throughout the course of the experiment. In the simplest design of such systems, two non-mixing fluid streams move over a population of cells, which subsequently differentiate or grow differently in response to the molecular cues in the media (Korin et al., 2009; Taylor and Jeon, 2011; Taylor et al., 2006; Weibel and Whitesides, 2006).

Initially, MEMS microfluidic systems were developed to “miniaturize” chemical and biochemical analyses (Weibel and Whitesides, 2006; Whitesides, 2006). Subsequently, investigtors employed inert polymers such as poly (dimethylsiloxane) (PDMS) to make increasingly complex and even multichambered and multilayered devices for a wide range of applications for the biomedical sciences (Peterson et al., 2005). For example, a novel array of U-shaped microwell chambers in a microfluidic device, which were chemically patterned using methacrylate to culture cortical neurons and observe the directional outgrowth of the neurites (He et al., 2004). The results of these experiments showed that the neurites formed a neuronal network on the chemically patterned microwells, demonstrating that the device could be a promising tool to produce morphologically and functionally regulated cultured neuronal networks (Goto et al., 2008; Takayama et al., 2011; Taylor et al., 2006). In another research and development, microfluidic devices were used in cell migration studies to investigate chemotaxis capacities of different cell types. These chemoattractant studies helped to accelerate the process of finding drug candidates and the response of different cell types to a large number of drug candidates in a high throughput process (Korin et al., 2009). A multicompartmen microfluidic neuronal cell culture chamber that was capable of fluidically isolating neuronal processes from cell bodies and in which neurites could extend through a series of microgrooves embedded in a physical barrier was designed to study the axonal migration and directional axon outgrowth (Taylor et al., 2006),(Taylor and Jeon, 2011). The use of a microfluidic device allows designs of the devices to be reproducibly programmed according to the researcher’s preferences and can be complex (ref). The fluidic environment surrounding the cells can be easily altered during experiments using a minimum of expensive media. Thus, microfluidic systems have been developed that are ideal for culturing neuronal cells and observing their behavior in “real time”. Microfluidic platforms have been used to isolate and direct the growth of
CNS axons even without the use of neurotrophins, providing a highly adaptable system for modelling many aspects of CNS neuro-degeneration and injury (Taylor and Jeon, 2011). Scientists have also cocultured oligodendrocytes and central nervous system axons in a compartmental microfluidic device to potentially investigate the mechanisms of myelination and demyelination (Taylor and Jeon, 2010). Although there are many interesting neural cell studies that have been performed using microfluidic devices, to date no one has studied embryonic stem cell (ES cell) differentiation into two different cell lineages from the same population of cells in a microfluidic device. In the studies described in this thesis, a mouse ESC population was differentiated into two different cell types—neurons and SCwann Cells—using two microfluidic streams carrying different media that are capable of inducing either neurons (Bank et al., submitted; Ramamurthy et al., submitted) or SCwann Cells (Roth et al., 2007). Such cultures were differentiated using the two inducing media in two parallel fluid streams over a common population of mouse ESC and then the two differentiated populations were allowed to interact in the microfluidic device and analyzed by microscopy (light/fluorescence/confocal) and biochemical, immunocytochemical and molecular assays during the more than three weeks in the channels over which we observed cell–cell interactions. We have therefore used microfluidic devices such as those seen in Chapter 2 to spare expensive biological reagents and media and to serve as a prelude to experiments in which the SCwann Cell like cells induced from the mESC were used to “coat” a cochlear implant and mESC-derived neurons were tested to see if they could interact with the mESC-derived SC. These studies are designed to establish the assays necessary for evaluating the interaction of the population of mESC-derived SGN like neurons; however, SAG and SGN can be placed in the devices to examine their interaction with the mESC-derived SCwann Cell population. These studies are fore-runners of experiments designed to test whether a) a mESC-derived neuronal population could be induced to become SGN-like with the “right” combination of MIF and DHA; b) whether such a population of “neuron” like cells could respond to a source of MIF, produced by SCwann Cell like cells induced from mESC by neuregulin. By observing the interactions between the two mESC derived populations in a microfluidic device, the ground work for these experiments can be established—the assays that document interaction between the two populations (e.g.
evidence of myelination or production of the appropriate neurotransmitter(s) or uptake systems) can be tested with a minimum of expensive reagent usage. The long-term goals of these experiments are to 1) determine if a SGN-like population capable of replacing injured or lost SGN in an animal model can be derived using MIF and DHA and 2) to determine if “coating” a CI with MIF producing SC-like cells will enhance the possibility that embryonic SAG neurons, adult SGN neurons (both derived from mice) or the putative mESC-derived SGN neuron like cells will a) form a larger number of contacts with the CI than an uncoated CI or a CI coated with another cell population (e.g. undifferentiated ESC) b) test whether such contacts are functional. These studies must precede any attempts to follow them in vivo. Such in vivo studies would ultimately involve implantation of the mouse-sized CIs into living animals, but such studies are well beyond the scope of the experiments presented in this thesis.

1.5 Our Research:

ES Cell based therapy for providing a SGN-like population of cells that is capable of responding to an appropriate target, an ESC-derived SC-like population. Our goal is two-fold: the first goal is to develop mESC-derived SGN-like cells by differentiating mouse embryonic stem (ES) cells using the specific neurotrophic cytokine that is unique to the development of the early inner ear, macrophage migration inhibitory factor (MIF) to produce SGN-like cells. Neural induction from stem cells depends on a) the environment, including extracellular matrix components of cells of the pathway (Pierret et al., 2010) and the target tissues present (Schmitz and Chew, 2008) b) soluble neurotrophic or cytokine factors released by the target tissue(s) (Schmitz and Chew, 2008) and c) maturation factors (such as DHA) which are also concentrated in the nervous system environment and which are known to promote neurite fasciculation and synaptogenesis (Cao et al., 2009). Neuronal precursor cells must become post-mitotic to develop into functional neurons (Okabe et al., 1996), which means that they must cease to proliferate and differentiate into cells that will no longer divide. To serve the role of missing SGN, the ES derived neurons must fulfill certain criteria: they must develop the appropriate neurotransmitter-synthesizing machinery to operate in the inner ear (that is they must become glutamatergic neurons). Assessment of whether ES cell-derived
neurons become glutamatergic is based on whether they express the vesicular glutamate transporters 1 and 2 (i.e. based on co-labeling for VGLUT1/2); they must express the appropriate MIF-responsive receptors (CD74 and possibly CXCR4/CXCR2), they must be able to take up glutamate in a recycling process (via the Vglut transporters), they must express the correct cytoskeletal proteins (e.g. TuJ1) and they must be able to release the correct neurotransmitter in response to stimulation. Just how far such ES-derived neurons will go in their maturation process, with and without DHA is unknown at this time.

Our laboratory was the first to identify a new role for MIF as that of a directional “neurotrophin-like” molecule produced by developing cells in the primitive inner ear (Shen et al., 2012; Bank et al, submitted). MIF serves as both a directional neurite outgrowth factor and a survival factor for both immature SAG neurons and mature SGN neurons (Bank et al., submitted; Holmes et al, 2011; Shen et al., 2012). In addition, other components such as DHA promote neurite outgrowth and synaptogenesis as well as the expression of synapsins and glutamate receptors, improving glutamatergic synaptic transmission (Cao et al., 2009), which is also found in SG neurons. However the combined effects of MIF and DHA in the process of neuronal development have not been studied prior to this work.

In addition, providing an in vitro microfluidic microenvironment for the cells could allow us to simulate the in vivo cellular environment, including the addition of extracellular matrix (ECM) components and the ability to expose the cells to factors at concentrations which are difficult to manipulate in traditional culture systems (by incorporating them into matrices or hydrogels of various compositions). By combining all of these approaches we can determine the effects on neuronal development and synaptogenesis, in this case with a CI in the culture. The significance of our study is that providing an artificially controlled microenvironment for mES cell differentiation into inner ear-like neurons, using a combination of cytokine (MIF) and DHA for neuronal maturation and developing ES-derived MIF producing SC-like cells with their neuronal attractant properties in the same microfluidic culture system could lead to development of strategies that could be employed in the future to replace or augment the survival of
damaged or ageing nerves in the human inner ear and a cell population that could enhance directed neurite outgrowth towards a CI simultaneously. Once the conditions for producing functional “neurons” from ESC are established, similar microfluidic platform conditions can be used for human stem cell differentiation or human iPSC differentiation into neurons or “SC” like populations for inner ear neuronal regeneration therapies. A microfluidic device is the ideal environment in which to test these features to mimic the fluidic environment of the inner ear, and spare the expensive reagents that are the inducing agents. We hope that these studies will eventually pave the way for studies of therapeutic approaches in humans, based on human ESC or iPS derived materials.

To develop SGN-like cells derived from mES cells, we used a combination of the inner ear’s neurotrophic cytokine MIF and DHA, which was added to the cultures as a differentiation and maturation factor during neuronal induction process. We have recently discovered that instead of “classical” neurotrophins already identified as playing roles in the development and regeneration of neurons at later stages in the auditory system, the immune system cytokine Macrophage inhibitory factor (MIF) acts as the inaugural “neurotrophin” and plays vital roles in the development and directional outgrowth of SGN neurons of the inner ear (Holmes et al, 2011; Shen et al, 2012; Bank et al, submitted). MIF can act as a neurotrophin for developing inner ear neurons and, most importantly, recombinant MIF (either mouse or human) can promote the development of mESC into neuron-like cells (Bank et al., submitted). In particular we are determining how a precise combination of the specific inner ear neurotrophic cytokine, MIF and DHA might regulate mES cell differentiation into neurons that resemble inner ear neurons. Our initial studies have been done in a microfluidic device by exposing a common population of mouse ESC to cytokines alone or to cytokines and DHA to determine their effect on neuronal differentiation, process extension and electrophysiological property maturation. The resulting “neuronal” properties have been characterized at the electrophysiological, morphologically and molecular levels.

The long-term objective of these studies is to enhance contact between the endogenous remaining spiral ganglion neurons (SGN) of the mature mammalian inner ear and a CI. To enhance a) the number of physical contacts between the remaining SGN
population and the CI and b) the number of functional contacts, creating a CI that “attracts” SGN during the repair process would be ideal. SCwann Cells (SC) are prominent among the tissues in the inner ear that express MIF and other supporting factors, as are the supporting cells (SuC) that underlie sensory Hair Cells (HC) in the O of C (Bank et al, submitted). However, in order for the CI to send meaningful acoustic information to the brain/CNS it must become closely coupled to the processes of as large and as healthy a population of SGN like neurons as possible (Hendricks et al., 2008). Providing a second “SGN neuron-like” population of cells derived from the same population of mESC by exposure to MIF and DHA will allow us to study the cell populations interactions both in a microfluidic device (Chapter 2) and in a tissue culture chamber more amenable to electrophysiological recordings. Improvement in the direct coupling of healthy neurons to the CI could increase the resolution of electrical stimulation and restore hearing, or at the very least, some form of speech perception (Hendricks et al., 2008). The contribution of the thesis are to develop neuron-like cells derived mES cells with mature neuronal characteristics and their ability to potentiate directional outgrowth towards the target. Also using the Schwann like cells derived from mES cells when coated on the CI provide molecular cues to support and establish directional outgrowth of the primary neurons.

1. Determine the degree of neuronal development and “inner ear neuron like” and study the SGN-like and cell –ES derived SCwann cell interaction in microfluidic systems.

2. Test the ability of DHA “enhanced” ES derived neurons to make functional contact with target cells/tissue including:

   2.1 Test the ability of ES derived SGN-like cell to make functional contact with target cells/tissue including:

       a. Isolated inner ear mouse sensory cell explants (O of C explants),
       b. Cochlear implants alone or
       c. CI carrying engineered mESC-derived “SC”.

   2.2 Test the ability of primary SGN to make functional contact with target cells/tissue including:
a. Isolated inner ear mouse sensory cell explants (O of C explants) (this has already been done; Ebisu et al, in prep),
b. Cochlear implants alone or
c. CI carrying engineered mESC-derived “SC”.
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Chapter II:

Differentiation of a common population of mouse embryonic stem cells into spiral ganglion neuron-like cells and Schwann Cell-like cells in a microfluidic device:

Studies of cell-cell interactions that might aid approaches to regenerative medicine

2.1 Abstract

This paper reports an important advance in the practical application of microfluidic technology to solving critical problems in cell biology—the application of microfluidics to achieve extremely slow flow (on the order of a few μm/s) and stable gradient generation for long-term (up to 3 week) culture and differentiation of a common population of mouse embryonic stem cells (mESC) into “neuron-like,” cells resembling those of the inner ear’s Spiral Ganglion (SG) and Schwann Cell-like cells. A combination of macrophage migration inhibitory factor (MIF), the ear’s developmental neurotrophin and docosahexaenoic acid (DHA) a fatty acid important for synaptic maturation, was introduced into one of two inlets in the device to induce “neurons”. Neuregulin was introduced into the other inlet to induce Schwann Cell-like cells. This technique, which facilitates retention of autocrine/paracrine growth factors, that allows the phenotypic development of two populations of mESC (one neuron-like, which is mature enough to display glutamatergic properties, and one Schwann Cell-like) from the same population of ESC, can also be used to study interactions between the two differentiated cell types. Studies of these interactions provide vital information that can be used to devise means to replace lost SG inner ear neurons, and thus, to restore hearing.
2.2 Introduction

Development of new stem cell-based approaches is bringing the era of regenerative medicine into closer proximity and more immediate focus. For example, a current area of intense interest to neuroscientists and bioengineers is the potential replacement of lost or diseased central and peripheral neurons or Schwann Cells with biologically engineered stem cells. Application of recent microfluidic technological advances and now practical proofs of principle to stem cell studies have helped to bring the onset of this era in regenerative medicine even closer. In this study, we take a microfluidic approach differentiate a common population of embryonic stem cells into two different types of cells—neurons and Schwann Cells, which could be used independently or together to provide therapeutic approaches to alleviate deafness.

The mammalian inner ear, including that of humans, is vulnerable to genetic disorders and aging, as well as to injuries caused by noise overstimulation, ototoxic drugs (cisplatin), and viral infections (Hu and Ulfendahl, 2006). Deafness typically results from irreversible cochlear sensory hair cell (HC) death followed by the death of the spiral ganglion neurons (SGNs) that innervate them (Alam et al., 2007); however, in humans this decline is gradual and can take years to occur. Hearing aids can partially restore lost hearing if HC function remains and neurons are not severely compromised, but CI implantation remains the only viable option in the event of severe HC loss or functional impairment in humans. There are limitations to CI functional success, which depends primarily on the number of healthy SGNs present in the patient’s cochlea and the extent of connections made by these SGN with the CI. Due to the lack of regeneration of both HC and SGN in the adult mammalian inner ear, cell replacement therapy strategies have been proposed to compensate for degeneration and loss of either sensory HC (Zheng and Gao, 2000) or neuronal cells (Martinez-Monedero and Edge, 2007) or both. Introduction of embryonic stem cell-derived neurons into the inner ear has been partially successful(Kesser and Lalwani, 2009) and small populations of such exogenous cells can
survive, migrate, differentiate, and even extend neuritic projections in the auditory system of adult mammals. These results suggest that cell replacement therapies could provide an effective future treatment alternative for hearing loss and other inner ear disorders (Hu and Ulfendahl, 2006) especially when coupled with a functioning CI.

Embryonic, adult, and induced pluripotent stem cells (iPSC) are being investigated as potential sources of regenerative cells that could be used to treat spinal cord injury and neurodegenerative diseases (Odorico et al., 2001). Effective neurological regeneration also requires the presence of soluble biomolecules such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), macrophage inhibitory factor (MIF), and docosahexaenoic acid (DHA), which promote cell differentiation of neural precursors (Calderon and Kim, 2004; Glomset, 2006; Ma et al., 2010). Cell-cell interactions with Schwann cells are also crucial to neural growth and regeneration (Golden et al., 2007) and importantly, the presence of Schwann cells and BDNF or NT-3 has a synergistic growth effect over either condition alone (Pettingill et al., 2008).

Better understanding the neural precursor cell microenvironment is critical to engineering platforms that can be applied to developing regeneration techniques. This is especially challenging in the context of neural differentiation because it requires long culture (days to weeks) and spatial control over cell positioning and soluble factor concentrations. Here we utilize a previously-reported microfluidic device to study the effects of both juxtacrine and soluble factor signaling on mESC differentiation. The osmotic pump is essential to achieving sufficiently slow flow that does not cause cell detachment and allows us to culture cells in devices for up to three weeks. The device was designed to generate overlapping gradients of neurotrophic factors from two different inlets that differentiated a common population of mESC into neuron-like and Schwann cell-like subpopulations. By stably maintaining flow over three weeks, we were able to correlate cell differentiation and neuronal outgrowth to computed concentrations of different factors. This strategy of developing and characterizing neuronal maturation is innovative and useful for a dual stem cell therapy approach for hearing restoration—deriving a SGN-like neuronal population to replace lost or damaged SGN and a Schwann
Cell population that, because of its attractive properties could be used to “coat” a CI, enhancing neuronal functional connections with the CI.

2.3 Materials and methods

**Differentiation of mES cell into neuron-like cells in vitro**

Cell lines: The cell line used for these studies was the D3 mES cell line (ATCC-CRL-1934). Media Preparation: Proliferating (ES): 81% DMEM (Invitrogen) without phenol red, 1% L-GLut, 1% Pen/Strep, 1% nonessential amino acids (Invitrogen), 15% FBS (Atlanta Biological, GA), 1% sodium pyruvate (2% stock Sigma Aldrich, USA), 7 µL/L mercaptoethanol (Sigma Aldrich, St. Louis, MO), 1,000 U/mL ESGRO (Chemicon, Temecula, CA). Neuronal differentiation: 95% F-12 (Invitrogen), 1% Pen/Strep (Invitrogen), 1% N2 (Invitrogen, CA), 2% B27 (Invitrogen, CA), 2% sodium pyruvate (2% stock), 0.5 µg/L bFGF (R&D Systems), 1 µg/L IL-7, 5 µg/L IGF-1 (Sigma), or 1 µg/L CNTF (R&D Systems) or 1 µg/L NGF (Chemicon, CA) or MIF 1 µg/mL (R&D Systems), ± 5 µM DHA (Nu-check Prep Inc., MN, USA). DHA supplemented media included: CNTF and 5 µM DHA, NGF and 5 µM DHA, MIF and 5 µM DHA.

**Differentiation conditions for directed differentiation**

10,000 cells were plated onto 0.1% porcine gel coated (Sigma) 48 well Primaria® plates (Falcon) and the media conditions shown in Table 1 were used for differentiation over a three-week period. Undifferentiated mES cells were fed daily, while cells in neuronal differentiation media were fed every third day (Table 1). After three weeks of growth, cells were labeled for expression of neurofilament marker (NF150) (neuronal differentiation marker) and cells were examined by RTqPCR for changes in gene expression of a variety of neuronal markers (Table 2). Cells were then fixed in 4% paraformaldehyde (Sigma), and followed by primary antibody NF150 (Chemicon, Temecula, CA) at 1:400 dilution and secondary horseradish peroxidase (HRP) conjugate or Alexa fluor 480 (Invitrogen, CA) at 1:500 dilution. Photomicrographs were taken with light microscope or using appropriate filters on a Leica inverted fluorescent microscope. Antibodies were diluted in 5% normal goat serum (Chemicon) in 0.1%
Isolation and culture of dissociated mouse primary Statouacoustic Ganglion Neurons (SAG)

SAG from 15.5 to 17.5 day Balb/C mouse embryos were removed from the dam’s uterine horns and the SAG dissected from the 2 ears and rinsed in PBS. The SAGs were isolated (Bianchi and Raz, 2004) and collected in 15mL of DMEM. The collected SAGs were then centrifuged at 1500 RPM for 10mins. DMEM media was aspirated and 1ml of 1:10 diluted trypsin EDTA added and incubated at 37°C for 10mins. 3mL of DMEM media was added to stop the trypsinisation and the SAGs centrifuged at 1500 RPM (Damon IEC centrifuge) for 10mins. The dissociated SAGs was resuspended in F12 media and cultured for 3 weeks. The cells were fixed in 4% PFA for ICC staining and were examined for gene expression by RTqPCR.

Differentiation of neuron-like cells and SCwann Cell-like cells in a microfluidic device

Schwann cell media: 500 mL alpha modified MEM(GIBCO 41061-029), 6% Day 11 chick embryo extract (prepared in house), 10% Heat Inactivated FBS, 58uL Gentamycin (Invitrogen, Federick, MD), 10uL Neuregulin1 (NRG1) (R&D Systems, MN). The microfluidic device was designed according to previous specifications.(Park et al., 2009) Microfluidic chambers and tubing were made hydrophilic and sterilized by oxygen plasma and UV treatment, respectively. Devices were pre-filled with PBS by inserting a 1000 μl pipette tip into the outlet and allowing PBS to fill the chamber by gravity-driven flow. Air bubbles were avoided by maintaining liquid (PBS, culture medium) on top of the device, thereby preventing bubble formation due to evaporation or due to connection of pipette tips. Devices were coated with 0.1% porcine gel for 2 h under UV light before cell seeding. mEScells were trypsinized and seeded into devices at a density of 10,000 cells/mL and allowed to adhere overnight at 37°C and 5% CO₂. When the mESC were fully attached, an osmotic pump was connected to tubing at the outlet channel and 1 ml pipette tip reservoirs containing either neuronal differentiation or
Schwann cell differentiation media were inserted into the appropriate inlets (Park et al., 2009). In the control experimental studies both the inlet were fed with the neuronal differentiation media and compared with the experimental conditions. Devices, including osmotic pumps and inlet and PEG reservoirs, were incubated at 37°C and 5% CO₂ over three weeks with the PEG and inlet reservoirs being replaced once every 7 days. Cells were fixed with 4% PFA and labeled with either a) antibody to neurofilament heavy chain protein (200kDa), a general marker for neurons, b) VgluT1, a marker for mature “auditory system-like” neurons, or c) myelin basic protein, a marker for Schwann cells. Fluorescent images were taken of 5 sections of the device to observe the overall pattern of neurite outgrowth from the neuron-like cells derived from the mESC and neurite extension over the mESC-derived Schwann Cell-like cell lawns in the other side of the device.

**Medium viscosity measurement**

Schwann Cell-inducing and F12 medium kinematic viscosities (ν) were measured using a Scott ViscoSystem® AVS 350 and Ubbelohde type viscometer (#713). Briefly, the viscometer was filled with 14 mL of either Schwann Cell or F12 medium, connected to the ViscoSystem®, and submerged in a 37°C water bath. Retention times of the liquids in the viscometer were measured in quadruplicate. Kinematic viscosities were calculated by multiplying the correction factor of the viscometer (0.0099 cSt/s) by the average retention times (s). Densities of each medium were measured by obtaining the mass of 1 mL of medium; measurements were made in triplicate and densities averaged. Dynamic viscosity (μ) was calculated by multiplying kinematic viscosity and fluid density: μ = νρ. Volumetric flow rate in the tubing (Q_{tubing} = 0.00318 mm³/s) was calculated by dividing the volume of the inner tubing (1700 mm x 1.13 mm² = 1922.66 mm³) by the retention time (7 d, 604800 s). Channel velocity (0.00397 mm/s) was calculated using conservation of mass principles: v_{channel} = \frac{Q_{tubing}}{A_{channel}}, where A_{channel} is the cross-sectional area of the channel (4 mm width x 0.2 mm height = 0.8 mm²). Because of the low aspect ratio of the main channel (0.05), flow is assumed to be Hele-Shaw flow and wall shear (τ_{wall} = \frac{6μν}{A})
is calculated from average dynamic viscosity \((\mu)\), flow velocity \((v)\), and channel height \((h)\).

**COMSOL simulation**

Microfluidic device geometry was imported into COMSOL Multiphysics 4.2 (COMSOL, Inc.). Fluid density and viscosity were set based on previous measurements and laminar flow average velocities were set at the two inlets and outlet based on volumetric flow measurements as described in “Medium viscosity measurement” To ensure a fully developed profile, entrance and exit lengths were set to 1000 times greater than \(0.06ReD\), where \(Re\) and \(D\) are Reynolds number of flow and tubing diameter, respectively. A laminar flow solver was used to generate flow profiles which were then called by the dilute species transport model to analyze concentration profiles of relevant growth factors and cytokines. Diffusion coefficients were selected to match previous results for molecules with similar molecular weights: \(1.5\times10^{-10}\), \(4.25\times10^{-10}\), and \(1.27\times10^{-10}\) m²/s for MIF (Durand et al., 2009), DHA, and NGF (or CNTF, since molecular weights are similar), respectively. A free quad mesh with minimum element size of \(3.1\times10^{-7}\) and maximum element size of \(0.154\) mm, 1.1 maximum element growth rate, 0.2 resolution of curvature, and 20 resolution of narrow regions was generated to fully capture flow and concentration profiles within the channel for steady flow.

**Neurite measurement**

The MetaMorph© “Neurite Outgrowth Measurement” program was used to measure number of cells, total neurite outgrowth, total number of processes, total number of branches, and total number of outgrowths. Briefly, thresholds were adjusted so intensity levels for fluorescent detection were 50% above the background grayscale (MetaMorph® instruction manual (Universal imaging corporation, documented: T20029)). Settings were then optimized for cell body size before calculating the above parameters.

**Quantitative RTqPCR for directed differentiated neuronal cells**
Gene expression levels for directed differentiation of neurons: RTqPCR was performed using primer pairs designed using the Beacon designer program (BioRad, Hercules, CA), with target Ta at 55°C, length 18–22 bp, and amplicon size 100–200.

**Neurogenic differentiation**

Gene expression was normalized to GAPDH. Quantification of marker expression was assayed by extracting total RNA from the samples using Qiagen mRNA Kit 74106 (Qiagen, Valencia, CA). RT-PCR was performed as follows: cDNA was synthesized from 2 µg total RNA by reverse transcription using Super Script III transcriptase (Invitrogen, Carlsbad, CA) and oligo dT primer. The PCR conditions included an initial denaturation at 94°C for 1 min, 94°C for 1 min, 55°C for 30 s and 74°C for 30 s and 34 cycles and final extension at 72°C for 5 min and 4°C holding temperature.

**Statistical analysis**

For quantification of the data, all the experiments were run in triplicates and the values are expressed as MEAN ± SD. Statistical significance was determined for each differentiation condition with and without DHA and compared with F12 basal media as control. The results were analyzed using Minitab16 statistical software by one way ANOVA followed by the Dunnett comparison method. p-values less than 0.05 were judged to be statistically significant.

**2.4 Results and discussion**

**Differentiation of mESC in co-cultures of “neurons” and “Schwann Cells” in a microfluidic device**

We differentiated mES cells into both SGN neuron-like and Schwann Cell-like cells on the same microfluidic platform to study cell-cell interactions between the Schwann cells and the neurite outgrowth from the neuron-like cells. The microfluidic system was designed to establish a gradient flow to nourish the cells with nutrients and growth factor or cytokines in order to facilitate the differentiation of two different cell populations (Fig. 2.1a) from undifferentiated mES cells (Fig. 2.1b) seeded on the device.
Figure 2.1: a, b, c, d. The mES cells differentiated into SGN-like cells and Schwann cells in the microfluidic device. The device is sectioned into 5 rows (I - V) to take the photomicrographs. The cells were stained for neurofilament 150kDa- neurites (green); Dapi-nucleus (blue); Myelin Basic Protein – Schwann cell (red). The top three rows show significant cell differentiation and direction outgrowth, whereas the bottom two rows with most diffused gradient of the media the cells didn’t differentiate into any specific lineage; b. Undifferentiated mES cells in the microfluidic device; c. The observation window overlay for photomicrographs; d. The microfluidic chip channel dimension and gradient flow illustration.

To see if there was preferential neurite outgrowth of the SGN like cells toward Schwann Cell like cells in the microfluidic device (Fig. 2.1a), we virtually sectioned the device into 5 rows (I, II, III, IV, V) and the middle rows II, III and IV were divided into
three columns (a, b, c) for taking photomicrographs (Fig. 2.1c). The mES cells showed concentration-dependent differentiation along the width of the channel (left inlet-neuronal differentiation media); and neurons near the left inlet (Ia) whereas the neurites projected toward the right side of the channel toward the Schwann-cell like cells because there was lower concentrations of cytokines and/or growth factors in the left region of the device. These SGN-like cells near the right inlet (Ib) had multiple instances where the neurites grew over the lawn of Schwann-like cells with multiple branch points (Fig. 3a). The SGN-like cells differentiated in the mid-section and third row (IIB and IIIa, b, c) had minimal numbers of neuronal processes; the few processes present in the IIIb and c sections grew directionally towards the Schwann Cell-like cells. The cells in the row IVa, stained for neurofilament but no processes developed in this region and the cells in the IVb and IVc stained mostly for MBP. The cells in the region V didn’t express any marker for Schwann cells or neurons. The gradient profile created in the device were getting enriched respective media near the inlets facilitate that the ES cells to differentiate them into SGN-like cells and Schwann-like cells with higher expression of their specific protein markers. The neurite outgrowth in the midsection had most fasciculation when compared with the other sections of the device. The experimental conditions was compared with the control experiment where both the inlet was fed with neuronal differentiation media and the cells experiment profound neuronal differentiation with neuronal processes, but no directional outgrowth (Supplemental Figure2.1). The cells grown in Petri dishes were static cultures which are differentiated into neuronal lineage with every other day of supplementing with fresh media whereas in the microfluidic device we created an microenvironment in which the cells can be differentiated into two lineage and further we could study the interaction of the two cell types. Particularly, the slow flow feature of our device (that is nearly similar to the interstitial flow regarding diffusion strength) is physiological to neuron cells. Neurons are usually developed and stay there for their whole life separated from high flows (blood flow or any other flows in body). It is important to notice that even in such flow-free environment; neurons still require nutrient supply and waste-clearing by minimal convective flow, which is also achieved in our slow flow device. Stability of the flow in the channel is guaranteed since the operation of the flow is based on simply osmosis which is known to be a very stable
phenomenon. The overall pattern of the two differentiated cell types on the device suggests that the SGN-like cells grown with the Schwann Cell-like cells exhibited directional outgrowth towards the target cells, the Schwann Cell-like cells.

Fig. 2.2a. Observations of SGN-like cells and Schwann Cell-like cell interactions in the mid-section of the microfluidic device. The cells were stained for neurofilament Heavy 200 kDa (NF-H) (blue) VgluT1 (red) Myelin Basic (green) Protein. (4b) The photomicrographs of the cells in the device were analyzed in the mid-section of the row II. Row II is shown in Fig 2.1.

For more specific analysis of neurite outgrowth in the devices, each device was virtually divided into 5 rows along the gradient axis (refer Fig. 2.1c) for photomicrographic sampling (Fig. 2.2). Although photographs were taken in all these virtual arbitrarily-assigned sections of neurite outgrowth, only the mid-section of the second row (IIb) in the device, which is marked with a black box in the figure was analyzed and compared among neuronal differentiation conditions using MetaMorph® neurite measurement software. Differentiated cells were stained for VgluT1 and Neurofilament 200 for neurons and myelin basic protein (MBP) for Schwann cells in the device to analyze SGN-like cell - Schwann Cell-like cell interactions (Fig. 2.2). ICC shows that the differentiation conditions under which cells were treated with neurotrophins, MIF, neurotrophin+DHA, or MIF+DHA have more neurite outgrowth than the F12 Basal condition. The differentiation conditions with neurotrophins or with MIF supplemented with DHA have fasciculated bundles of neurites and all stained for
both VgluT1 and Nfi1200kDa whereas few neurites stained for both VgluT1 and Nfi1200 under conditions with CNTF, NGF, MIF and DHA alone. All the differentiated SGN-like cells in the mid region of the device showed healthy neurite outgrowth over the lawn of Schwann cell-like cells whereas cells treated with CNTF+DHA, NGF+DHA and MIF+DHA showed robust neurite outgrowth and created a meshwork of neurites on the lawns of Schwann cell-like cells (Fig. 2.2). The robust neurite outgrowth with multiple branches and the VgluT staining of neurites in the device suggest that SGN-like cells when differentiated with supporting Schwann cell-like cells have substantial improvement in their glutamatergic properties.

Figure 2.3: Neurite measurements of the SGN-like cells differentiated in the microfluidic device. The neurites of SGN-like cells cocultured with Schwann Cell-like cells in the microfluidic device were measured using MetaMorph® software for total processes, total outgrowth and total branches for the mid-section of the photomicrograph image. Number of replicates (N)=3.

The photomicrographs of the neurite measurement of differentiated SGN-like cells in the microfluidic device were analyzed using MetaMorph® software to measure total “length” of outgrowth and total numbers of branch points. Even though the neurites were grown in micrometer length in the microfluidic device, but the pictures were taken at magnification of 20x in the Leica inverted microscope and using the MetaMorph® software we analyzed the pictures at the maximum pixel size which represents the neurite measurement at millimeters in length. The mean outgrowth was not normalized to a cell number because the neurite outgrowth normally tends to differentiate from the cluster of ES cells. The conditions including treatment with CNTF alone, CNTF+DHA,
NGF+DHA and MIF+DHA had significant increases in neurite outgrowth when compared with F12 treated condition whereas only NGF+DHA and MIF+DHA had a significant increase in neurite outgrowth and total branch numbers compared to conditions without DHA (Fig. 2.3). The increase in neurite outgrowth and branches suggest that Schwann Cell-like cells had a greater influence on SGN-like cells’ maturation than neurotrophins or cytokines alone.

**Differentiation of SGN-like cells**

We first differentiated mES cells with varying concentrations of cytokines (MIF) compared to other growth factors (CNTF, NGF) with and without DHA in a conventional tissue culture system to find the optimum concentrations of factors to be added to the media to obtain more than 75% neuron-like cells based on Neurofilament150kDa ICC staining (Supplemental Fig.2.1). After three weeks of mES cell growth under the different neuronal differentiation conditions the cells had the morphology of neural cell body clusters with emerging long neurites. MIF alone can act as a neurotrophin as can CNTF or NGF. However, 5 μM DHA additions to the growth factor or cytokine in combination induced both extensive fasciculation and branching.

**Neurite measurement during directed differentiation**

We differentiated the cells under the conditions represented in Table 1; NGF-treated cells were found to have thinner and shorter neurites and fewer branch points in contrast to conditions supplemented with DHA which exhibited fasciculated bundles of neurites with extensive branch points. MIF alone or MIF+DHA can differentiate mES cells into neuron-like cells as do NGF or CNTF (Supplemental Fig.2. 1). The photomicrographs were analyzed using MetaMorph© software to measure (1) the total number of processes (units: 1 pixel = 1 micron); (2) the total outgrowth length and (3) the total number of branch points. The mean values were calculated for the above three parameters by counting the cell body (cell clusters) in the photomicrograph and averaging the parameters.
Figure 2.4a,b,c: Neurite measurements using MetaMorph® software on the differentiated SGN-like cells. F12: Neuronal basal media, C: CNTF differentiation condition, N: NGF differentiation condition, M: MIF differentiation condition, D: DHA differentiation condition, CD: CNTF+DHA differentiation condition, ND: NGF+DHA differentiation condition, MD: MIF+DHA differentiation condition. * indicates a significant difference between differentiation conditions with DHA addition compared with F12 condition. Number of repetitions (N)= 3.

The neurite measurements showed increases in all three parameters under differentiation conditions with cytokine (MIF), growth factors (GF) (e.g. CNTF, NGF), or cytokine/GF supplemented with DHA when compared with cells grown in F12 basal medium. The total branches under the conditions with DHA alone, MIF alone, CNTF+DHA, NGF+DHA and MIF+DHA show significant increases in neurite branching compared to F12 conditions. However, only CNTF+DHA and NGF+DHA showed significant increases in the total branches compared to NTs alone (Fig.2. 4a).
These measurements indicate that there is a statistically significant increase in the total neurite outgrowth under all neuronal differentiation conditions compared with F12 condition. CNTF+DHA, NGF+DHA and MIF+DHA showed significant increases in neurite outgrowth compared with conditions without DHA addition (Fig. 2.4b). DHA alone, CNTF+DHA, NGF+DHA and MIF+DHA produced significant increases in total processes compared to F12 condition but there was not a significant difference between conditions with DHA and without DHA (Fig.2.4c). All the neurotrophins including MIF promoted neurite outgrowth and differentiated mES cells into neuron-like cells.

**Gene expression studies for the differentiated SGN-like cells**

In addition to neurofilament protein staining, we examined the expression of different gene markers specific for SGN (Table 2.2). These gene expression studies were performed for Oct4, a key factor of an expanded transcriptional network that governs pluripotency and self-renewal in embryonic stem cells and in the inner cell mass from which ESCs are derived (Zuccotti et al., 2011) neurogenin-1 (Ngn-1), which appears to control the decision of embryonic neural stem cells to enter the neuronal differentiation program with a bias towards sensory neurons (marker of early differentiation);(Korzh and Strahle, 2002) and markers for mature neurons: TrkB(Lim et al., 2011; Martinez et al., 1998; Otal et al., 2005), TrkC (Bartkowska et al., 2007), and Peripherin (Liu et al., 2010; Martinez-Monedero et al., 2008; Nayagam and Minter, 2011).
Figure 2.5: RTqPCR shows OCT4 expression in undifferentiated mES cells and SGN-like neurons differentiated from mES cells. D3: Undifferentiated condition, SAG: Satouacoustic spiral ganglion neuron culture. 2b. Expression of proneural marker genes in SGN-like cells. Ngn1, NeuroD and Nfil expression is much higher in neuronal differentiated cells, but not detected in undifferentiated condition (D3ES). 2c. Expression of mature neuronal marker genes TrkB, TrkC, Prph in the SGN-like cells are upregulated in all the neuronal differentiated conditions and much higher expression in the conditions with DHA addition. F12: Neuronal basal media, C: CNTF differentiation condition, N: NGF differentiation condition, M: MIF differentiation condition, D: DHA differentiation condition, CD: CNTF+DHA differentiation condition, ND: NGF+DHA differentiation condition, MD: MIF+DHA differentiation condition. Expression of auditory neuronal markers, GabaAR, VgluT1 and SP19 are upregulated highly in the neuronal differentiated conditions than the undifferentiated condition and F12 condition. VgluT1 expression is much higher in the MIF+DHA condition than the other differentiated conditions.

Oct4 expression was upregulated under proliferative conditions and was downregulated under differentiation conditions (Fig. 2.5a). Cells treated with cytokines and
growth factors alone showed 2-fold down-regulation of OCT4 gene expression and cells treated with DHA along with cytokine and growth factors showed four-fold OCT4 down-regulation when compared with the undifferentiated conditions.

In general, neurotrophins are thought to provide molecular signals that mediate the survival of neurons. (Reichardt, 2006) In mice, formation of ganglion neuron precursors and developing cochler neurons express the proneural gene Ngn-1, NeuroD and neurofilament (Nfil) is an early event in ear development. (Fritzsch et al., 2002; Kim et al., 2001; Ma et al., 1998) Ngn-1 expression was upregulated under all neuronal differentiation conditions when compared with F12 basal media and the undifferentiated conditions (Fig.2.5b). Neurogenin1 had no effect when we compared conditions with and without DHA. Except for MIF, CNTF+DHA, and NGF+DHA conditions, Ngn-1 expression under all other differentiation conditions showed a significant upregulation. The expression of Ngn-1 under all neuronal differentiation conditions suggests that the cells were becoming neuron-like. Expression of NeuroD was upregulated in cells treated with MIF, CNTF+DHA, NGF+DHA, MIF+DHA (Fig.2.5b). NeuroD expression suggests that MIF alone can support the differentiation and survival of mES derived-neurons. CNTF, MIF, CNTF+DHA, NGF+DHA, MIF+DHA significantly increased NeuroD expression when compared with the F12 basal condition. Nfil expression was upregulated significantly under all the differentiation conditions compared with F12 basal condition. Nfil expression was up-regulated in cells treated with CNTF+DHA NGF+DHA and MIF+DHA when compared with conditions without DHA (Fig.2.5b). Proneuronal gene marker upregulation in cells treated with MIF alone and MIF+DHA suggests that the cells’ expression profile is consistent with what can be expected of expression in SAGs.

A number of transcription factors (GATA3, Brn3a, Ngn1, NeuroD, islet1) as well as receptors for neurotrophins (TrkB and TrkC) have been defined as mature markers for early auditory and vestibular neurons (Farinas et al., 1998). Peripherin is also expressed concomitantly with axonal growth during development, and its synthesis appears necessary for axonal regeneration in the adult (Portier et al., 1993). TrkB, TrkC and Prphn were significantly upregulated under conditions of DHA supplementation of growth factors or cytokine compared to cells treated with no DHA (Fig.2.5c). Increase in
expression of mature gene markers in cells treated with neuronal differentiation factors along with DHA supplementation suggests that, as expected from the studies of others, DHA enhances the maturation of neurons. The cells differentiated with MIF+DHA have mature neuronal properties demonstrated by expression of these mature neuronal gene markers.

Neurotrophins influence the expression of voltage-gated ion channels and regulate the expression of inhibitory and excitatory neurotransmitter receptors (Sun, 2009). For example, NT-3 promotes the expression of GABA receptors on SGN and enhances the amplitude of GABA-induced currents in neonatal SGNs (Sun, 2009). The GABAergic system is thought to modulate the response of these auditory neurons and functional GABA-A receptor is present on primary afferent auditory neurons (Malgrange et al., 1997) Vesicular glutamate transporter 1 (VGLUT1) is strongly expressed by most if not all glutamatergic neurons in the auditory cortex and at variable levels among auditory subcortical nuclei (Hackett et al., 2011). SP19 or NaV1.1 is weakly expressed in the SGN cell body with strong axonal localization.

GABA-A R was significantly upregulated under conditions of DHA supplementation of growth factor or cytokines when compared with conditions without DHA addition. Vglut1 and Sp19 expression were significantly upregulated under all the conditions tested when compared with F12 alone. NGF+DHA and MIF+DHA produced more significant upregulation of Vglut1 and Sp19 gene markers than NGF and MIF alone (Fig. 2.5d). Increases in expression of all three auditory markers’ expression levels under MIF+DHA conditions suggests that differentiated SGN neuron-like cells have at least some of the properties of auditory neurons, in addition to electrical and biochemical properties of SGN (data not shown).

**Shear stress**

Fluid properties of SCwann Cell-inducing and F12 medium are recorded in Table 3. Because the resulting dynamic viscosities of SCwann Cell and F12 medium were very similar, an assumed uniform dynamic viscosity of 0.008 g/cm·s was used in channel shear calculations. Wall shear \( \tau_{wall} = \frac{6 \mu \nu}{h} \) is calculated as 0.000954 dyn/cm².
2.5 Conclusion

We have differentiated mESC into cells with some properties of SGN using the cytokine MIF as a neurotrophic factor and DHA as an enhancer to produce mature neurons. The extremely slow flow maintained in the microfluidic device over 3 weeks allows for the accumulation of autocrine and paracrine factors that would ordinarily be washed through the device if the flow rate were accelerated. This also allows for interactions between the two differentiated cell populations. Differentiation with a combination of MIF+DHA produced a neuron-like cell population with many of the attributes of the spiral ganglion. MIF and not classical neurotrophins serves as the first neurotrophin on which inner ear neurogenesis and development depends. (Holmes et al., 2011) MIF also supports a robust increase in neurite outgrowth, and an increase in number of processes and branch points as well as neuronal fasciculation, which could ensure, at the very least, that more contacts would ultimately be made with a CI. The down-regulation of proliferative gene markers like OCT4 and increase of expression of pro neural and mature neuronal differentiation markers is consistent with MIF directing these cells to differentiate into neurons. DHA addition under neuronal differentiation conditions increases the chance of directing/influencing or inducing a majority of the cells to differentiate into mature neuron-like cells. The expression of auditory system gene markers suggests that these cells have gone some way towards differentiation into SGN-like cells, suggesting that these differentiated cells will have SGN-like electrophysiological properties, which preliminary data (not shown here) confirm. SGN-like-cells especially when differentiated with supporting Schwann cell-like cells in the microfluidic device exhibit directional outgrowth towards the Schwann Cell-like cells and have substantial improvement in their glutamatergic properties. The increase in neurite outgrowth and number of branches suggests that the Schwann Cell like cells had greater influence on SGN cell-cell interactions. mES cells can be differentiated into SGN-like cells using the cytokine MIF and with DHA addition, the neurites become functionally mature. When SGN-like cells grow along with the Schwann Cell like cells, a directional outgrowth towards the target
tissue is seen, suggesting that if the Schwann Cell-like cells were used to “coat” a CI, contacts with the CI would be enhanced.
Supplemental Figures:

Supplemental Figure 2. 1. mES cell neuronal differentiation conditions with and without DHA. The cells were stained for Neurofilament 150kDa protein (red)
Supplemental Figure 2.2: mES cells differentiated using different growth factors (NGF, CNTF) and the Cytokine (MIF) with and without DHA. The left column show ICC staining for Nfil 150kDa protein (green) in the SGN-like cells. Right column indicates the MetaMorph® neurite measurement overlay of the neurites.
Supplemental Figure 2.3: Control experiment - neuronal differentiation media in both the inlet.

Supplemental Figure 2.4: The close up view of the directional outgrowth in the channel.
Table 2.1: Differentiation conditions.

<table>
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<th>Proliferation media</th>
<th>Differentiation media</th>
<th>Differentiation media with DHA supplement</th>
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<tbody>
<tr>
<td>ES media</td>
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<td>F12 base + CNTF + 5mM DHA</td>
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<tr>
<td>F12 base</td>
<td>F12 base + MIF</td>
<td>F12 base + MIF + 5mM DHA</td>
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<tr>
<td></td>
<td>F12 base + NGF</td>
<td>F12 base + NGF + 5mM DHA</td>
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<td></td>
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<td>F12 base + 10mM DHA</td>
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<td>Tyrosine kinase receptor3 (TrkC)</td>
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<td>Vesicular glutamate transporter 1 (VgluT1)</td>
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Table 2.3: Shear stress measurement.

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<th>Density (g/cm³)</th>
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2.6 References


Chapter III:

Development of two Stem cell-based therapies to ameliorate hearing impairment or potentiation of a Cochlear Implant

3.1 Abstract

Sensory neural hearing loss (SNHL) occurs when the sensory neurons of the inner ear are deprived of peripheral targets including sensory Hair cells (HC) of the inner ear. If sensory neurons are lost due to ageing (presbycusis), disease or loss of peripheral targets, therapies to remedy this situation are very limited. An adequate number of functional spiral ganglion neuronals (SGN) is required to provide input to the only known “cure” for deafness in the case of mechanosensory HC loss—a cochlear implant (CI). There are several limiting factors involved in restoration of hearing and/or speech recognition in patients who receive a CI. The main problem is that the number of healthy existing SGNs available to grow towards the CI may be limited and secondarily, achieving close enough proximity of SGN neuritic processes to the electrode of the CI in order to make contact and establish lasting functional connections is critical; if few SGN reach the CI, this can limit optimal CI performance. To improve CI efficacy in patients who receive this therapy—1. Either preservation of existing SGN in sufficient numbers to make and sustain functional contacts needs to be achieved, possibly through enhanced electrical activity or provision of neurotrophins to the SGN either at the site or at the CI site itself or 2. An indigenous (e.g. inner ear stem cell) or an exogenous cell substitute for lost SGN should be found. The possibility of implanting SGN-like cells derived from external cell sources—including embryonic or adult stem cells or iPS cells, which, if they sufficiently resembled the lost SGN chemically, electrophysiologically and morphologically has been a goal of recent research in many laboratories, including ours. Here we demonstrate, in in vitro studies, that neuron-like cells with some significant
A feature of SGN can be derived from (mouse) embryonic stem cells (mESC). Characterization of such populations of mESC-derived cells is a prelude to studying their interactions with suitably designed CI in vitro, with a view to implanting both mESC-derived “neurons” with specific properties resembling SGN as one potential future cell replacement therapy. However, a second approach, also presented in this report, involves a second mESC based therapeutic strategy, in which a CI is coated with a hydrogel containing mESC-derived Schwann Cell-like cells mESC-SC. What is important about the mESC-SC is that they produce a neurotrophic cytokine, commonly produced by all vertebrate Supporting cell (SuC), which we have shown to be the first neurotrophin produced by the developing inner ear (Holmes et al., 2011; Shen et al., 2012; Bank et al, submitted). Both the neuron-like cells that could replace SGN and the SC-like cells that could provide directional cues for a remaining population of SGN or the mESC-derived neuron like cells are derived from the same population of mESC (Ramamurthy et al, submitted). A SC-coated CI could provide trophic support and neuron-directing molecular cues to attract existing SGNs towards the CI.

These two-pronged studies might presage a future improvement in hearing restoration in SNHL patients, who either receive replacement ESC-derived or iPS-derived “neuronal” populations, “SC” “coated” CI or both.

3.2 Introduction:

The inner ear spiral ganglion’s bipolar neurons provide the conduit for sensory messages from peripheral sensory receptors of mechanosensory hair cells to the central neurons of auditory brain stems nuclei. When the HCs of the Organ of Corti (O of C) are lost, SGN lose their peripheral targets and can progressively degenerate, so that the remaining SGN are less capable of functional interaction with a CI. Although SGN loss in humans is less pronounced than in animal models of HC loss or Knock out models of HC loss (Bermingham-McDonogh and Rubel, 2003; Bodmer, 2008; Breuskin et al., 2008), it appears that even if SGN remain as long as 40 years post onset of deafness, their functionality is questionable (Sato et al., 2006; Wise et al., 2010a) (Jiang et al., 2006). In order to interact productively with a CI, the surviving SGNs must be healthy and sufficiently well-distributed along the cochlea to cover the range of frequency
distribution required to deliver the necessary information for speech discrimination (Parkins, 1985). Although conductive hearing loss can be remediated by using hearing aids, middle ear implants and bone anchored hearing aids, a direct stimulation of the auditory nerve is required when SNHL is severe. Such stimulation can be achieved by implanting cochlear prostheses in the scala tympani of the cochlea.

Several animal models have been used to study restoration of hearing function by treating deafened animals with electrical stimulation and providing neurotrophin to the remaining SGNs (Thompson et al., 2010; Winter et al., 2007; Wise et al., 2011a). To restore hearing function and prolong survival of existing SGNs, some researchers have developed paradigms in which a single neurotrophins or a cocktail of neurotrophins were administered to deafened animals using mini osmotic pumps or by via cochlear implants coated with various gels/hydrogels that can slowly release such neurotrophins (Jhaveri et al., 2009; Jun et al., 2008; Winter et al., 2007; Winter et al., 2008). However, these approaches have met with limited success and these treatment options have not yet progressed to clinical trials in SNHL patients (Miller et al., 2002; O’Leary et al., 2009a; Pettingill et al., 2007a, b). The only clinical therapy presently approved by the FDA is surgically implanting “conventional” cochlear prostheses in patients judged to have a sufficient healthy population of SGNs to at least partially restore hearing or speech recognition. Avoiding current decay and in order to use only minimal electrical stimulation of CI can be achieved by placing electrodes closer to the target neurons (O’Leary et al., 2009b). After the cochlear processes implantation, it’s required to improve neural connectivity with the electrodes, and also to increase the number of electrodes on the intracochlear array to drive smaller numbers of neurons independently (O’Leary et al., 2009b). To improve CI performance scientists and engineers are trying different strategies to improve hearing perception; among these are: 1. Advanced engineering of CI devices, which can communicate well with the brain stem, 2. Cell replacement therapies to augment or substitute for lost or malfunctioning SGN; 3. Regrowing spiral ganglion neuronal processes to improve connections with the CI and concomitantly to reduce the distance between them. To improve electrode selectivity, various strategies to retain as healthy and numerous a population of the extant SGNs could be devised or exogenous primary (e.g. DRG Hu and (Hu et al., 2004a, b, 2005b))
or stem cell derived neuronal cells which have similar functions to primary SGNs could be used (Altschuler et al., 1999; Altschuler et al., 2008).

The research described in this report is focused on using a common population of ESC to derive either a “neuron-like” population that has some significant resemblance to the SGN or a population of MIF-producing SCwann Cell like cells that provide directional molecular cues for either SGN or the ESC-derived “neurons”. Such a SC like population can be used to “coat” a CI, when they are enmeshed in hydrogels. In the neuronal replacement approach, SGN-like cells are derived from mES cells using the inner ear specific cytokine MIF which can act as neurotrophin (Holmes et al., 2011a; Shen et al., 2011; Ramamurthy et al., submitted; Bank et al, submitted). Such neuron-like cells’ maturation is enhanced with docosahexaenoic acid (DHA), which is capable of enhancing electrophysiological functional maturation (Khedr et al., 2004; Uauy et al., 2001). The mESC-derived neuron-like cells where characterized for electrophysiological functional maturation by patch clamping and for transporters, neurotransmitters and appropriate ion channel expression by immunohistochemistry. The CI electrodes in this study were coated with polymer-trapped neurotrophin producing SCC-like cells that continuously produce MIF rather than with polymer alone that releases neurotrophins in a controlled manner (Jhaveri et al., 2009; Winter et al., 2007). In the NT-releasing approach, neurotrophic support is not achieved well or sustained. Concentration of actual released NT is unknown and there is a limit to the period of application of neurotrophins which is temporary for a 2 weeks (Gillespie and Shepherd, 2005). Providing neurotrophic support along with electrical stimulation provided by the CI to bring neurites into close proximity with the implanted electrode has had some significant success (Evans et al., 2009a; O’Leary et al., 2009a). Providing NTs to regrow spinal cord neurons has had some success in therapeutic applications in animal models for spinal cord injury (Lu et al., 2007). In another study, neurotrophins were also used for the regeneration of chronically injured spinal neurons (Houle and Ye, 1999). Several studies have been done (Suter, 2011; Vavrek et al., 2007; Xu et al., 1997; Xu et al., 1995) to determine if guidance cues from neurotrophin expressing cells can be used to regrow neuronal processes towards the electrode surface (O’Leary et al., 2009a, b; Roehm and Hansen, 2005b). This approach relies upon choosing a cell model that provides cells that are similar to the supporting
cells that exist in vivo and the diffusion of molecular cues from implanted cells through the perilymph to the auditory neurons. We have found that the SC of the inner ear (Bank et al, submitted) as well as all Schwann Cells (Roth et al., 2007, 2008) and the mESC-derived SCC produce MIF. We have employed this strategy in these studies rather than to use implanted cells expressing classical neurotrophins which were encapsulated in a hydrogel in the coheal region, a strategy (Pettingill et al., 2008) previously reported (Inaoka et al., 2009; Ito et al., 2005). Genetically modified Schwann cells that can over express BDNF were encapsulated in hydrogels and implanted in the intracochlear region of the deaf guinea pig showed survival-promoting effects of auditory neurons (Pettingill et al., 2011). Cohoroid plexus cells from neonatal pigs which are able to produce cocktail of neurotrophins were encapsulated in hydrogels and implanted in the intracochlear region showed increased SGN survival (Wise et al., 2011b) PC12 cells showed sustained “neurite” outgrowth when grown with neurotrophin-expressing fibroblast cells (Wissel et al., 2008). The approach detailed in this report is novel due in that neurotrophic cytokine producing/ (MIF-producing SCwann Cell-like cells derived from mES (Roth et al., 2007) are “coated” on a CI, which attract explanted SG neurites in a directional manner towards the CI. Many studies (Paino et al., 1994; Xu et al., 1995; Zhang et al., 2007; Zhang et al., 2010) have demonstrated that SCwann cells and neurons can make and maintain cell-cell interactions, including myelination of neurons for conduction of electrical signals (Bahr et al., 1991; Bunge, 1994). Schwann cells also provide neurotrophins and other supporting molecular guidance cues to improve directional neurite outgrowth (Bunge et al., 1981; Bunge, 1991).

Various combinations of these approaches, e.g. implanting neuron-like (and potentially SGN-like) mESC-derived cells into an inner ear in which HC degeneration has not occurred or regrowing neurites directionally from the existing SGN toward mESC-derived Schwann Cell like cells capable of sustained delivery of supporting factors from the target site on a CI are both promising avenues of research. These research studies are limited to in vitro conditions; in the future this approach will be applied in animal models in in vivo studies.
In the first study in this report, the neuron-like cells developed some SGN-like properties, including electrophysiological properties and sodium and potassium ion channels similar to those on SGN. In the second part of the study, SGN-like cells derived from mES cells were cocultured with mouse O of C explants to determine if the directional outgrowth of neurites towards the target could be documented. In additional experiments, MIF-producing Schwann cells were encapsulated in hydrogels used to coat a CI and directional outgrowth of neurites of a primary SGN explant isolated from mouse toward the implant was observed by time-lapse cinematography. This study could eventually benefit two types of patients--those with low SGN counts who are not candidates for CI therapy, for whom a replacement population of SGN-like exogenous cells could enhance functional contacts with their own native O of C sensory HC and also patients for whom MIF-producing mESC-derived SCC coated CIs could markedly improve directional outgrowth of SGN neurites to a CI.

3.3 Materials and Methods:

Culturing mES and differentiation of SCC-like cells: Mouse embryonic stem cells were cultured in Schwann cell differentiation media for three weeks as previously described (Roth.T).

Differentiation of mES cell into neuron-like cells in vitro: For neuronal differentiation: 95% F-12 (Invitrogen), 1% Pen/Strep (Invitrogen), 1% N2 (Invitrogen, CA), 2% B27 (Invitrogen,CA), 2% sodium pyruvate (2% stock), 0.5 µg/L bFGF (R&D Systems ), 1 µg/L IL-7, 5 µg/L IGF-1(Sigma), MIF 1 µg/mL (R&D Systems), or MIF alone± 5 µM DHA (Nu-check Prep Inc., MN, USA).DHA supplemented media included: 10 µM MIF and 5µM DHA.

Differentiation conditions for directed differentiation:

10,000 mESC cells were plated onto 0.1% porcine gel coated (Sigma) 48 well Primaria® plates (Falcon) and were differentiated over a three-week period. Undifferentiated mES cells (controls) were fed daily, while cells in neuronal differentiation media were fed every third day. After three weeks of growth, cells were labeled for expression of neurofilament marker (NF150) (neuronal differentiation
marker). Antibodies (Chemican, MA) were diluted in 5% normal goat serum (Chemicon) in 0.1% tween20/phosphate buffered saline (PBS) (Sigma). Cells were fixed in 4% paraformaldehyde (Sigma), and treated with primary antibody NF150 (R&D systems) at 1:500 dilution followed by secondary antibody Alexa fluor 590 at 1:200 dilution (Invitrogen, CA) and DAPI (1:1000 dilution) stain for cell nucleus. Photomicrographs were taken with a light microscope or using appropriate filters on a Leica inverted fluorescence microscope. We repeated each set of experimental conditions at least three times in separate experiments. The differentiated cells were labeled by our previously reported ICC techniques (Roth et al., 2007) for ion channel protein expression with antibodies (Abcam, CA) to sodium channel (SP19) and potassium channel (Kv3.1).

**Isolation and culture of mouse primary Statouacoustic Ganglion Neurons (SAG)**

SAG from 15.5 to 17.5 day Balb/C mouse embryos were removed from the dam’s uterine horns and the SAG dissected from the 2 ears and rinsed in PBS (Bianchi and Raz, 2004b). The SAGs were then plated in a 0.1% porcine gel treated Primeria plates with the addition of 0.5ml F12 neuronal basal media (source?) and incubated overnight for the attachment at 37°C in a cell culture incubator with 5% CO².

**Electrical recordings-patch clamp whole cell recording**

Whole-cell current- and voltage-clamp recordings were performed on bipolar SGN-like cells with long processes at room temperature using an Axopatch 200B amplifier with a Digidata 1200 or 1322A digitizer (Molecular Devices, Sunnyvale, CA). Data were acquired using pCLAMP 9 software (Molecular Devices). Glass electrodes were pulled from borosilicate capillaries (World Precision Instruments, Sarasota, FL) to achieve an electrical resistance of 3-6 M. Electrodes were filled with an internal solution containing (in mM): 112 KCl, 2 MgCl2, 0.1 CaCl2, 11 EGTA, 10 HEPES, 5 Na2+ ATP, and buffered to pH 7.2 with KOH. The external solution contained (in mM): 137 NaCl, 5 KCl, 1.7 CaCl2, 1 MgCl2, 10 HEPES, 16 glucose and buffered to pH 7.2 with NaOH.

**Isolation of Organ of Corti and explant culture(Parker et al., 2010):**
Day 6 neonatal Balb/C mouse were first decapitated and the outer skin was removed using a scalpel blade. The skull and the brain was bisected to expose the temporal bone and the tissue and the bones surrounding the temporal bone were removed. The temporal bone was rinsed in sterile PBS and transferred to a new what kind of dish and the cochlea was removed under a surgical microscope. The bony labryinth was carefully separated and the apex of the organ of Corti will be exposed to see the basal cochlea not the apical region after the removal of the labyrinth. Using fine forceps the base was held and the O of C carefully removed and unwound. The isolated O of C explants were placed carefully in 1% porcine gel coated Primeria (Falcon) plates with 0.5mL F12 basal media and incubated at 5%CO$_2$ in a tissue culture incubator overnight.

**Isolation of primary SGN:**

Once the O of C explant was isolated, the modilius part of the cochlea was cut in half and the tissue was minced using a scalpel blade. The SGN tissue explant was placed on 0.1% porcine gel coated primeria tissue culture plates with 0.5mL F12 media and incubated overnight in order to promote attachment.

**Coating CI with hydrogel-encapsulated SC cells(Winter et al., 2007):**

The Schwann cell like cells were differentiated following our previous published work (Roth et al., 2007) and trypsinized using 0.5% trypsin EDTA. The cells were counted and diluted to 10,000cells/mL and centrifuged at 1000RPM for 5 mins. The cell pellet was resuspended in 1 mL of Schwann Cell differentiation media and 1 mL 2% Sodium alginate (hydrogel) tissue culture grade (Pronovo SLG-100, Novomatrix) to bring the hydrogel concentration to 1%. The cells were thoroughly mixed with alginate and placed in an ice cold bath. The CI was coated with hydrogel in order to support SCC cell growth and was monitored daily under a light microscope to determine if SC growth was limited to the vicinity of the electrodes. The electrode was initially dipped in an ice cold 200mM CaCl$_2$ solution, followed by dipping in the hydrogel – cell mixture solution. This step was repeated to cover hydrogel-cell mixture on the CI up to 2-3mms upto 5-6 layers of coating. Then the whole hydrogel – cell and CI setup was placed in an ice cold 200mM CaCl$_2$ solution for 10 mins. Once polymerization was complete, the electrode
was placed in a tissue culture plate (35mm Falcon) and the encapsulated SCC allowed to grow for two days with SCC media supplementation (Roth et al., 2007). After we observed that the SC cell encapsulation was successful and the Schwann cells population was tested for survivability. The encapsulated cells were checked for viability using the Live/Dead stain (Invitrogen, CA) following the manufactures protocol. The cells were stained for Ethidium homodimer orange for dead cells and Calcein green for the live cells. The Schwann cell coated CI was then used for co-culture studies.

**Coculture of SGN-like cells- with O of C explant, with bare CI and (SC)CI**

The neuron-like cells were differentiated from mES cells on a 35mm Primeria (Falcon) plate for three weeks and the O of C explant was placed in the same plate at 1 cm apart to study directional neurite outgrowth towards the target (O of C). The cells were cultured and fed with F12 basal media for 5 days and neurite outgrowth was observed by ICC staining for TuJ1 for neurofilament protein expression. ICC was performed by fixing the preparation in 4%PFA followed by neurofilament marker as primary antibody followed by? Tuj1 and followed by Alexa fluor 488-conjugated secondary? Species?? (Invitrogen, CA). Photomicrographs were taken using a Leica inverted microscope. Interactions between the neuron-like cells and a (SCC) coated CI was monitored by placing the mESC-derived SC C-like cell coated electrode or bare electrode on the differentiated neuron-like cells and for 5 days. Directional outgrowth was observed and photomicrographs (time lapse?) were taken using light microscope.

**Coculture of SGN with bare CI, (ES)-CI and (SC) CI**

SGN explants were cultured alone for first 2 days to promote strong attachment to the 0.1% porcine gel-coated plate and then a bare CI or the SCC encapsulated CI was placed 1 cm apart from the SG explant. Neurite outgrowth and neurite contact with (SCC)CI or bare CI were monitoried for 5 days and photomicroographs were taken at 24 hr intervals using a light microscope. SC(CI) and SGN interactions and SGN neurite migration was also monitored in live time lapse imaging for two days post positioning of the SGN using a live cell imaging station -Delta vision microscope. The camera were assigned for different locations around the SGN explant and revolves around the explant
and captured time lapse images at 20 mins interval. The actual setup of the experiment is showed in Figure:5 in Chapter 1 of this thesis.

3.4 Results:

Electrophysiological properties of Neuron-like cells:

In this study we labelled the MIF and/or DHA-induced neuron-like cells for ion channel protein expression using immunohistochemistry, with antibodies for sodium ion channel SP19 and potassium ion channel Kv3.1 on the same cell preparation (double labeling). SP19 was distributed all over the neurite membrane and along the neurite, as well as internal to the cell soma (potentially nuclear staining); K3.1 was also expressed in the cell body and along the process, although the staining was less defined (Fig1).

Additionally we used whole-cell patch clamp electrophysiological recordings to determine whether the neuron-like cells derived from mES cells have excitable properties that could be similar to those of the SGN, and therefore sufficiently SGN-like to be capable of encoding frequency information from a CI electrode and conveying it to the central nervous system (CNS). Such functionally mature neuron-like cells when implanted in vivo could improve the efficacy and accessibility of these CI devices. After 3 weeks exposure we observe more than 75% of the cells exhibit neuronal processes upon differentiation of mES in culture to MIF- and DHA supplemented in the F12 basal neuronal media, an excitable population of neuron-like cells was identified. In contrast, if the mESC were exposed to the F12 basal neuronal without any differentiation factors media alone, no such properties could be documented. To explore the underlying cause of these changes, we investigated the voltage-dependent properties of Na+ and K+ currents using whole-cell voltage-clamp electrophysiology. We detected voltage dependent inward and outward current with long active response times in the MIF/DHA exposed “neurons” (Fig2) whereas the cells treated with F12 media alone (basal medium) show no active responses or any inward or outward currents.
Figure 3.1: Ion channel protein expression was detected on the Neuron-like cells by staining them for sodium SP19 and potassium Kv3.1 ion channel by ICC which is stained as red and green respectively. The photographs are of the same field, with different filters used to visualize the two fluorophores.

Figure 3.2: Patch clamp studies performed on the neuron-like cells. a. The electrode was filled with ionic fluorescent rhodamine dye and was used to patch clamp the cell membrane of the differentiated cells. b. Phase contrast image of a group of differentiated Neuron-like cells, the cells with bipolar neurons were patch-clamped using a fire polished glass pipette electrode. The MIF and DHA treated ES cells showed voltage dependent inward and outward current with long active response times, demonstrating voltage-dependent currents in a neuron-like bipolar cell. Voltage dependent changes were seen in response to step depolarization from a holding potential of – 80 mV. Both transient inward currents and maintained outward currents were observed.

**Coating of SC cells on CI using hydrogel**
To optimize cell growth in alginate gels we tested different concentrations of sodium alginate from 0.5 – 2.0% , which was used for encapsulating the SCCs at 10,000 cells/mL. We also tested gel polymerization achieved with different concentrations of calcium chloride (50mM – 300mM). Optimized conditions for SCC viability and gel firmness was determined to be 1% sodium alginate and 200mM calcium chloride for gel polymerization. Two different types of platinum iridium electrodes (the kind provision of Dr. Bryan Pfingst, Dept. Otolaryngology Head and Neck Surgery, University of Michigan) were “coated” with SCCs or undifferentiated mESC; both cell types were grown in culture for two weeks, supplementing the cultures fresh media every week (Fig4a,b). The encapsulated cells were differentially stained for live and dead cells with ethidium bromide and calcein (LIVE/DEAD® Cell Viability Assays, Invitrogen, CA). Large populations of healthy cells and very few dead cells were found with both the SCC-differentiated ES cells or undifferentiated ES cell conditions (Fig4c). At the optimized conditions encapsulated cells were also cocultured with the Neuron-like cells and with primary SGN explants in our directional outgrowth study.
Figure 3.3: CI coated hydrogels with encapsulated SCC-like cells: Two different types of electrodes coated with encapsulated SCC-like cells. a. Platinum iridium eight array electrode. b. Platinum iridium electrode with a ball at the end. The differentiated SCC cells were grown on the electrode in the sodium alginate hydrogel for a 2-week period; a healthy population SCC cell clusters was seen in the hydrogel. c: Photograph of Live/Dead staining (ethidium homodimer for dead cells - orange/calcein AM for live cells-green) of CI coated with hydrogel-encapsulated SCC cells, which were maintained for 2 weeks in the encapsulated form and then checked for viability. Insert shows a phase contrast image of the SCC cells encapsulated on the CI.

Neuron-like cells’ directional outgrowth towards targets:

The neuron-like cells were assessed after a week of coculture for directional migration and directional outgrowth towards any of the following: a target O of C derived from 6 day old post natal mouse cochlea as described in the Material and Methods section; a bare CI or a SCC in hydrogel coated CI. Neuron-like cells exhibited no directional neurite outgrowth toward the bare CI and fewer processes emerge from the cell bodies towards the bare CI (Fig3a) whereas neuron-like cells cocultured with the (SCC)CI showed long processes and migration and directional outgrowth towards the CI (Fig3b). Neuron-like cells co-cultured with the 6 day old postnatal mouse O of C showed clusters of neurites with growth cones and multiple branches growing towards the O of C (Fig3c). These studies indicate that the MIF/DHA-induced Neuron-like cells derived after 3 weeks exposure to the neurotrophic cytokine MIF and DHA are mature and functional and can migrate towards the target in vitro. This remains to be thoroughly tested when they are implanted in vivo in animal models.
Figure 3.4: Neuron-like cells derived from mES cells exposed to MIF as a differentiation factor and DHA as a maturation factor exhibited directional outgrowth towards a number of different targets. a: Neuron-like cells co-cultured with bare CI showed no directional neurite outgrowth and exhibited fewer processes extending from the cell body (control for the experiment); b: Neuron-like cells with the (SCC)CI showed long processes and directional outgrowth of these processes towards the CI when compared with bare CI/SGN-like cell coculture (a) and c: Neuron-like cells with the wild type O of C cocultured for a week showed clusters of neurites with branches growing towards the O of C after a week of coculture.

Migration of SAG/SGN towards targets:

Neurite migration and directional outgrowth trajectories were investigated in our studies every day after addition of the CI to the culture by morphological observation in the light microscope and by immunohistological staining for neurofilament protein marker, by image analysis to determine neurite extension and directional changes, some of which were monitored by time-lapse cinematography. The long-term coculture of (SCC)CI and SAG for up to 13 days showed directional outgrowth of SAG neurites and extensive contact of neuronal processes on the (SCC)CI but not on “bare” CI. Directional outgrowth was not noted until 5 days after establishment of the coculture; random trajectory outgrowth of short processes was observed prior to that time. From the
5th day onwards, however, the neuronal processes emerging from the SAG were observed to turn in the direction of the (SC)CI and to migrate towards it (Fig5a,b). The migrating cells could be positively stained for neurofilament marker protein (Figc). A control study was performed with a bare CI, which showed no directional outgrowth or migration of the processes (Fig 5d). These migration and directional outgrowth studies demonstrate that the SCC-encapsulated CI could potentially deliver directional molecular cues to attract neurites of an endogenous SAG or SGN.

Figure3.5: Migration of embryonic mouse (Eday12.5) SAG neurites after 2 weeks in culture. Note: a and b are separate photographs of the same SAG but different fields of view of the same cultured SAG. In B, the CI is labeled with an arrow. To register the two images, two landmarks have been circled on the photo with ovals. The top oval is vertical and the bottom oval is horizontal. a: The processes of the SAG, which were originally extending 360° around the cell bodies in the explant, have, by day 13, all turned and are extending in the direction of the CI (see arrow in B). SAG exhibit directional migration towards the CI coated with SCC-like cells. b: Register the ovals in a with those in b. Extensive processes from the SAG are seen to be extending directionally toward the CI. c: Photograph of the CI itself (same CI as seen in (b). After 13 days, neurites in the vicinity of the CI were labelled for Neurofilament 150kDa (Nfil) protein marker by immunocytochemistry in which a secondary antibody (AlexaFlor 580 (red) labels the processes extensively touching the CI. Dapi stained nuclei of the SCwann Cell like cells are seen in blue within the hydrogel “coat”. The dotted line shows the electrode
area. The insert shows the phase contrast image of the electrode. d: No directional SAG neurite migration is seen if the SAG is co-cultured with a bare CI. Short processes extend 360° from the cell body cluster in the center of the explant.

**Directional outgrowth of neurites from SGN towards targets:**

SGNs isolated from the 6 day old post natal mouse cochlea were cocultured with bare CI or with (SCC)CI to observe the neurite migration and directional outgrowth under the light microscope and by time-lapse live cell imaging. These studies were performed for 5 days after the addition of the CIs and showed directional outgrowth only towards the (SCC)CI. Initially, as with the SAG, outgrowth of neurites was observed 360° around the cell bodies, but after 3rd day of co culture the majority of the neurites showed directional outgrowth towards the CI in the experimental condition, ie towards the SCC-coated CI (Fig6b,c). Neurite outgrowth from SGN in the presence of a bare CI showed no migration or directional outgrowth pattern of neurites even after 5 days of coculture (Fig6a). These directional outgrowth studies with mature primary SGN demonstrate that the SCCs encapsulated on CI could potentially deliver supporting cues to attract the neurites in an *in vivo* condition.
Figure 3.6: SGN isolated from 6 day BalbC mouse pups interacting with variously prepared CI. a: Neurite migration in the control experiment with a bare CI: neurites were short, uniformly extending 360° around the SGN and did not reach the electrode even after day 5 in co-culture. b: In the experimental conditions, in which SGN were cultured with SCC-coated CIs, we observed directional outgrowth of neurites towards the encapsulated SCC coated CI. The dotted circle in the image shows the processes on top of the hydrogel containing the SCC. c: The enlarged picture of the circled areas in image b shows the neurites had migrated and were growing on top of the hydrogel, which contained the SCC.

Live cell migration was monitored during an 2 day period by time-lapse imaging to attempt to document any directional outgrowth of neurites over time towards the CI. The cocultured explants and CI were placed 1 cm apart and the camera was manipulated to capture images at different locations at 20 min intervals over 2 days. We then subsequently analyzed the different images to observe neurite extension and any directional outgrowth. Most of the neuritic processes eventually took the direction towards the (SCC)CI and we also observed that the explant itself in some cases split in half and some of the SGN themselves (with and without underlying glial cells) migrated in the CI direction. This study suggests that directional outgrowth of neuronal processes and the growth cone bearing tip of the extending “axon” could be influenced by the molecular cues provided by the SCCs present on the CI.

3.5 Conclusion

Two separate stem cell-based therapeutic approaches have been used in these studies to explore the possibility of novel mechanisms to alleviate deafness. A common population of mouse embryonic stem cells, which we previously demonstrated could interact with each other after being differentiated into neuron-like cells or SCwann Cell like cells (Ramamurty et al, submitted; chapter 2, this thesis) in microfluidic devices have been used to develop these strategies. In one case, a population of mESC-derived neurons has been developed that might eventually be used in strategies to replace lost SGN in SNHL. In the second strategy, the same population of mESC was used to create a population of MIF-secreting Schwann Cell like cells, following on from our pioneering
creation of the first embryonic stem cell derived models of the myelinating and non-myelinating Schwann Cell (Roth et al., 2007; Roth et al., 2008b). Work from the group in Melbourne, AU, has already used a Schwann Cell approach, using an engineered population of Schwann Cells to coal a CI, with limited success (Pettingill et al., 2008).

Neuron-like cells derived from MIF and DHA-induced ES cells could potentially be used in the future to design therapies to replace lost sensory neurons of the cochlear spiral ganglion. However, if this paradigm is to be successful, several important hurdles will have to be met, some of which would be incurred in optimizing the cell implant procedure itself and some of which involve meeting critical criteria for monitoring functional connections of the “neuronal population to the O of C if the implant is successful.

First, any implanted substitute “neuronal” population would have to remain viable and capable of extending “neurites” in vivo, towards a source of molecular cues (the native O of C or a CI). The number of surviving “neuron-like” cells has to be sufficient to have any functionally viable after however many days are required for their neuronal processes to reach the target tissue. If the target tissue is the native O of C, the implant site would have to be optimized and two additional critical criteria would have to be met: the implanted cells cannot form a tumor or dedifferentiate and proliferate, even if the cell bolus that eventually forms is benign and the possibility of infection must be minimized. Of course, the most important criteria to be realized is that the implanted surviving population of neuron-like cells must be sufficiently “like” the SGN they replace to be viable and functional. This means that they must have cellular, molecular and electrophysiological properties sufficiently similar to the cells they replace to be functionally “competent” in situ.

How far have we come towards realizing these MIF and DHA are capable of inducing some properties in the mESC-derived neurons that are similar to those of the SGN under the conditions of the experiment. These include: VGlut transporter expression (Ramamurthy et al., submitted; chapter 2) was documented in these MIF and DHA induced neurons. These transmitters are also expressed to the same extent in NGF or CNTF induced mESC. Electrophysiological recordings were also made in the NGF+DHA condition. However, no inward or outward currents were found. We have not yet
documented action potentials in any of these conditions; such recordings will be attempted in the future. Researchers had attempted to develop glutamatergic neurons from ES cells by transient expression of neurogening 1 and supplementing with BDNF and GDNF and implanted them in animal model (Reyes et al., 2008). These studies were not successful in cell replacement therapy for auditory system. We chose to use MIF as the inducing neurotrophin because we have previously demonstrated that MIF is the first neurotrophin that functions in the developing inner ear (Holmes et al, 2011; Shen et al., 2012, Ramamurthy et al., submitted and Bank et al., submitted) and that SGN retain the appropriate receptors for MIF into adulthood (Bank et al., submitted).

Differentiated neuron-like cells whose synaptic properties were enhanced with DHA were tested for a functional neuronal phenotype resembling SGN by performing immunostaining analyses for ion channels and testing their physiological properties by whole cell electrophysiology. The results of these experiments showed that the cells expressed ion channels that are expected for SGN (Greenwood et al., 2007), but sodium channel protein expression was better defined in the immunohistochemistry labeling experiments than potassium ion channel expression on the neurites. These experiments demonstrate that these neuron-like cells express some functionally mature properties in common with SGN under the in vitro conditions. These neuron-like cells already exhibit electrophysiological properties in vitro, which encourages us to believe that they could eventually be implanted in animal models and could potentially transmit active electrical responses to the brain stem after being implanted in the inner ear. These functionally mature neuron-like mESC-derived cells were experimentally tested for directional neurite outgrowth when cocultured with CI or primary explanted O of C targets in the in vitro conditions. The migration patterns documented in these studies demonstrate that the neuron-like cells could also respond to chemoattractant-producing SCCs derived from the same population of mESC and take directional outgrowth towards the (SCC)CI or O of C. Neuron-like cells derived from mESC using MIF and DHA could be implanted as an mESC-based therapy in patients with low SGN counts who are not candidates for CI therapy.
The second embryonic stem cell based therapeutic approach initiated in these studies and described in this report, is to produce a population of cells, in this case, mESC-derived Schwann Cell like cells, capable of producing and continuing to produce molecular cues capable of improving connections between the remaining SGN and the CI. In these studies, SCC-coated CIs in vitro were studied for their ability to “attract” both the embryonic SAG and the mature SGN, using primary explants of these ganglia. Eventually, such a strategy might be employed to enhance the functionality of a CI and encourage the outgrowth of any remaining SGN neurite processes towards the CI. If successful, this could lead to improved hearing or better speech perception in patients experiencing hearing loss.

Schwann cells (SC) have proven important for peripheral nerve regeneration (Bhatheja and Field, 2006) and also exhibit in culture or in implants, properties they also demonstrate within the environment of the peripheral nerve, which depend on their state of differentiation (e.g. quiescent, proliferating or mature SCs,) (Baehr and Bunge, 1989). In studies by other investigators, the effects of factors secreted by SCwann cells were examined on a quasi-neuronal model composed of PC12 cells, which are, in some investigators’ estimations a model for neuronal cells, which were tested for cell survival and “neurite” outgrowth(Bampton and Taylor, 2005). In these experiments, SCwann Cell conditioned medium was tested on “neurite” outgrowth from PC12 cells against a range of isolated factors known to be secreted by SCs. This conditioned medium showed clear neuritogenic effects and hence suggested that SCwann Cells are likely candidates for promoting neuronal regeneration(Bampton and Taylor, 2005). Other groups, have reported enhanced survival of SGN in hearing loss animal models by implanting the SCwann Cells, which were molecularly engineered to provide sustained delivery of neurotrophins(Bunge, 1993; Girard et al., 2005; Golden et al., 2007; Pettingill et al., 2008). However, whether the neurotrophins released by the engineered SCwann cells improve directional outgrowth of the SGN neurites towards a CI sufficiently to result in increased CI contact, so that meaningful acoustic signals could reach the brainstem has not been addressed in any of the research studies published to date.
To encourage directional neurite outgrowth towards the CI, our approach employs encapsulated SCC-like cells on the CI, which could potentially provide chemotactic cues to direct the primary SAG/SGN to the CI and also provide sustained delivery of the inner ear’s own neurotrophin, MIF, to which both the SAG and SGN retain the appropriate receptors (Bank et al, submitted; Shen et al., 2012) in order to promote the survival of the native SGN. The SCwann Cell-like cells derived from mES cells were tested for neurotrophin release (Ramamurthy et al. submitted) and such neurotrophins could promote the directional outgrowth of neurites. SAG and SGN cocultured with (SCC)CI showed greater numbers of processes growing towards the CI in *in vitro* studies than to either a “bare” CI or towards a CI “coated” with hydrogels bearing undifferentiated mESC. It is expected that when this approach is tested *in vivo* in animal models, SCC coated CI could potentially provide an optimal target for the existing SGN to grow towards the CI. If this strategy is successful, initially after CI implantation electrical impedance will be higher due to the presence of hydrogel encapsulated cells, but the biocompatible and biodegradable hydrogel might release the cells in the perilymph or area surrounding the CI, which could potentially provide sustained neurotrophin release, which would also serve to protect the SGN. Once the hydrogel has completely degraded after releasing all the cells, the CI’s own electrical stimulation would presumably function normally.
3.6 References


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Chapter IV

Conclusion

The thesis research work presented here had new approach in developing stem cell based therapies to regenerate functional SGNs in the inner by using regeneration cell therapy. The challenges involved in stem cell based therapy are identifying the specific signal molecule that could direct the differentiation to acquire specific lineage. Our laboratory has done extensive research studies on MIF a neurotrophic cytokine and showed MIF is an important factor in inner ear development. Using MIF as signaling molecule for differentiation ES cells into SGN-like cells is a novel approach to overcome challenges in choosing the appropriate signaling factor. DHA is proven to be neuronal maturation factor in several studies. Additional to MIF as differentiation factor, DHA was supplemented in the media to the differentiating neurons to exhibit maturation of the neurons. The SGN-like cells we developed will behave like functional phenotype when implanted in vivo and could potentiate the proper neuronal connections with functional properties of matured neurons that could replace the lost SGNs in the SNHL patients. The primary limiting factor in CI therapy for SHNL patients is the availability of functional SGNs in the inner ear. Using our SGN-like cell for regeneration therapy in patients, the implanted cells make functional neuronal network connections, mimic the native SGNs. The SGN-like cells were characterized in our studies for functional maturation, cell-cell interaction and for directional outgrowth towards the target.

SGN-like cells exhibit functional maturation by expressing mature auditory markers along with neurofilament and VgluT1 protein marker. The cell–cell interaction studies was performed in a novel microfluidic device where we differentiated two cells types (SGN-like cell and Schwann-like cells) in the same channel from the same population of ES cells. The SC cells expressed myelin basic protein which indicates that there was a cell-cell interaction between the neuron and SC cells. SGN-like-cells especially when differentiated with supporting Schwann cell-like cells in the microfluidic
device to mimic the microenvironment of the inner ear exhibit directional outgrowth towards the Schwann Cell-like cells and have substantial improvement in their glutamatergic properties. The increase in neurite outgrowth and number of branches suggests that the SCwann Cell like cells had greater influence on SGN cell-cell interactions. This study is promising that our SGN-like cells establish functional connections when used in vivo.

The hurdles behind the CI therapy efficacy are the functional connection of the remaining SGNs and the distance between the CI and the SGNs. One of the exciting approaches is when SGN-like cells grow along with the Schwann Cell like cells, a directional outgrowth towards the target tissue is seen, suggesting that if the Schwann Cell-like cells were used to “coat” a CI, contacts with the CI would be enhanced. The increase in neurite outgrowth and number of branches suggests that the Schwann Cell-like cells had greater influence on SGN cell-cell interactions. In our previous research we developed ES derived Schwann-like cells which could secrete MIF and other supporting molecular cues for the survival of neurons when coated on CI, the remaining SGNs will survive and establish contacts would ultimately be made with a CI. These in vitro studies, SC coated CIs were tested for their interactions with both the embryonic SAG and the mature SGN, using primary explants of these ganglia. The neurotrophins released by the Schwann-cell like cells improve directional outgrowth of the SGN neurites towards a CI to send meaningful acoustic signals. Eventually, such a strategy might be employed to enhance the functionality of a CI and encourage the outgrowth of any remaining SGN neuronal processes towards the CI. If this is approach is successful, this could lead to improved hearing or better speech perception in patients experiencing hearing loss. If it were a successful strategy, initially after CI implantation the electrical impedance will be higher due to the presence of hydrogel encapsulated cells, but the biocompatible and biodegradable hydrogel might release the cells in the perilymph or area surrounding the CI, which could potentially provide sustained neurotrophin release, which would also serve to protect the SGN. Once the hydrogel has completely degraded after releasing all the cells, the CI’s own electrical stimulation would function normally.