Role of the Cytokine Macrophage Migration Inhibitory Factor (MIF) in inner ear neuronal and sensory cell development

by

Fumi Ebisu

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2010

Doctoral Committee:

Professor Kate F. Barald, Chair
Professor Richard A. Altschuler
Professor Peter F. Hitchcock
Assistant Professor R. Keith Duncan
Research Professor Emerita Margaret I. Lomax

Dedication

To my family, for your truly unconditional love and support.

Acknowledgments

I have many people to thank for contributing to my dissertation in various ways.

First and foremost, I would like to thank my mentor, Kate Barald. It has been such an honor to be a “scientific child” of hers. She has given me an opportunity to learn the theory behind the work as well as scientific experimental approaches and techniques that I will need to be an independent scientist. The joy and enthusiasm she has for her research and teaching were always motivational for me. There is no doubt that I would not be at this position without her guidance, understanding and patience as well as financial support. Her devotion to each of us was far beyond just that of a professor. I always remember that she and her husband, Doug, picked me up at 4 o’clock in the morning on the day of my surgery and stayed with me to comfort me until the doctors brought me to the surgery room. For this and so many other things you have done for me, Kate, I thank you.

My sincere thanks go to all the members of Barald lab, both past and present, for their friendship and assistance. Special thanks to my “taking walk” buddies, Yu-chi Shen, Poorna Ramamurthy and Therese Roth, who listened to my occasional worries and frustrations and finally geared me back in the right

direction. Thank you so much to Steffi Linn for her willingness to proofread and listen to my practice talks. Her comments were always valuable and appreciated. Thank you so much to Andy Chervenak and Matt Wyatt for their help in many ways when I needed it most. Thanks so much to Dov Lerman-Sinkoff for his unstinting and always available technical help. Thanks much to John Hennessy for working with me helping me with the western blotting assays for these last several months and for sharing with me his interests in science. They all are wonderful individuals not only for working with but also being with. I hope that we will always remain in contact.

I would like to thank all the members of my dissertation committee, Rick Altschuler, Keith Duncan, Peter Hitchcock and Margaret Lomax. Rick, thank you very much for your insightful comments and suggestions. Your smiling face made me feel that I was always welcome to bring my questions to you even without an appointment, and you always had the answers for me. Keith, first of all, thank you so much for becoming part of my committee last year. Your questions and suggestions during the meetings were truly valuable and helped me focus what I needed to do next. I would also like to thank you for your time and patience while my training in the basilar papilla excision technique. Peter, I would like to express my sincere thanks for your continuous encouragement and support not only as a member of my committee but also as the former director of the Neuroscience (NS) program. Your tough questions and feedback during the meetings were just amazing and helped me realize what I needed to understand to be a “scientist”. But I am ready to take that journey. Margaret, thank you so

much for your helpful comments and suggestions. I am also thankful for your excellent example as a successful female biologist and professor.

I would like to special thank my additional committee member, Beth Moore, who spent a great deal of her time teaching me the basics of Immunology. I would also like to thank my previous academic advisor and committee member, Rudy Richardson, for his time and support during the last four years.

I would like to express my deep thanks to all the people in the NS program. Special thanks go to the director of the program, Steve Maren, and the associate director, Jill Becker, for their guidance and support. Thank you so much for Valerie Smith and Rachel Flaten for your unending assistance and help whenever I needed it. Thank you very much to all NS students, especially the fourth and the fifth year students who I shared a lot of my time with, for their continuous support and friendship.

Thank you very much to my past mentor at Eastern Michigan University, Steve Pernecky, who accepted me as one of his students and taught me how enjoyable and challenging scientific research could be.

Thank you so much to Su-Hua Sha and Fu-Quan Chen for their time and efforts to train me in the organ of Corti excision process, and more importantly for their friendship.

Thank you to Mary Caponite Hurley at the Michigan Proteome Consortium for performing proteomic analyses for our studies and helping us interpret them. Thank you so much to the stuff of the CDB department, Karen Lang, Karen

Meeks, Lori Longeway, Kristen Hug and Ryan Schell, for their assistance. Thank you to all the people at the MIL and Morph Cores in CDB, particularly Chris Edwards.

Thanks so much to all my friends for their support and friendship. Very special thanks go to Beth Valka, who was formerly my Bible teacher and is now my best friend, for her unending support, patience and encouragement. More importantly, thank you so much for just being with me when I needed someone most to listen to my concerns. Another special thanks to Naoko Murakami, who cooked Japanese food for me when I was sick or too busy to cook. Very many thanks go to Tomiyo Wemert, Noriko Yi, Satoko Motohara, Ayako Hayami, Christie Kim, Becky (Heekyong) Bae, Harsha jayatillake and Hemali Amunugama for their companionship.

Finally and most heartfelt, I thank my family: my parents, Yukifumi Ebisu and Harumi Ebisu, for their unconditional love and endless support and encouragement to pursue my interests, and my brother, Kazuyuki Ebisu, for being close to our parents during my time in the U.S. and for believing in me

Table of contents

Dedication……ii

Acknowledgements……iii

List of Figures……..……x

List of Tables……...xii

List of Abbreviations……xiii

Abstract…….……xviii

Chapter

I. Introduction

ODF is not one of the previously identified neural growth factors……..2

The neuroimmunoaxis……...5

Physiological basis of hearing and balance in the vertebrate ear……..6

Mammalian inner ear development……..……10

Organogenesis……10

Neurogenesis……..11

Spiral ganglion neurons…….……..12

Sensorineural hearing loss (SNHL)……...…15

How hair cell loss leads secondarily to the loss of sensory neurons in the inner ear. Why this causes deafness……16

Why sensory neurons last longer than hair dells in the inner ear?.....18

How a cochlear implant works……20

Trophic factors on which each of the stages of neurons depends…...22

Otocyst derived factor(s) (ODF) enhance(s) directional neurite outgrowth and neuronal survival of the early stage Statoacoustic ganglion (SAG) neurons……..……28

Proteomic studies demonstrate that the major components of ODF are immune system cytokines……...32

Development of immortalized mouse inner ear cell lines to characterize ODF……..…….....32

Macrophage migration inhibitory factor (MIF), an inflammatory cytokine, plays the role of a "neurotrophin" in inner ear development.……35

Enzymatic activity of MIF/MIF as a therapeutic target …….....35

The pathogenesis and progression of acute inflammation in the body……..40

The pathogenesis and progression of chronic inflammatory diseases (Autoimmune diseases)……42

Inflammation and cancer……..……..47

Role of MIF in development……..……51

Role of MIF in neuronal regeneration……..…56

Mechanisms of MIF……..…62

References……67

II. The cytokine, macrophage migration inhibitory factor (MIF) acts as a neurotrophin for neurons in the developing inner ear…….85

Abstract……..85

Introduction…….…...86

Materials and experimental methods……88

Results……...……...102

Discussion…….……128

References……..…136

III. Conclusion and future work……143

References……..159

Appendix……163

Abstract……163

Introduction…….….164

Materials and experimental methods……..168

Results……...……...175

Discussion…….……..183

References……..…188

List of Figures

Chapter I

Figure 1.1: Schematic representations of the human ear 9

Figure 1.2: Schematic view of the cochlea and spiral ganglion neurons 14

Figure 1.3: Schematic view of a cochlear implant system……...21

Figure 1.4: Three-dimensional ribbon diagram of human MIF 38

Figure 1.5: MIF mRNA expression in the embryonic mouse and Xenopus 54

Figure 1.6: Zebrafish mif expression and role in inner ear 56

Figure 1.7: Intracellular mechanisms of MIF 63

Chapter II

Figure 2.1: Chick SAG neurite extension and survival in otocyst derived factor (ODF) 104

Figure 2.2: Proteomic analysis and identification of cytokines 106

Figure 2.3: Recombinant MIF (rMIF) enhances both chick SAG neurite outgrowth and survival 108

Figure 2.4: rMIF enhances postnatal mouse spiral ganglion (SG) neurite outgrowth 109

Figure 2.5: Mouse D3 embryonic stem (ES) cells treated with rMIF promotes the neuronal differentiation 111

Figure 2.6: Blockade of MIF activity reduces SAG neurite outgrowth and survival 113

Figure 2.7: MIF mRNA expression in the cochlear tissues and SG neurons (SGNs) 114

Figure 2.8: MIF protein expression in the embryonic day 15.5 (E15.5) mouse inner ear 115

Figure 2.9: Expression of MIF and MCP1 proteins in the postnatal cochlea..... 116

Figure 2.10: Formation of ear domes from cultured E13-14 mouse inner ear cells..... 118

Figure 2.11: CD74, a MIF receptor, expression in the developing SAG and postnatal SG neurons at mRNA level..... 119

Figure 2.12: Density of SGNs in wild-type (WT), MIF and MCP1 knockout (KO) mice 122

Figure 2.13: immunostaining of organ of Corti (OC) in WT, MIF and MCP1 KO mice 123

Figure 2.14: Epifluorescence of OC for neuronal and HC in WT, MIF and MCP1 KO mice..... 124

Figure 2.15: Mean OHC loss percentage in MIF KO mice..... 125

Figure 2.16: Co-culture assays of OC with SG explants..... 127

Appendix

Figure A.1: Western blotting assays for mouse MIF siRNA and its missense .. 177

Figure A.2: Western blotting assays for avian CD74 siRNA and its missense . 181

Figure A.3: Blockade of avian CD74 expression reduces chick SAG neurite outgrowth..... 182

List of Tables

Chapter I

Table 1.1: Trophic factors on which each of the stages of neurons depends…..24

Chapter II

Table 2.1: Summary of the two major cytokines found in ODF and experimental approaches for identification and verification of these cytokines……..…...107

Table 2.2: ABR thresholds in WT and MIF KO mice at 4 weeks of age……..121

Appendix

Table A.1: The ratio of mouse MIF siRNA to its missense control……...177

Table A.2: The ratio of avian CD74 siRNA to its missense control……..181

.

List of Abbreviations

MIF	macrophage migration inhibitory factor
SAG	statoacoustic ganglion
SG	spiral ganglion
SGNs	spiral ganglion neurons
MCP1	monocyte chemotactic protein 1
IMO	immortalized otocyst
mESC	mouse embryonic stem cells
HC	hair cells
SC	supporting cells
ODF	otocyst derived factor
OC	organ of Corti
WT	wild type
KO	knock-out
FBS	fetal bovine serum
CEF	chick embryo fibroblast
rMIF	recombinant MIF
DME	Dulbecco's modified eagle

CM	conditioned medium
E	embryonic day
PBS	phosphate buffered saline
P	postnatal day
PFA	paraformaldehyde
EDTA	ethylenediaminetetraacetic acid
ABR	auditory brainstem responses
ICC	immunocytochemistry
BSA	bovine serum albumin
NGS	normal goat serum
HRP	horse radish peroxide
TMB	tetramethylbenzidine
IHC	inner hair cells
OHC	outer hair cells
StV	stria vascularis
SL	spiral limbus
SpL	spiral ligament
SP	spiral prominence
RM	reissner's membrane
SM	scala media
SV	scala vestibuli

TM	tectorial membrane
CVG	cochleovestibular ganglion
AG	auditory ganglion
VG	vestibular ganglion
CI	cochlear implants
SNHL	sensorineural hearing loss
Da	dalton
DDT	D-dopachrome-tautomerase
DHICA	5,6-dihydroxyindole-2-carboxylic acid
ISO-1	(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester
4-IPP	4-Iodo-6-phenylpyrimidine
LPS	lipopolysaccharide
CLP	cecal ligation and puncture
LBP	LPS-binding protein
TLR-4	toll-like receptor 4
SLE	systemic lupus erythematosis
IL	interleukin
RA	rheumatoid arthritis
FLS	fibroblast-like synoviocytes
COX2	cyclooxygenase 2
PGE2	prostaglandin E2

AIA	antigen-induced arthritis
DEX	dexamethasone
ERK	extracellular-signal-regulated kinase
MAPK	mitogen-activated protein kinase
ApoE	apolipoprotein E
NK	natural killer
Tg	transgenic
AH	aqueous humor
MOs	morpholino antisense oligonucleotides
MEFs	mouse embryonic fibroblasts
HFDFs	human foreskin dermal fibroblasts
GPCR	G protein coupled receptor
Jab1	Jun activation domain-binding protein
AP-1	activation protein 1
BDNF	brain-derived neurotrophic factor
GDNF	glial cell line-derived neurotrophic factor
CNTF	ciliary neurotrophic factor
NGF	nerve growth factor
NT	neurotrophin
FGF	fibroblast growth factor
IGF	insulin-like growth factor

TGF	transforming growth factor
TNF	tumor necrosis factor
PDGF	platelet-derived growth factor
Rb	retinoblastoma
SDF-1	stromal cell-derived factor-1
hpf	hours post fertilization

Abstract

Role of the cytokine of macrophage migration inhibitory factor (MIF) in inner ear neuronal and sensory cell development

By

Fumi Ebisu

Chair: Kate F. Barald

Spiral ganglion neuron (SGN) loss, either dependent or independent of sensory hair cell (HC) loss, is a major cause of deafness, particularly in the ageing population. Cochlear implants (CI) are presently the only known "cure" for many forms of deafness. Nevertheless, successful function of a CI depends on the preservation of SGNs. In the early developing inner ear, the otocyst secretes a factor called Otocyst Derived Factor (ODF). ODF promotes directional neurite outgrowth and neuronal survival of the statoacoustic ganglion (SAG), the precursor of the auditory portion of the SAG that eventually forms the SG. Cytokine arrays and proteomic studies demonstrated that the bioactive components of ODF include Macrophage Migration Inhibitory Factor (MIF), which has been described as “pleiotropic” cytokine because of its multiple roles, including roles in the immune system and in neuronal development and

regeneration. Based on its known roles and our preliminary data, we hypothesized that MIF plays a key instructional role, acting as a neurotrophin, in inner ear development. The goal of this dissertation project is to elucidate the role of MIF in inner ear neuronal development as well as the possibility of using this developmental information to study and to enhance inner ear neuronal regeneration. We found that, at low concentrations (5 pg/ml and 5 ng/ml), recombinant MIF alone supports both mouse and chick SAG directional neurite outgrowth and neuronal survival, and evokes a neuronal phenotype from mouse embryonic stem cells (ESC); at higher concentrations (500 ng/ml), MIF inhibits these functions. We also found that MIF is expressed in supporting cells (SC) of the inner ear and its receptor, CD74 is expressed on both SAG and SGN. In the MIF knock-out (KO) mice, abnormal development of both SC and hair cells (HC) as well as a significant hearing impairment in the high frequency region of the cochlea are seen with concomitant loss of SGN in this region of the cochlea. In addition, we observed that the neurites from SG explants extend directionally in culture toward the wild-type (WT) Organ of Corti (OC), but not in the isolated MIF KO OC. Finally, we found that blockade of either MIF or its receptor with antibody or RNAi respectively suppresses SAG neurite outgrowth and survival. Our study indicates that MIF functions as an essential component of normal inner ear neuronal development and innervation and could potentially be used for SGN retention or re-growth as well as to potentiate the function of a cochlear implant in the injured or diseased mammalian inner ear.

Chapter I

Introduction

More than 25 years ago, scientists and physicians interested in the intricacies of inner ear development discovered that the developing inner ear, particularly cells in the region of the anterior ventral otocyst called the otic crest, produced a factor or factors, called the Otocyst Derived Factor (ODF) that served as a "neurotrophin" for the neurons of the developing statoacoustic ganglion (SAG) (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, Hemond and Morest, 1991, Bianchi and Cohan, 1993). Neurotrophins, which are produced by target tissues, are responsible for causing directional outgrowth of the neurons that innervate them toward the source. Neurotrophins also support the survival of such target-bound neurons. In the intervening years, many groups, including ours, have sought to identify the molecular component(s) of ODF (Thompson et al., 2003, Germiller et al., 2004, Bianchi et al., 2005). Investigations into the identification of ODF were initially more useful for identifying which "classical" previously identified "neurotrophins" were either not in ODF or did not recapitulate ODF bioactivity in bioassays.

ODF is not one of the previously identified neural growth factors

Bianchi and Cohan (Bianchi and Cohan, 1991, 1993, Bianchi et al., 1998) investigated the role of classical neurotrophins in SAG development. Very early stage SAG neurons do not survive or extend neurites when cultured in the presence of one or more of the identified neurotrophic growth factors including: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), fibroblast growth factors (FGFs), transforming growth factors (TGFs) or ciliary neurotrophic factor (CNTF) (Bianchi and Cohan, 1993). The ODF is released at early stages corresponding to the period of initial neurite outgrowth (E4-6 in the chick, E11-14 in the mouse and rat; Bianchi and Cohan 1991, Bianchi and Cohan 1993a, Bianchi and Cohan 1993b). Other tissues such as the limb bud, heart and liver do **not** release proteins that influence the outgrowth or survival of early stage SAG although they can influence the outgrowth and survival of other neural cell types (Bianchi and Cohan, 1991).

At later stages of inner ear development, corresponding to the period of synaptogenesis, other growth factors such as the NT-3 and BDNF are necessary for **maintaining the survival** of cochlear and vestibular neurons, respectively (Bianchi et al., 1998, Wyatt et al., 1998). However, these neurotrophins are **not required** at the early stage of initial neurite outgrowth (Bianchi and Cohan, 1993). Furthermore, re-supplying NT3, BDNF or glial cell line-derived neurotrophic

factor (GDNF) has only limited positive effects on spiral ganglion neuron (SGN) regeneration (Altschuler et al., 1999).

Combinations of various concentrations of these growth factors also failed to mimic the activity of ODF, which was produced by incubating 8 chick or mouse otocysts in 250 µl of serum-free medium for several days (Bianchi and Cohan, 1991, 1993). Furthermore, other tissues do not release factors that promote outgrowth of early embryonic SAG. These results suggested that the otocyst releases a novel growth factor or combination of factors to promote the initial outgrowth and survival of SAG.

If ODF components are to have any potential therapeutic value in maintaining adult neuron survival, maintaining or re-growing axons, or in potentiating a cochlear implant (CI), three conditions must be met: 1) adult neurons must either retain or re-express embryonic receptors for these factors; 2) ODF must support neuron survival and *directional* neurite re-growth in the adult; and 3) the surviving or re-grown neurites must be able to reconnect with their target tissues, or interact with the electrodes of a CI. We have been able to document many of these criteria, and this thesis seeks to evaluate many of the others.

Our recent surprising finding that ODF is a mixture of growth factors called cytokines or chemokines, known for their roles in both the immune system and in neuronal development and regeneration (Bajetto et al., 2002) has laid to rest only part of the mystery (the components of ODF). This finding has raised a number of significant questions, which we address in the experiments described here.

We have identified 3 of these functionally significant components of ODF activity, through cytokine array and proteomic approaches: monocyte chemoattractant protein 1 (MCP1) (Bianchi et al., 2005), which is made by inner ear hair cells (HC) (Bianchi et al., 2005), macrophage migration inhibitory factor (MIF), which is made by supporting cells (SC) (see preliminary data; Table 2.1; Gerlach-Bank, Ebisu et al., in preparation), and RANTES (Bianchi et al., 2005), which is known to be a neurotrophic factor for dorsal root ganglion neurons (Bolin et al., 1998).

The research in this thesis focuses on the role of the one of the identified cytokines, MIF, in the basic developmental mechanisms of inner ear formation and innervation as well as the possibility of using this developmental information to study inner ear regeneration.

We hypothesize that the ODF cytokines play a key instructional role in the very earliest stages of inner ear development and demonstrate in the chapters in this thesis that adult neurons continue to express receptors for these critical components of the embryonic ODF, raising the possibility that a receptor-expressing adult neuron in the spiral ganglion (SG) could respond therapeutically to one or more of the identified cytokines, which, if introduced at appropriate concentrations might be able to preserve remaining SG neurons and restore function, or at least potentiate the function of a cochlear prosthesis/implant. It is known that the larger the number of functional SGNs remaining at the time of implant, the better the prognosis for a patient's restoration of function (Pfingst and Rai, 1990, Miller et al., 2000).

The neuroimmunoaxis

Discovering that cytokines play important roles in development of the inner ear is fully in accord with a major new focus in cytokine biology and neuroimmunology (Siemion et al., 2005, Wrona, 2006). Although cytokines and chemokines (cytokines with known attractant properties) were initially identified and characterized for their critical roles in the immune system, particularly in mediating T cell function (Gonzalo et al., 1996), the roles of such molecules in the nervous system have been known for many years.

Cytokines/chemokines are relatively small molecular weight proteins within a large super-family that are classified based on the relative location of the cysteine residues in the N-terminus of the molecule (Mantovani et al., 2003). Chemokines were initially identified for their ability to attract and activate immune cells by interacting with specific cytokine receptors (Coughlan et al., 2000). Recent studies have demonstrated that cytokines/chemokines are also present in **developing and adult neural systems**. These proteins are now being implicated in a variety of roles including neuronal migration, outgrowth, and survival (Meng et al., 1999, Bajetto et al., 2001, Chalasani et al., 2003a, Chalasani et al., 2003b, Belmadani et al., 2005). In some cases chemokines were found to regulate axonal outgrowth by inhibiting the repellent effects of other molecules such as semaphorins and slit-2 (Chalasani et al., 2003b). Not only are cytokines/chemokines absolutely required for neuronal development in

Xenopus (Suzuki et al., 2004), they are also critical in neuronal repair processes after axotomy in both the peripheral and central nervous systems (Bajetto et al., 2001, Koda et al., 2004).

In order to set the experiments in this thesis in context, we will first discuss the major tenets of inner ear development and then the hypothesized role of the specific ODF cytokines in the process. In this thesis the cytokine of interest is MIF, which we believe we have demonstrated is both necessary and sufficient to fulfill all the roles of the early neurotrophin in inner ear development.

Physiological basis of hearing and balance in the vertebrate ear

The ear consists of three functional parts; the external ear, the middle ear and the inner ear (Figure 1.1-A) (Friedman and Avraham, 2009). The external ear, called the external pinna or auricle, captures and focuses sound into the external auditory meatus or ear canal, which ends at the tympanum, also called the tympanic membrane or eardrum. The increases and decreases in air pressure that correspond to sound wave energy cause the tympanum to vibrate. These vibrations are conveyed across the air-filled middle ear by three tiny ossicles (bones), which in humans together are smaller than the size of a dime, called the malleus (the hammer), incus (anvil) and the stapes (stirrup) (Dror and Avraham, 2009). Vibration of the stapes, which makes direct contact with the oval window, generates pressure waves in the fluids of the snail-shaped cochlea, which, in humans, houses the auditory organ of the inner ear. Inner ear anatomy differs in mammals, birds and reptiles/amphibians and those differences will be

discussed in this dissertation where appropriate.

The interior of the mammalian cochlea contains three co-spiraling fluid-filled tubes within what is called the cochlear duct. The duct is separated into three parts by membranes. This is seen most easily in a cross section through the tube at right angles to its long axis. The three tubes are the scala vestibule, the scala media (SM) and the scala tympani and are shown in Figure 1.1-B (Friedman and Avraham, 2009). The organ of Corti (OC), which includes the sensory epithelium of the cochlea, is located on the "floor" of the SM, and contains a variety of cell types, which play both functional and structural supporting roles. Two of the critical cell types in this organ are the non-sensory SC called Deiters cells (Spicer and Schulte, 1994b) and the mechanosensory receptor cells called hair cells (HC), which the underlying Deiters SC "support", cupping the inner hair cells (IHC) physically. HC acquired their name due to the hair-like stereocilia, which are really long actin-containing projections from the cell surface called microvilli, on the apical surface of these polarized epithelial cells (Figure 1.1-C) (Friedman and Avraham, 2009). The HC and underlying SC are believed to be derived from a common progenitor cell (Fekete et al., 1998, Rivolta and Holley, 2002) and arranged in a precise, intricate and polarized cellular pattern, which is essential for proper mechanotransduction to occur.

There are three rows of outer hair cells (OHC) and a single row of IHC, surrounded by SC that include Pillar cells, Hensen's cells, and Claudius and Boettcher cells (Raphael and Altschuler, 2003). Hensen, Claudius and Boettcher

cells are located lateral to the HC, and not directly underneath them. They are also thought to be part of the supporting system of the OC (Spicer and Schulte, 1994b, a). Deiters cells, which have a very high metabolic capacity, are thought to provide more than just structural support (Spicer and Schulte, 1994b, a) although their exact role is still undefined.

The Pillar cells, with their apposing cellular apices, structurally form the tunnel of Corti (Figure 1.1-D) (Audition Promenade 'round the Cochlea). The stereocilia on the outer HC are embedded in an overlying flexible structure called the tectorial membrane (TM) (Richardson et al., 2008). Together the tiny bones of the middle ear and the tympanic membrane convert auditory signals to wave energy in the cochlear fluid (Rosowski et al., 1985). The basilar membrane, on which the HC and SC sit, moves in response to the fluid movement in the inner ear canals; HC also move up and down, in turn instigating movement in the stereocilia of the cochlear HC which causes ion channels on the stereocilia plasma membrane to open (Corey and Hudspeth, 1983b, a, Pickles and Corey, 1992, Denk et al., 1995, Littlewood Evans and Muller, 2000, Gillespie, 2004, Vollrath et al., 2007, Grillet et al., 2009). Thus, the cochlear HC convert auditory-derived mechanical stimuli (vibrations/waves) to electrical signals (Raphael and Altschuler, 2003). The electrical signals are transmitted from the HC to cochlear nuclei in the brain *via* the auditory nerve (Shepherd and Javel, 1997).

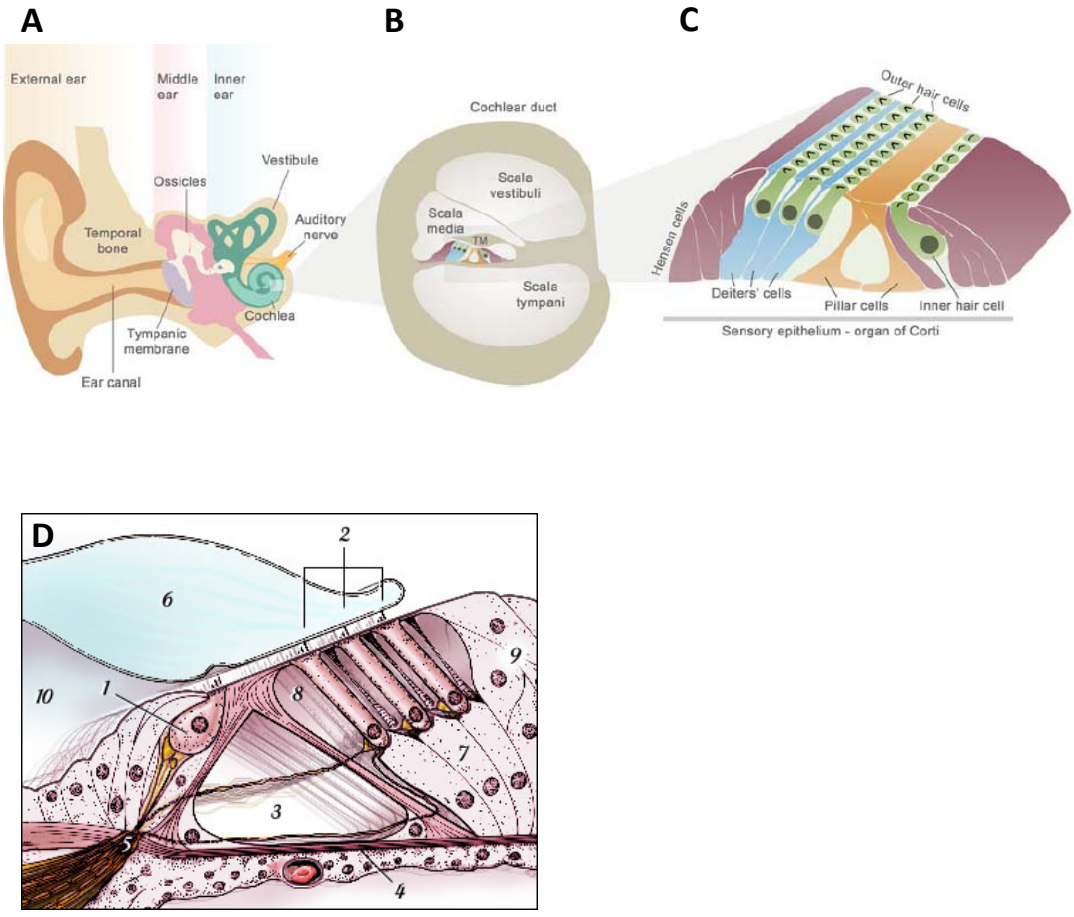


Figure 1.1: Schematic representations of the human ear. **A:** The ear is composed of three parts: the external, middle, and inner ear. **B:** Cross section of the cochlear duct. The three tubes, including the scala vestibule, the scala media (SM) and the scala tympani are shown. The cochlear sensory epithelium is also illustrated, with the tectorial membrane (TM) situated above the HC in the SM. **C:** Enlarged view of the sensory HC (green) and SC (blue) in the cochlear sensory epithelium-the OC. Stereocilia (long microvilli) are seen on the apical surface of HCs (Friedman and Avraham, 2009). **D:** The Pillar cells, with their apposing cellular apices, structurally form the tunnel of Corti (number 3) (From: Audition Promenade 'round the Cochlea: http://www.neuroreille.com/promenade/english/corti/fcorti.htm).

Mammalian inner ear development

Organogenesis:

The mammalian inner ear is one of the most complex organs in the body and its formation is considered as one of the most remarkable and sophisticated events in vertebrate organogenesis (Barald and Kelley, 2004). The development of this highly complex inner ear structure is the result of consecutive inductive signals emanating from neighboring tissues (the brain, the neural crest and the periotic mesenchyme are the best defined sources of signals) as well as the developing inner ear itself (Ohyama et al., 2007). The developing inner ear is the source of ODF (see discussion above).

The earliest morphologically visible step of inner ear development is the formation of the otic placode, a thickened disc of ectoderm located adjacent to rhombomeres 5 and 6 of the hindbrain in the early embryo (Barald and Kelley, 2004, Ohyama et al., 2007), typically seen once the first 5-10 pairs of somites have been generated (Ohyama et al., 2007). As development proceeds, the otic placode invaginates in birds and mammals to produce a hollow open pit, called the otic pit, which post-invagination, closes to form an epithelial cystic vesicle referred to as the otocyst (Barald and Kelley, 2004, Kelley, 2006, Ohyama et al., 2007). The otocyst undergoes elaborate morphogenetic changes. The part of the developing otocyst that originates in the dorsomedial region and extends towards the brain forms the endolymphatic duct and sac in the dorsal region (Riccomagno et al., 2002, Zheng et al., 2003), whereas the ventral region of the

otocyst extends to form the cochlear duct (Barald and Kelley, 2004, Kelley, 2006). Different regions of the developing inner ear continue to grow and differentiate and give rise to the different components of the inner ear. These include the cochlea, the semicircular canals and their associated sensory organs called cristae, the organs of the vestibular system (utricle, saccule and, in the bird, the lagena) as well as the vestibulo-acoustic ganglion (Ohyama et al., 2007) or SAG.

Neurogenesis:

Soon after closure of the otocyst, which occurs at embryonic day 9 (E9) in the mouse, neuroblasts delaminate from the anteroventral surface of the otocyst, also called the “otic crest” and migrate, giving rise to the developing SAG [(Ard et al., 1985, Hemond and Morest, 1991); reviewed in (Barald and Kelley, 2004, Kelley, 2006, Ohyama et al., 2007)], also called the vestibuloacoustic ganglion (VAG) that transmits hearing and balance information to the brain during early development. The SAG/VAG which is also termed the cochleovestibular ganglion (CVG) later gives rise to both the SG auditory ganglion (AG) that innervates the auditory organs and the vestibular ganglion (VG) that innervates the vestibular organs of the inner ear (Puligilla et al., Barald and Kelley, 2004, Ohyama et al., 2007).

Spiral ganglion neurons (SGNs)

As mentioned above, the SGNs are a mature form of the neurons in the part of the SAG destined to assume an auditory function. The SG is defined as the collection of neuronal cell bodies that provide the afferent or sensory innervation to the OC (Spoendlin, 1985, Spoendlin and Schrott, 1988, Rusznak and Szucs, 2009, Shibata et al., 2010). The central processes of these ganglionic neurons, which project toward the brain, comprise a major part of the acoustic nerve (Rusznak and Szucs, 2009). The cell bodies of the SGNs themselves are located in a canal, called Rosenthal's canal that spirals around the axis of the cochlea as shown in Figure 1.2-A.

SGNs are of two different types, type I and type II, based on their morphology, the nature of their synaptic connections and their functions (Spoendlin, 1971, Morrison et al., 1975, Ota and Kimura, 1980, Spoendlin, 1981, Rusznak and Szucs, 2009) (Figure 1.2-B). Type I SGNs have larger cell bodies than Type II and are responsible for the sensory innervation of the IHC. IHC function as the primary sensory cells of the auditory system. Activation of the IHC results in neurotransmitter release at the IHC type I SGN synapse. The action potential firing of the acoustic nerve causes activation of the central auditory pathways (Rusznak and Szucs, 2009), which ascend from the cochlear nucleus to the auditory cortex via three main pathways, the dorsal acoustic stria, the intermediate acoustic stria and the trapezoid body. The first binaural interactions take place in the superior olivary nucleus which receives input from the trapezoid body. Both the medial and lateral divisions of the superior olivary

nucleus are involved in the localization of sounds in space. Postsynaptic axons from the superior olivary nucleus and axons from the cochlear nuclei extend to the inferior colliculus in the midbrain via the lateral lemniscus, which contains axons relaying input from both ears. The axons in the colliculus project to the medial geniculate nucleus in the thalamus. The geniculate axons terminate in the primary auditory cortex (Lee and Sherman, 2010). Type II SGNs are smaller and provide the afferent innervation of the OHC (Spoendlin, 1969, 1971, 1972, Robertson et al., 1999). Although the precise function of OHC has not been determined, there is a general consensus that activation of the OHC can adjust or modify some of the mechanical properties of the basilar membrane of the OC, and thus, may influence the level of activation of the IHC (Rusznak and Szucs, 2009).

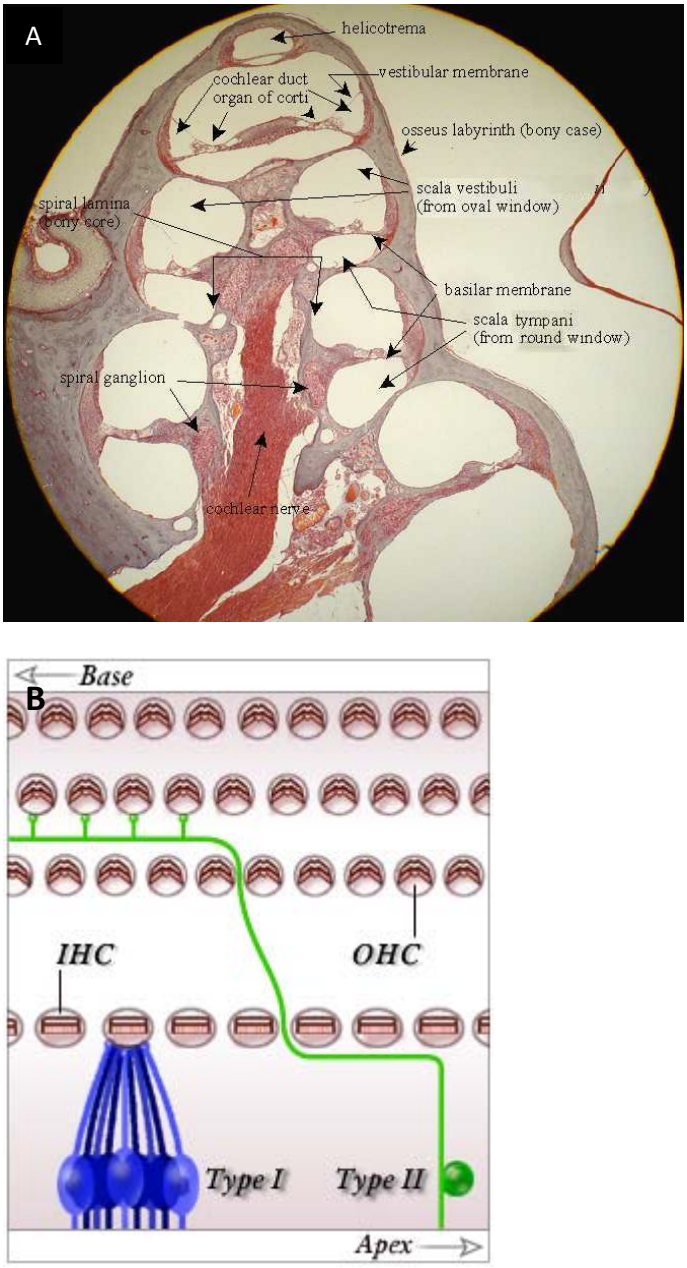


Figure 1.2: Schematic view of the cochlea and SGNs. A: The cell bodies of the SGNs are located in a canal, called Rosenthal's canal, that spirals around the axis of the cochlea. **B**: Two types of SGNs, type I and type II are present in the SG. Type I SGNs have larger cell bodies than Type II and are responsible for the sensory innervation of the IHC, while Type II SGNs are generally smaller and provide the afferent innervation of the OHC. (A:http://biology.clc.uc.edu/fankhauser/Labs/Anatomy_&_Physiology/A&P202/Special_Senses/Histology_Ear.htm) (B: Audition Promenade 'round the Cochlea; http://www.neuroreille.com/promenade/english/corti/fcorti.htm).

Sensorineural hearing loss (SNHL)

Hearing loss or deafness is among the leading disabilities in the United States (US); approximately 16.1% of the US population is affected by some form of hearing loss (Dinh and Van De Water, 2009). This percentage increases with age and about 33% of individuals 70 years of age and older are hearing-impaired (Desai et al., 2001, Dinh and Van De Water, 2009). Hearing impairment diminishes the quality of life of affected individuals by leading to social isolation and cognitive decline (Dinh and Van De Water, 2009).

SNHL is the most common form of deafness in humans and derives, in most cases, from primary degeneration of the SGNs or from secondary degeneration of these neurons, which is seen after cochlear sensory HC loss (Martinez-Monedero et al., 2006, Friedman and Avraham, 2009). Genetic predisposition as well as environmental factors, such as exposure to high-volume sound, infections, inadvertent ototoxic treatments including with aminoglycoside antibiotics and the anti-cancer drug cisplatin, contribute to SNHL (Cheng et al., 2005, Beisel et al., 2008), and those insults are further exacerbated by age (Pichora-Fuller and Singh, 2006, Kricos, 2007). While new HC production is quite common among cold-blooded vertebrates including fish, amphibia and reptiles as well as in birds after HC lesion (Corwin and Cotanche, 1988, Ryals and Rubel, 1988, Balak et al., 1990, Lombarte et al., 1993, Raphael et al., 2007, Brignull et al., 2009), the mammalian cochlea has no ability to spontaneously

regenerate sensory auditory HC after birth (Matsui and Cotanche, 2004, Raphael et al., 2007, Stone and Cotanche, 2007, Beisel et al., 2008, Brignull et al., 2009).

How HC loss leads secondarily to the loss of sensory neurons in the inner ear. Why this causes deafness.

As mentioned above, HC in the cochlea transduce acoustic energy into electrical signals that are transmitted to the central auditory system through the auditory nerves in normal ears. SGNs provide the afferent innervation to the hair cells; type I SGNs are in synaptic contact with the inner HC and type II SGNs are responsible for the sensory innervation of the outer HC (Rusznak and Szucs, 2009).

Sensory HC in the OC provide neurotrophic support to SGNs (Fritzsch et al., 1997a). Thus, loss of HC may lead to degeneration of nerve fibers from the sensory epithelium and eventually to degeneration of the SGNs due to a lack of this trophic support (Webster and Webster, 1981, Fritzsch et al., 1997a), although some studies argued that SC also secrete the neurotrophins necessary for SGN survival (Fritzsch et al., 1997a). Since mammalian auditory HC and neurons do not spontaneously regenerate, the hearing impairment associated with these cells' degeneration is permanent (Spoendlin, 1975).

Among many neurotrophins, brain-derived neurotrophic factor (BDNF) is one of the most extensively studied neurotrophins in inner ear development and regeneration. Expression of BDNF has been detected both in SC (Tan and Shepherd, 2006) and HC (Wiechers et al., 1999, Tan and Shepherd, 2006).

BDNF binds with high affinity to tropomyosin-related kinase B receptor (TrkB) and TrkB expression has been found in SGNs. One study found that adult mutant mice with severely reduced TrkB developed significant hearing loss (Schimmang et al., 2003). The p75 neurotrophin receptor (NTR) is a member of the TNF receptor superfamily and has also been detected in SGNs (Gestwa et al., 1999). Both phosphoinositide 3-kinase and the mitogen-activated protein kinase (MAPK) signaling pathways mediate Trk-activated survival responses in neurons (Kaplan and Miller, 2000, Huang and Reichardt, 2003), whereas p75NTR has been shown to be aberrantly upregulated under both pathological and inflammatory conditions (Roux et al., 1999, Lee et al., 2001a). Furthermore, some precursors of neurotrophins or pro-neurotrophins have been demonstrated to mediate cell apoptosis by their binding to p75NTR (Lee et al., 2001b, Nykjaer et al., 2004).

Based on the fact that Trk receptors and p75NTR are often co-expressed in the same neuron, Tan and Shepherd examined the expression patterns of these receptors in SGNs after destroying both HC and SC by introducing aminoglycoside antibiotics. The authors observed an augmentation of p75NTR and a reduced TrkB expression in degenerating SGNs, along with a decline in SGN density in Rosenthal's canal where these molecular alternations occur. Coincidentally, the authors observed the upregulation of phosphorylated c-Jun, which is expressed in degenerating neurons and is a target of p75NTR-mediated pathway, whereas the downregulation of phosphorylated cyclic AMP response element binding protein (CREB) that is expressed in neurons and is a target of

TrkB-mediated pathway (Tan and Shepherd, 2006). In addition, they identified an increase in a truncated form of pro-BDNF and a decrease in mature BDNF in aminoglycoside-deafened cochleae, indicating that BDNF and its reception are altered under pathological conditions (Tan and Shepherd, 2006). Together, these findings also demonstrated an antagonistic interplay of p75NTR and TrkB receptor signaling as an important event in SGN degeneration by modulating the signaling pathway.

Why sensory neurons last longer than hair cells in the inner ear?

It is well known that degradation of HC leads to subsequent loss of peripheral nerve fibers from the region of the OC due to a lack of electrical stimulation or to loss of the neurotrophin(s) normally provided by HC (Shibata and Raphael). However, neuronal degradation, as a result of HC degeneration is a very slow process and spiral ganglion neurons can survival for many years after the loss of most or all HC (Bichler et al., 1983).

Because of this reciprocity between neurons and HC, but with neuronal longevity far exceeding HC longevity, earlier studies focused on whether neuronal degeneration and loss depends only on the loss of sensory hair cells or whether there is also a correlation between the presence of supporting cells in the OC and the extent of neuronal degeneration (Bichler et al., 1983).

Bichler et al. investigated the degeneration of SGNs after degeneration of the OC by treating rats with amikacin (Bichler et al., 1983). The authors found

that there was no significant difference in the extent of neuronal degeneration in the presence or absence of supporting cells. In addition, the authors found that the most rapid reduction of the number of ganglion cells occurred within the first two months after the onset of the treatment and that degeneration progression slowed after that period. About 90% of SGNs disappeared by 12 months; however, the remaining neurons could survive for many years, except for the natural degradation due to aging (Bichler et al., 1983). Whether SC produce some trophic support for SGN was not examined or discussed in this report.

In contrast, Otte et al. (1978) found that the degree of retrograde degeneration of SGNs was clearly dependent on the state of the supporting cells, particularly Deiters' cells and pillar cells, rather than the state of sensory HC in humans (Otte et al., 1978). These authors observed that even if the OC was devoid of hair cells, but if the pillar cells and Deiters' cells remained, there was little or no SGN loss. They also observed that some of the SGNs survived after SC injury, however, these surviving neurons frequently lacked a peripheral or dendritic fiber, a condition which may have affected the sensitivity or selectivity in response to electrical stimulation by cochlear electrodes in implants (Otte et al., 1978).

Neurotrophins are secreted from both sensory HC and SC (Fritzsch et al., 1997a); thus, as long as SC still survive, SGNs may be able to receive the neurotrophin(s) from SC for their survival. The cytokine, macrophage migration

inhibitory factor (MIF), is indeed made by supporting cells of the OC (discussed in Chapter II, see Figure 2.9).

How a cochlear implant works

Cochlear implants (CI) (auditory prostheses) are surgically implanted devices that bypass a nonfunctional cochlea and function by directly stimulating the auditory nerves in the cochlea with patterns of electrical currents (Altschuler et al., 2008, Shibata et al., 2010) and the essential components in a CI system are shown in Figure 1.3 (Wilson and Dorman, 2008). The external parts of the CI include a microphone, a speech processor and a transmitter, while the internal parts include a receiver and a stimulator and the electrodes. The microphone picks up sound from the environment and the speech processor selectively filters sound to prioritize audible speech and sends the electrical sound signals to the transmitter. The transmitter transmits the processed sound signals to the internal device by electromagnetic induction. The implanted receiver and stimulator convert the signals into electric impulses and send them to electrodes through an internal cable. The electrodes, which are inserted and wound through the cochlea, then send these impulses to the nerves followed by transmittal to the brain through the auditory nerve system.

Since no HC regeneration occurs in mammals, cochlear prostheses are currently the only treatment for SNHL (Roehm and Hansen, 2005, Altschuler et

al., 2008, Shibata et al., 2010). Cochlear prostheses provide an increasingly successful therapy to restore hearing, particularly speech recognition, for those suffering from profound deafness (Altschuler et al., 2008, Shibata et al., 2010). Nevertheless, the successful function of these cochlear prostheses depends on activation of auditory nerves, so the presence of these nerves and conserved functionality are essential for CI function (Altschuler et al., 2008, Serin et al., 2009, Shibata et al., 2010). Preserving SGN or finding a cellular replacement for lost or damaged SGN therefore is a priority if CI function is to be enhanced.

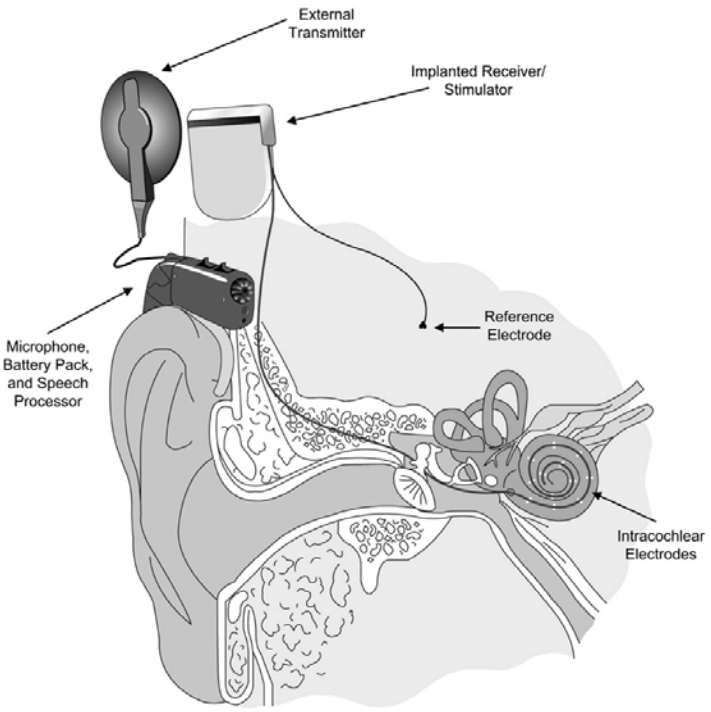


Figure 1.3: Schematic view of the essential components in a cochlear implant system (Wilson and Dorman, 2008).

Trophic factors on which each of the stages of neurons depends

Various approaches to maintaining and activating the remaining SGNs and whole nerves have also been reported (Kong et al., Miller et al., 2002, Gillespie and Shepherd, 2005, Martinez-Monedero et al., 2007, Miller et al., 2007, Agterberg et al., 2008, Altschuler et al., 2008, Shibata et al., 2010). Some investigators introduce exogenous intracochlear growth factors including BDNF (Kong et al., Miller et al., 2002, Miller et al., 2007, Agterberg et al., 2008, Shibata et al., 2010), GDNF (Ylikoski et al., 1998), a combination of GDNF with BDNF (Iguchi et al., 2003), as well as such combinations of growth factors/neurotrophins combined with antioxidants such as ascorbic acid and Trolox (Maruyama et al., 2008) or a combination of growth factors with continuous electrical stimulation (Scheper et al., 2009). The latter approach is based on the observation that secondary degeneration usually follows sensory epithelial degradation and neuronal cell death takes place due to a lack of trophic factors that could maintain the adult ganglion and nerve in functional condition (Fritzsch et al., 1997a, Martinez-Monedero et al., 2007, Shibata et al., 2010). Cells of the otocyst and later the cochlea secrete trophic factor(s) that support both the early development and growth of the developing neurons and later, the chemo-attraction, pathfinding and innervation of these neurons, which synapse on HC in the cochlea (Gillespie, 2003, Fekete and Campero, 2007) and still later, the continued maintenance of these neurons. The trophic factors on which some of the stages of neurons appears to depend is presented in Table 1.1. This table

presents what was known when we began the studies described in this thesis (and prior to the publication of Bianchi et al., 2005).

Ganglionic stage	Day of development	Functional or structural milestone	Trophic dependence	references
SAG	E12.5 (mouse)	Vestibular neuron survival	BDNF	(Tessarollo et al., 2004)
	E12.5 (mouse)	Cochlear neuron survival	NT-3	(Tessarollo et al., 2004)
	E10.5 (mouse)	Neuritogenesis and neuronal population	NGF	(Staecker et al., 1996b)
	E11 (mouse) E3.5 (chick)	Migration and neurite outgrowth	FGF-1 and FGF-2 FGF-2	(Hossain and Morest, 2000) (Brumwell et al., 2000)
	Otic cup stage (E2 in chick)	Cell survival, proliferation and neurogenesis	IGF-1	(Leon et al., 1995, Frago et al., 2000, Camarero et al., 2003)
SGN	Early postnatal	SGN development (maturation) and survival	BDNF	(Malgrange et al., 1996, Staecker et al., 1996a, Marzella et al., 1999)
	Early postnatal	SGN development (maturation) and survival	NT-3	(Malgrange et al., 1996, Staecker et al., 1996a, Marzella et al., 1999)
	Neonatal and mature	SGN survival	GDNF	(Ylikoski et al., 1998)
	Early postnatal	SGN survival	NT4/5	(Zheng et al., 1995, Zheng and Gao, 1996)
	E11-P14	Establishment of cochlear innervations	FGF-1	(Luo et al., 1993)
	Postnatal mice	SGN survival, differentiation and maturation	IGF-1	(Camarero et al., 2001, Camarero et al., 2003)
	Early postnatal rat (cell culture)	SGN survival	TGF-β3	(Marzella et al., 1998, Marzella et al., 1999)
	Early postnatal rat (cell culture)	SGN survival	TGF-β5	(Marzella et al., 1999)

Table 1.1: Trophic factors on which each of the stages of neurons depends.

In the developing inner ear, the SAG contains neuroblasts/neurons, which are the precursors of both SGNs and vestibular ganglion (VG) neurons. Several studies have reported that two neurotrophins, NT-3 and BDNF, play critical roles in inner ear neurite innervation and survival at a point in development when the SAG neurons ultimately separate into these distinct neuronal types. Each of these neuronal populations begins to rely on specific, identified trophic factors (Fritzsch et al., 1997b). Fritzsch et al. also demonstrated that the majority of mature SGNs relies on NT-3 for survival. Mice lacking either NT-3 or its associated receptor, TrkC, were found to have a dramatic loss of SGNs. VG neurons, on the other hand, become dependent on BDNF and mice lacking either BDNF or its receptor, TrkB, showed a tremendous loss of VG neurons (Fritzsch et al., 1997b, Fritzsch et al., 1997c).

However, prior to this stage, when afferent fibers initiate their extension towards the target cells in the otocyst in the periphery, these neurotrophins had little effect on outgrowth of SAG neurons (Bianchi and Cohan, 1991, 1993, Tessarollo et al., 2004). Moreover, the biologically active components on which the earliest stage initiation of SAG neurite outgrowth depends were not fully characterized until our recent work identified a quartet of “inflammatory” cytokine factors, known to function in the immune system, but which behave as neurotrophins in early inner ear development. More details about the most influential of these factors, MIF, in terms of being able to play both a necessary and sufficient role in SAG neurite outgrowth and survival will be presented in the following sections of this thesis.

At the SGN stage, several neurotrophins have been reported to play important roles in survival, maturation and maintenance in experimental animal models (Gillespie and Shepherd, 2005). Early studies demonstrated that both BDNF and NT-3 promoted survival of dissociated neuronal cells in cultures of early postnatal rat SGNs (Malgrange et al., 1996, Marzella et al., 1999). A synergic effect was seen when these neurotrophins were added together (Marzella et al., 1999). Zheng et al. found that NT-4/5 also promoted SGN survival, and its effect on survival was equivalent to that of BDNF and stronger than that of NT-3 (Zheng et al., 1995). In addition to their survival effects, each of these factors (BDNF, NT-3 and NT-4/5) has been reported to protect the SGNs from ototoxic agents including therapeutic drugs such as salicylates (Zheng and Gao, 1996) and chemotherapeutic drugs used in cancer treatment, such as cisplatin (Zheng et al., 1995), suggesting that these neurotrophins might have therapeutic value in preventing hearing impairment caused by damage to primary auditory neurons. In situ hybridization analysis detected the expression of GDNF in the inner ear HC of both the neonatal and mature rat cochlea, while its receptor, GDNF receptor-α, was found on the SGNs (Ylikoski et al., 1998). Several studies demonstrated that GDNF enhanced the survival in early postnatal rat SGNs both *in vitro* (Ylikoski et al., 1998, Qun et al., 1999) and *in vivo* following IHC lesions (Ylikoski et al., 1998).

In addition to the neurotrophins, other families of growth factors, including cytokines, have demonstrated effects on SGN survival and maintenance. Those include cytokines, TGF-β3, TGF-β5, FGF-1 and insulin-like growth factor (IGF-1).

Marzella et al. showed that both TGF-β3 and TGF-β5 promoted postnatal rat SGN survival *in vitro* (Marzella et al., 1999). An effect of TGF- β5 on SGN survival was dose-dependent, with a significant increase in survival seen at 0.01 ng/ml of exogenous TGF- β5 compared to control. Maximum neuronal survival was observed with 1 ng/ml TGF- β5; however, higher concentrations didn't show further survival. The expression of FGF-1 at the mRNA level was detected in the rat cochlea from E16 to P14 and in the HC during the first postnatal week (Luo et al., 1993). Based on its temporal expression patterns, the authors suggested its possible role in maintenance of SG neurons and the establishment of cochlear innervation (Luo et al., 1993). Further studies demonstrated a role for FGF-1 in enhancement of SG neuron survival and maintenance *in vitro* (Dazert et al., 1998, Aletsee et al., 2000) as well as induction of SG neuronal branching *in vitro* (Aletsee et al., 2003). The detection of IGF-1 expression has also been reported in both the developing inner ear and the postnatal cochlear as well as vestibular ganglia in chicks (Leon et al., 1995) and in mice (Varela-Nieto et al., 2004).

Early studies of KO and transgenic (Tg) mouse models for the *igf-1* gene have demonstrated an essential role for this cytokine in nervous system development (Powell-Braxton et al., 1993a, Powell-Braxton et al., 1993b). The *igf-1* null mice died shortly after birth, concomitantly showing growth retardation, drastically reduced brain size and loss of specific neuronal populations. Similarly, the *igf-1* deficient postnatal mice demonstrated reduction in the size of the cochlea, a highly immature tectorial membrane (TM) and SG neurons, and a significant decline in the number of and the size of the SG neuronal soma

(Camarero et al., 2001). In addition, the authors demonstrated that a decrease in the number of SGNs found in mutants was due to an increase in caspase-3 mediated apoptosis. Furthermore, an abnormal synaptophysin expression pattern was observed in the somata of both SGNs and sensory HC in the mutants (Camarero et al., 2001). An additional study reported that the myelin sheaths were severely affected in these mutants (Camarero et al., 2003). These results strongly suggest that IGF-1 plays an important role in inner ear neuronal and sensory cell maturation, and also in the survival and differentiation of the postnatal mouse inner ear.

Otocyst derived factor(s) (ODF) enhance(s) directional neurite outgrowth and neuronal survival of the early stage SAG neurons

Innervation target-derived or cellular pathway-derived growth and survival factors are essential for the normal development and survival of neurons (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993, Bianchi et al., 1998, Bianchi et al., 2005). Early studies in both the rat and the chick (E4-6 in the chick, E11-14 in the mouse and rat) demonstrated that during the early stages of auditory development, cells in the anteroventral region of the otocyst secrete soluble and diffusible factor(s), which have been collectively termed Otocyst Derived Factor(s) (ODF), which influence both directional neurite outgrowth and survival of innervating SAG neurons of the eighth (VIII) cranial nerve in the nascent auditory-vestibular system (Bianchi and Cohan, 1991). Further attempts at characterization demonstrated that this bioactivity was not

provided by classical growth factors, including the neurotrophins (NGF, BDNF, NT-3 and NT-4) or CNTF, none of which was present at the time of early development, although the SAG comes to rely on these factors at later developmental stages (see discussion above). Furthermore, these factors, exogenously applied "off the shelf", either alone or in combination, had little or no effect on the survival and neurite outgrowth of early stages SAG neurons (Bianchi and Cohan, 1993).

Moreover, ODF, produced from either early stage mouse or chick otocysts failed to promote neurite outgrowth from different types of placodally-derived or neural crest derived peripheral neurons, including trigeminal, ciliary, sympathetic, or dorsal root ganglion neurons, suggesting that ODF's effects were specific to inner ear neuronal development (Bianchi and Cohan, 1993). Additional experiments showed that antisera to nerve growth factors (e.g. NGF) were not able to block ODF activity (Bianchi and Cohan, 1993). These results suggest that the classical neurotrophic factors are not the primary components of ODF and that ODF may have different molecular properties from other identified growth factors commonly thought to have effects in the nervous system. Further study confirmed Bianchi and Cohan's work by providing the precise spatiotemporal pattern of expression of NT-3 and BDNF during the process of inner ear innervation (Tessarollo et al., 2004). This study was carried out with specific NT KO mice; wild-type (WT), mice null at the Bdnf locus ($BDNF^{-/-}$), BDNF model mice carrying a knock-in of Bdnf into the NT-3 locus for the replacement of

NT-3 with BDNF (NT-3$^{tgBDNF-/-}$; BDNF$^{-/-}$ and NT-3$^{tgBDNF+/-}$; BDNF$^{-/-}$). This elegant study characterized the spatiotemporal role of these factors in early otic development, prior to the time when embryonic lethality was notable. The authors observed neuronal innervation patterns both in WT and all the mutant mice towards the appropriate regions of the otocyst at E11.5, indicating that both NT-3 and BDNF are not necessary for the initial outgrowth of SAG neuronal processes toward their targets. On the contrary, the authors observed aberrant axonal branches and projections to the area, where BDNF was ectopically expressed in domains normally specific for NT-3 by E13.5.

However, in contrast, other studies reported an important role for NGF, FGF-1 and FGF-2 and IGF-1 in initiation of SAG neurite outgrowth (Hossain et al., 1996, Staecker et al., 1996a, Staecker et al., 1996b, Hossain et al., 1997, Hossain and Morest, 2000). Staecker et al. detected the expression of NGF as early as E10.5 in the mouse otocyst and purported to demonstrate its essential role in inner ear development (Staecker et al., 1996b). Their confocal microscopic analysis appear to demonstrate that downregulation of NGF in otocyst-SAG explants resulted in an inhibition of neuritogenesis as well as a reduction in the population of the SAG neurons compared to the control.

Other studies demonstrated what the authors believe is an essential role for FGF-2 in initiating migration and neurite outgrowth of early stage SAG neurons (Hossain et al., 1996, Hossain et al., 1997), while BDNF was found to enhance the effect of FGF-2 in SAG neuronal development at later stages (Brumwell et al., 2000) both in chicks (Hossain et al., 1996, Hossain et al., 1997,

Brumwell et al., 2000) and in mice (Hossain and Morest, 2000). In addition to FGF-2, a study showed that exogenous recombinant FGF-1 also enhanced early SAG migration and outgrowth in mice and the effect on migration and outgrowth was greater when combined it with FGF-2 than with either growth factor alone (Hossain and Morest, 2000).

Both IGF-1 and its receptor, IGF-1R, are expressed during the otic cup stage in chick embryos (Leon et al., 1995, Camarero et al., 2003). A central function of IGF-1 at the otic stage is to maintain cell survival and proliferation (Sanz et al., 1999, Frago et al., 2003). Frago et al. demonstrated that exogenous IGF-1 protected otic vesicles/otocysts from cell death caused by pro-apoptotic stimuli such as serum deprivation (Frago et al., 2003), and its mechanism in cell proliferation was apparently due to activation of the mitogen-activated protein kinase (MAPK) cascade as well as to upregulation of the early genes, c-Jun, c-Fos and proliferative cell nuclear antigen after IGF-1 binding to the IGF-1R (Leon et al., 1998, Sanz et al., 1999). Recent work by Camarero et al. identified an additional role for IGF-1 in neurogenesis in chick embryos (Camarero et al., 2003). The authors demonstrated that IGF-1 promoted transition of neuroblasts into the post-mitotic stage of neuronal development using stage 19-20 (70-72 hours) chick SAGs. They observed that 1 nM IGF-1 alone enhanced chick SAG neurite outgrowth, along with high G4 immunoreactivity, the early neuronal marker. Blockade of IGF-1 action with the receptor competitive antagonist, JB1, blocked IGF-1 induced cell proliferation and differentiation (Camarero et al., 2003).

Proteomic studies demonstrate that the major components of ODF are immune system cytokines

Our recent proteomic studies (included in Chapter 2) identified the bioactive components of ODF as immune system cytokines, including MIF (Gerlach-Bank, Ebisu et al., in preparation). These studies also confirmed our earlier study that demonstrated a second immune system cytokine, MCP1 or JE/CCL2, was also a bioactive component of the ODF (Bianchi et al., 2005). This work will be discussed in detail in chapter 2.

Development of immortalized mouse inner ear cell lines to characterize ODF

In order to analyze and characterize the cytokines in ODF, large quantities of material were and will still be required. ODF was originally generated by incubating 8 otocysts/ 250 µl of serum-free culture medium (Bianchi and (Bianchi and Cohan, 1991, 1993, Bianchi et al., 1998). Therefore, cultures of otocysts do not provide sufficient quantities of medium for biochemical or proteomic analysis. To overcome this limitation, other sources of ODF-like activity have been sought. A number of years ago, we attempted to identify and isolate clones of cells that retained some plasticity and pluripotentiality by immortalizing early developing inner ear otocyst cells from the earliest stage of the otocyst that could be reliably isolated free of periotic mesenchyme and before emigration of the cells of the otic crest (Barald et al., 1997).

Inner ear sensory HC and SCs as well as sensory neurons (SNs) are hypothesized to develop from common precursors in the early embryonic otocyst

(Germiller et al., 2004). However, cellular and molecular studies of the developing and adult inner ear in mammals have been hampered by the increasingly difficult access to developing sensory organs and especially to adult organs as the temporal bone, which houses these delicate structures, ossifies during inner ear maturation.

Lack of appropriate organ and cell culture systems of the early inner ear also hindered more extensive molecular studies, due to the limited availability of the cellular material in the inner ear. Thus, our laboratory (Barald et al., 1997) as well as others (Holley and Lawlor, 1997, Kalinec et al., 1999) developed conditionally immortalized cell lines from the embryonic mouse inner ear. The cell lines in our lab were derived from the otocysts of 9.5-day embryos, the earliest stage at which the otocyst can easily be separated from surrounding mesenchymal, nervous system and neural crest cells (Germiller et al., 2004). Moreover, at this stage, many of the cells in the otocyst are still pluripotent, allowing us to identify uncommitted precursors as well as both HC and SN precursors.

The H2kbtsA58 Tg, which carries a temperature sensitive variant of the SV40 large T antigen under the control of a gamma-interferon sensitive promoter, was used to isolate the otocyst cells that provided these IMO clonal cell lines (Barald et al., 1997, Barald and Kelley, 2004, Germiller et al., 2004). Conditionally IMO cells from this transgenic animal proliferate in the presence of gamma-interferon at 32-33°C, the permissive temperature for transgene

expression, but they stop propagating and differentiate after a temperature shift to 37-39°C (the non-permissive temperature for the temperature-sensitive transgene expression) and removal of gamma-interferon from the media (Barald et al., 1997, Germiller et al., 2004).

One notable difference between chicks and mice is that mature HCs in chicks express BMP4, while SCs do not. The opposite is true in the mature mouse ear, where SCs but not HCs express BMP4 (Germiller et al., 2004). In the chick, "new" HC arise from the SC population through transdifferentiation (Corwin and Cotanche, 1988, Balak et al., 1990, Corwin et al., 1991, Bhave et al., 1995, Jones and Corwin, 1996, Stone et al., 1996, Warchol and Corwin, 1996, Molea et al., 1999, Stone and Rubel, 1999, 2000b, a, Bermingham-McDonogh et al., 2001, Stone and Cotanche, 2007, Ma et al., 2008, McCullar and Oesterle, 2009). These IMO cell lines provide a unique model system for studying early stages of inner ear development to determine the consequences of affecting key molecular events in their differentiation.

At least 2 of the IMO cell lines (IMO-2B1, -2D2) that we have developed (Barald et al., 1997, Thompson et al., 2003, Germiller et al., 2004, Bianchi et al., 2005) provide neurite-outgrowth and survival promoting activity for early stage SAG (Chapter 2). Because the IMO cells are grown in large numbers under serum-free conditions in larger volumes (10 ml per 100 mm culture dish), the otocyst cell line-conditioned medium provides sufficient material for biochemical

characterization of the neurite-outgrowth promoting and survival factor(s) released by IMO cell lines.

In this thesis, we have taken advantage of the IMO cells' ability to *produce an* ODF-like bioactivity to use these cells to identify the composition of ODF (Bianchi et al., 2005). However, only by extending the information obtained from studies of IMO-generated ODF otocyst-derived ODF can we validate IMO cells as a model of inner ear development. In the future, we will employ both gain and loss of function studies to examine the bioactivity of the cytokine components of ODF and the molecular rules that govern their potential synergy or antagonism. So far, we have used protein and nucleic acid probes to determine the expression patterns of the bioactive cytokine components in the developing and mature inner ear and eventually plan to extend these studies to determine if such factors could influence regeneration of neurons in a damaged or diseased inner ear.

MIF, an inflammatory cytokine, plays the role of a "neurotrophin" in early inner ear development

As discussed in the brief introductory paragraphs to this chapter, MIF has been found to be a major component (in terms of activity) in ODF. MIF is a highly conserved cytokine with a molecular weight of 12.5 kilodaltons (kDa) (Weiser et al., 1989, Baugh and Bucala, 2002b), was originally described as an "activity" of cognate T cell supernatants that inhibited macrophage migration as the name suggests (David, 1966). Much research has been done since then,

yielding evidence that MIF is expressed in various cell types including monocytes/macrophages, endothelial cells, eosinophils, neurotrophils, epithelial cells, lymphocytes and smooth muscle cells under physiological conditions (El-Turk et al., 2008, Cooke et al., 2009, Santos and Morand, 2009) and is secreted by the anterior pituitary gland in systemic stress responses or in response to low concentrations of glucocorticoids (El-Turk et al., 2008). MIF is now described as a "pleiotropic" cytokine since it plays multiple roles including enzymatic activity, the pathogenesis and progression of both acute and chronic inflammatory, and autoimmune diseases including systemic and organ-specific autoimmune diseases (Bernhagen et al., 1993, Calandra and Roger, 2003, El-Turk et al., 2008). Further studies demonstrated that MIF is implicated in carcinogenesis, linking chronic inflammation and cancer (Lue et al., 2007).

More recently, several studies found a vital additional role for MIF in the development and regeneration of peripheral nerves and skin after injury (Nishio et al., 1999, Abe et al., 2000, Nishio et al., 2002, Suzuki et al., 2004, Shimizu, 2005, Dewor et al., 2007, Velnar et al., 2009) (discussed below).

Enzymatic activity of MIF/MIF as a therapeutic target:

Structural analysis of MIF by X-ray crystallographic and NMR studies demonstrate that MIF exists as a homo-trimer as shown in figure 1.4 (Sun et al., 1996, Baugh and Bucala, 2002b, Leng and Bucala, 2006), and that is homologous to an enzyme called D-dopachrome-tautomerase (DDT) (Sugimoto et al., 1997, Bach et al., 2009). Based on the structural similarity between MIF

and DDT, Rosengren et al. found that MIF has tautomerase activity and is able to convert D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Rosengren et al., 1996). Since D-dopachrome is not a naturally-occurring substrate, the authors were not able to determine what the actual biological effect of MIF is in the body that might be a consequence of this type of conversion. Nevertheless, the results could offer a potential approach for designing pharmacological inhibitors of MIF that might modulate its potent immunological effects *in vivo* (Rosengren et al., 1996). Further studies identified additional enzymatic activities of MIF, including its action as a phenylpyruvate tautomerase with thiol-protein oxidoreductase (Kleemann et al., 1998). More recently, El-Turk et al. have demonstrated that inter-subunit interactions involving carboxy terminal residues 105-114 of MIF play critical roles in modulating tertiary structure stabilization, enzymatic activity and the thermodynamic stability of MIF, and suggested the possible use of strategies to develop novel inhibitors of MIF tautomerase activity (El-Turk et al., 2008), although the precise role for MIF enzymatic activity in either normal function or in clinical disease has not been clearly defined at the present time.

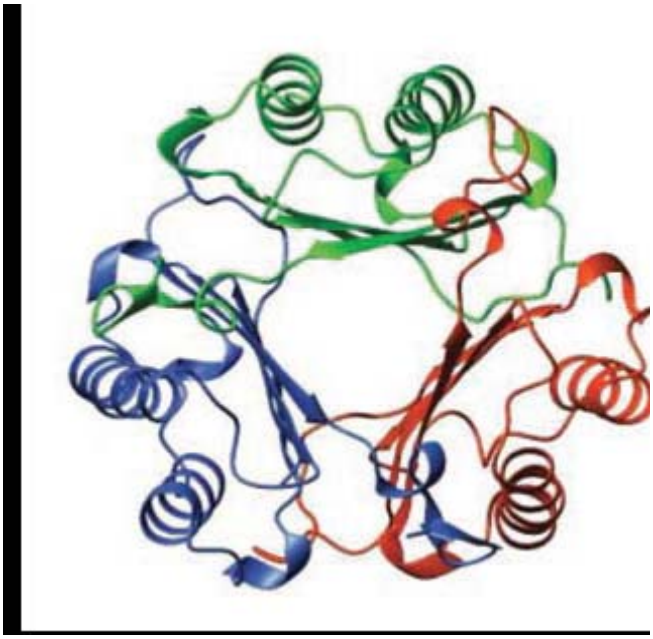


Figure 1.4: Three-dimensional ribbon diagram of human MIF. MIF exists as a homo-trimer; each molecule is represented in this diagram in a different color (green, red, blue) (Leng and Bucala, 2006).

Since tautomerase activity is an evolutionarily ancient phenomenon and expendable, Al-Abed et al. developed a molecule called (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) that binds the catalytic site to inhibit MIF tautomerase activity (Al-Abed et al., 2005). In this study, the authors first treated mice with either lipopolysaccharide (LPS) or the cecal ligation and puncture (CLP) to induce endotoxemia, a condition that leads to rising levels of endotoxins in the blood, and which leads to septic shock, or to polymicrobial sepsis respectively. Twenty four hours post injection, they treated mice with either ISO-1 or dimethyl sulfoxide (vehicle) to examine the effects of ISO-1 on MIF cytokine activity *in vitro* as well as tumor necrosis factor (TNF) release from macrophages and, in *in vivo* studies, the animals' survival.

The authors observed that ISO-1 inhibited MIF cytokine activity and TNF release from macrophages isolated from LPS-treated mice (Al-Abed et al., 2005, Cooke et al., 2009). They also observed that ISO-1 significantly enhanced survival in mice treated with LPS/CLP when compared to control. These studies showed that MIF activity could be therapeutically regulated by a molecule specifically targeting its tautomerase-active site. Moreover, they raise the possibility that the deleterious effects of MIF in disease is due to excess of MIF expression and that this may be abolished by treatments with ISO-1.

More recently, Winner et al. identified an additional irreversible inhibitor of MIF, 4-iodo-6-phenylpyrimidine (4-IPP) that serves as a suicide substrate for MIF, resulting in the covalent modification of the catalytically active NH_2 terminal proline (Winner et al., 2008). The group demonstrated that 4-IPP is 5 to 10 times more potent in blocking MIF-dependent catalysis and lung adenocarcinoma cell invasion/migration and anchorage-independent tumor cell growth than ISO-1, although further studies are required to determine the antitumor efficacy of this novel compound.

Our laboratory has begun studies of MIF-inhibition by 4-IPP, among other inhibitor assessments (Shen et al., submitted), to block MIF activity to determine its effects on inner ear development. We have observed that zebrafish embryos treated with this small molecule had a curved body, slightly smaller head, eye and ears, which is to the phenotype of mif morpholino antisense oligonucleotides (MOs)-treated embryos compared to the DMSO treated controls (Shen et al., in preparation). Additionally, delayed or incomplete semicircular canal formation

was observed in the 4-IPP treated embryos. Furthermore, the saccular macula (the zebrafish hearing organ, which bears sensory HC) in these embryos was slightly smaller than the control embryos (Shen et al., in preparation). The effect of 4-IPP on HC numbers is presently being evaluated.

The pathogenesis and progression of acute inflammation in the body:

The innate immune system provides host defense mechanisms against microbial pathogens in a non-specific manner (Calandra, 2003). Macrophages play an essential role in the innate immune system by recognizing and eliminating invasive microbial pathogens (Aderem and Underhill, 1999, Roger et al., 2001). When microbial products bind to pathogen-recognition receptors, macrophages are activated and release many kinds of cytokines, including MIF, to orchestrate the host's innate immune response. MIF has emerged as a pivotal mediator of acute inflammatory responses, such as those that occur during bacterial infections (Wiersinga et al., Geldhoff et al., 2009).

Bacterial infection: Roger et al. demonstrated that MIF is an essential regulator of macrophage responses to endotoxin, such as those engendered by LPS, and gram-negative bacteria. They compared NF- κB activity, which is involved in cellular responses to various stimuli, and pro-inflammatory cytokine production, including the production of TNF-α and interleukin (IL)-6, in MIF-deficient mouse macrophages compared with WT macrophages (Roger et al., 2001). The authors observed a large reduction in NF-κB activity and TNF-α and IL-6

production in MIF-deficient macrophages, compared to WT macrophages. The vital steps in the recognition of LPS or gram-negative bacteria by the host require interaction between LPS-binding protein (LBP) (Schumann et al., 1990), CD14, a ligand-binding GPI-anchored protein (Wright et al., 1990), and Toll-like receptor 4 (TLR4) (Poltorak et al., 1998), the signal-transducing molecule of the LPS receptor complex required to activate NF-κB and proinflammatory cytokines (Roger et al., 2001). LBP binds to LPS-containing particles and transfers them to a receptor complex composed of CD14 and TLR4. Additional experiments performed by Schumann et al. (1990) demonstrated that the reduction of NF-κB activity and pro-inflammatory expression were due to the downregulation of TLR4, which is associated with decreased activity of PU.1, a transcription factor required for optimal expression of the Tlr4 gene in myeloid cells. Their findings provided important information that MIF promotes both the recognition of LPS and gram-negative bacteria by immune cells and development of the host defensive system through upregulation of TLR4. In addition, TLR4 upregulation mediated by MIF demonstrated a molecular basis for the increased resistance of MIF KO mice to LPS induced endotoxic shock. Numerous studies have reported that neutralization of MIF either with anti-MIF antibodies (Calandra et al., 2000) or small molecule MIF inhibitors, including ISO-1 (Al-Abed et al., 2005), protected mice from various infections, including that resulting from CLP, which induced both peritonitis and sepsis, and which was previously described. Recent studies showed that patients with the bacterial infections, such as melioidosis, had higher MIF expression in both plasma and in their peripheral blood leukocytes than

healthy controls. Furthermore, elevation of MIF expression was associated with an adverse outcome for such patients (Wiersinga et al.), further suggesting MIF's essential role in acute inflammation. MIF is therefore a prime candidate target molecule for treatment of acute inflammation as well as many other diseases with inflammatory sequellae (Al-Abed et al., 2005).

The pathogenesis and progression of chronic inflammatory diseases (Autoimmune diseases):

Autoimmunity results from either dysregulation of central tolerance in the thymus or the lack of appropriate peripheral tolerance (Stosic-Grujicic et al., 2009). Once the immune response is triggered, autoimmune diseases are sustained through a permanent activation of the inflammatory process, which usually protects the host against infection. As a consequence of such an immune response, immune cells can induce cell death in the target tissue. Numerous studies have reported that pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 are involved in both systemic and organ-specific autoimmune diseases and that MIF is prominently located upstream of the events that lead to such autoimmune responses (Stosic-Grujicic et al., 2009). Inhibition of MIF has proven beneficial in the treatment of autoimmune diseases in both animal and human studies (Mikulowska et al., 1997, Santos et al., 2001, Burger-Kentischer et al., 2006, Hoi et al., 2006, Leech et al., 2008, Santos et al., 2008).

a. Systemic autoimmune diseases: Systemic lupus erythematosis (SLE) is a chronic multisystem immune disorder characterized by a loss of tolerance to host antigens. It is associated with the development of pathogenic autoantibodies that can harm target organs, including the skin, joints, the brain and the kidney (Hoi et al., 2003, Santos and Morand, 2009). Key immune response factors, which contribute to these disease states have been identified: TLRs, CD28-B7 family members, TNF-TNF receptor family members and inflammatory cytokines such as TNF, IL-6, IFN-α, IFN-γ and IL-10 (Santos and Morand, 2009). Unlike other autoimmune diseases, cytokine-target therapy against the cytokines mentioned above is not applicable to SLE because of the strong possibility that they are required for the active maintenance of immune system tolerance. An association of MIF with SLE severity was first reported by Rovenskey et al. over three decades ago (Rovensky et al., 1975). More recent clinical studies have identified MIF as a therapeutic target in SLE. The reasons for implicating MIF include: MIF is expressed in the cell cytoplasm of immune system and blood cells and is and released only on stimulation of these cells (Calandra et al., 1994). Second, MIF directly affects key effector cells including macrophages and can generate other proinflammatory stimuli (Cunha et al., 1993, Calandra et al., 1994). In addition, MIF can act as a chemokine and help facilitate the interaction between leukocytes and endothelial cells. It can also inhibit apoptosis and promote cell proliferation. These properties suggest that a blockade of MIF might have only partial effects once inflammation cascades had been triggered (Santos and Morand, 2009). Investigators who examined the expression and function of MIF

in SLE using the lupus-prone MRL/*lpr* mice (Hoi et al., 2006). Found that renal MIF expression was significantly higher in MRL/*lpr* mice compared with control mice. These findings also paralleled the markedly up-regulated MIF levels found in skin lesions of MRL/*lpr* mice. In this study, the authors generated MRL/*lpr* mice with a targeted disruption of the *MIF* gene ($MIF^{-/-}$MRL/*lpr*) to examine an effect of MIF on development of SLE. They demonstrated that $MIF^{-/-}$MRL/*lpr* mice exhibited significantly prolonged survival and a reduction of both the renal and skin effects of SLE compared with their unaffected littermates. Furthermore, no major changes in T and B cell markers or alterations in autoantibody production were observed, suggesting that MIF is a critical effector of organ injury in SLE (Hoi et al., 2006).

b. Organ-specific autoimmune diseases: MIF has been implicated in the pathogenesis of multiple organ-specific autoimmune diseases as well, including rheumatoid arthritis (RA) and multiple sclerosis (Stosic-Grujicic et al., 2009).

b-1. Rheumatoid arthritis (RA): RA is characterized by chronic inflammatory destructive polyarthritis (Santos and Morand, 2009). MIF is upregulated in RA synovial fluid and serum compared to its levels in healthy controls (Leech et al., 1999, Onodera et al., 1999) and appears to contribute directly to the destructive progression of this disease (Onodera et al., 2000). MIF stimulates macrophages to release other proinflammatory cytokines critical in RA, such as TNF, IL-1, IL-6 and IL-8 (Onodera et al., 1999, Onodera et al., 2004, Santos et al., 2004). In addition, MIF has been reported to promote the expression of other

proinflammatory genes, including fibroblast-like synoviocytes (FLS) phospholipase A_2, cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) (Sampey et al., 2001, Santos et al., 2004). Antagonizing MIF delays onset and decreases the frequency of collagen-induced arthritis (Mikulowska et al., 1997) and murine antigen-induced arthritis (AIA) (Santos et al., 2001). In the latter study, the authors first induced AIA by intra-articular injection of methylated bovine serum albumin in presensitized mice, followed by treatment of these mice with an anti-MIF antibody, recombinant MIF (rMIF) and/or dexamethasone (DEX). The severity of arthritis was measured in a blinded study quantitating synovial cellularity by histological assessments. They observed that that AIA was significantly inhibited by the anti-MIF antibody, whereas DEX treatment inhibited AIA in a dose-dependent manner. Exogenous MIF treatment reversed the effect of therapeutic DEX on AIA. Further studies confirmed that, in MIF KO mice, AIA severity was reduced (Leech et al., 2003, Santos et al., 2008). This was also associated with an increased synovial p53 expression and apoptosis in fibroblasts (Leech et al., 2003) as well as decreased T-cell activation and the extracellular-signal regulated kinase (ERK) phosphorylation, which is also associated with cell proliferation in splenocytes (Santos et al., 2008).

b-2. Atherosclerosis: Atherosclerosis is now recognized as not only a lipid disorder but also a chronic inflammatory disease of the arterial wall characterized by chemokine-mediated influx of immunocompetent mononuclear cells (Kleemann et al., 2008, Santos and Morand, 2009). Inflammation is a major

contributor to artherogenic progress through adverse effects on lipoprotein metabolism and the health and maintenance of the arterial wall. Both the innate and acquired immune systems are involved in this process (Hansson and Libby, 2006, Bernhagen et al., 2007). Monocytes and T-cells migrate into the innermost arterial wall where they differentiate into macrophages (Kleemann et al., 2008). Monocyte-derived macrophages are found in large numbers during all stages of the disease process (Mangge et al., 2004, Hansson and Libby, 2006) and act as critical effectors through release of a series of proinflammatory molecules including cytokines (MIF, TNF-α, IFN-γ, etc.), chemokines and co-stimulatory factors (Nilsson and Hansson, 2008, Zernecke et al., 2008). MIF has been demonstrated in all cell types present in atherosclerotic lesions and its expression is upregulated during the progression of atherosclerosis in humans (Burger-Kentischer et al., 2002) and in an animal (rabbit) model (Lin et al., 2000). An *in vivo* study confirmed an essential role for MIF in atherogenesis by examining the inflammation status, macrophage content and plaque areas in the aortas and in the heart. The study compared apolipoprotein E deficient ($ApoE^{-/-}$) mice treated with an anti-MIF antibody and the IgG treated controls (Burger-Kentischer et al., 2006). The authors observed that MIF expression was elevated in the aortic wall during atherogenesis by foam cells, a major source of atherosclerotic plaque formation in vessels. Blockade of MIF showed a marked reduction of macrophages associated with the endothelial layer of the intima and a variety of inflammatory mediators associated with atherosclerosis, including MIF and the inflammatory proatherogenic vascular genes, such as the circulating

fibrinogen and TNF. Reduction of inflammation by anti-MIF antibody treatment was associated with a small but non-significant reduction in the aortic plaque area, suggesting the involvement of MIF in arthrosclerosis.

Inflammation and cancer:

Chronic inflammation is an essential component of tumor progression. Recent studies have raised a possible role for MIF in providing a critical link between chronic inflammation and carcinogenesis based on the observation of MIF overexpression in various tumors including those of the breast, colon, liver, lung, prostate and in neuroblastoma (Cooke et al., 2009). MIF influences tumor growth and development by inducing tumor associated angiogenesis, by promoting cell cycle progression in cells of the tumor, by inhibiting apoptosis in tumor cells through suppression of the p53 tumor suppressor gene and by inhibiting tumor cell lysis by natural killer (NK) cells (Bach et al., 2009).

a. Tumor growth and tumor-associated angiogenesis: Tumors require neo-vascularization (angiogenesis) to provide the innermost cells of the tumor with oxygen and nutrients for cell survival (Mitchell and Bucala, 2000). Higher expression levels of MIF were observed in human melanoma cell lines than in normal cultured melanocytes and blockade of MIF with an antisense human MIF plasmid markedly suppressed the cells' growth rates, implicating MIF in the mechanism of proliferation of melanoma cells (Shimizu et al., 1999). These authors also examined the function of MIF in tumor cell migration and found that its effects were MIF dose-dependent. Administration of anti-MIF antibody to

tumor cells *in vivo* significantly suppressed tumor-associated angiogenesis (Shimizu et al., 1999). A different group compared tumor-induced angiogenesis in Tg mice carrying MIF cDNA driven by a cytomegalovirus enhancer and a β-actin/β-globin promoter with non-Tg mice. The mice were subcutaneously inoculated with cells of a murine sarcoma cell line, S-180 (Nishihira et al., 2003). The investigators found that tumor-induced angiogenesis was significantly enhanced in the MIF Tg mice compared to non-Tg mice. They also found that tumor-induced angiogenesis was significantly suppressed by an anti-MIF antibody in the subcutaneous fascia that had been injected with the antibody. Taken together, these results indicate that MIF can stimulate both the growth and invasion of tumor cells and promote neo-vascularization of a growing tumor.

b. Proliferation and cell cycle progression: Proper regulation of the cell cycle is essential for normal development and tumor prevention. The earliest direct evidence suggesting a role of MIF in cell cycle activation was reported in studies of T lymphocyte activation (Bacher et al., 1996, Mitchell and Bucala, 2000). These authors demonstrated that immunoneutralization of MIF by antibodies during antigen or mitogenic challenge of T lymphocytes reduced the proliferative response by more than 80%. Induction of both interleukin-2 and its receptor (Bacher et al., 1996) were also blocked. These findings indicate that MIF could be an important factor acting in an autocrine manner to mediate upstream signaling required for activated T lymphocyte proliferation (Bacher et al., 1996, Mitchell and Bucala, 2000). Other investigators have also examined in more precise detail, the role of MIF in cell proliferation. Takahashi et al. examined MIF

expression and its effects in a colon carcinoma cell line (Takahashi et al., 1998). They observed that MIF expression was induced by several growth factors including TGF-β, basic-FGF (b-FGF) and platelet-derived growth factor (PDGF), in these cells. Depleting endogenous MIF with an anti-sense MIF plasmid resulted in greater than a 40% reduction in tumor cell growth. A molecular basis for these observations was provided by studies of serum-mediated growth in quiescent murine fibroblasts (Mitchell et al., 1999). These experiments were designed to investigate the role of MIF in serum-induced cell cycle progression in the NIH/3T3 fibroblast cell line. The studies showed that serum induced a rapid release of preformed MIF (about 6 ng/ml 4 hours post MIF plasmid administration) from the resting fibroblasts (about 2.5 ng/ml), while inhibition of secreted MIF by an antibody during serum-induced cell cycle progression resulted in a decrease of DNA synthesis by as much as 50% when compared to controls (Mitchell et al., 1999). These studies showed that serum-deprived quiescent cells were stimulated to undergo cell cycle progression and DNA synthesis in response to exogenous MIF (Liao et al., 2003), further suggesting a role for MIF in cell proliferation by directly stimulating cell cycle progression.

c. Inhibition of apoptosis by suppressing p53 tumor suppressor gene:

Proper cell proliferation and maintenance are critical for normal development and cancer prevention (Nemajerova et al., 2007). Cells continuously experience DNA damage caused by base alternations due to reactive oxygen species (ROS)-induced oxidative stress or genotoxic agents including ultraviolet and ionizing radiation (Nemajerova et al., 2007). Failure to complete proper DNA repair leads

to various disorders including cancer (Whitcomb, 2004). P53 has been classified as a tumor suppressor. It plays a very important role in the regulation of cell proliferation and maintenance of genomic stability as well as in the suppression of cellular transformation and tumorigenesis. The high frequencies of mutations in the p53 gene that are found in human tumors (Vogelstein et al., 2000) and the apparent correlation of p53 loss with tumor aggressiveness (Levine, 1997) emphasize the importance of p53 as a "gatekeeper' in the development of neoplastic disease (Fingerle-Rowson et al., 2003). Recent studies have suggested a possible broader role of MIF in growth regulation through its role in antagonizing p53-mediated gene activation and apoptosis (Hudson et al., 1999, Fingerle-Rowson et al., 2003, Honda et al., 2009). By performing gene-targeting studies with embryonic fibroblasts from MIF KO mice ($MIF^{-/-}$MEFs), Fingerle-Rowson et al. observed p53-dependent premature growth arrest, and an increase in p53 transcriptional. These parameters were associated with an observed resistance to ras-mediated transformation in the $MIF^{-/-}$MEFs. However, if the p53 gene was also deleted, these phenotypes were reversed (Fingerle-Rowson et al., 2003). Further, the authors demonstrated that benzo[α]-pyrene-induced fibrosarcomas were smaller in size and had a lower mitotic index in MIF KO mice compared to their littermates *in vivo*. More recently, Honda et al. found a higher incidence of tumors as well as a substantial decrease in the levels of p53, Bax and p21 proteins in Tg mice in which MIF was overexpressed (MIF Tg) than in WT mice after chronic UV irradiation (Honda et al., 2009), further supporting the idea that MIF promotes tumorigenesis by suppressing p53 activity.

d. Inhibition of tumor cell lysis by NK cells: Various cell types in the immune system collaborate in an orchestrated, multifactorial process to execute their cytotoxic functions (Chavez-Galan et al., 2009). NK cells are among the major cytotoxic cell types since their most critical functions are to remove abnormal or infected cells from the body, preventing the development of malignancies and eliminating intracellular pathogens (Chavez-Galan et al., 2009). Studies have demonstrated a functional link between MIF and NK cells, in that MIF inhibits the lysis of tumor cells by NK cells in at least one organ-- the eye (Apte and Niederkorn, 1996, Apte et al., 1998, Repp et al., 2000). Apte et al. found that aqueous humor (AH), which bathes the anterior chamber of the eye bounded by the corneal endothelium, contains a protein that inhibits the NK mediated lysis of corneal endothelial cells (Apte et al., 1998). Their amino acid sequence analysis showed that this protein shared more than 90% homology with MIF, was present in AH and can inhibit NK cell mediated cytolysis *in vitro*. The same group showed that mouse rMIF produced a similar effect by inhibiting NK cell activity. Inhibition of MIF activity with goat anti-human MIF antibody neutralized the NK inhibitory effect of AH (Apte et al., 1998).

Role of MIF in development:

Cytokines are now being implicated in a variety of roles including neuronal migration, outgrowth and survival (Meng et al., 1999, Bajetto et al., 2001, Chalasani et al., 2003a, Chalasani et al., 2003b, Belmadani et al., 2005). Among

them, MIF is the most extensively investigated cytokine in nervous system development and regeneration.

a. MIF expression during embryogenesis: A number of studies demonstrated that MIF is ubiquitously expressed in various cell types and involved in both intracellular and extracellular events as mentioned previously (Abe et al., 2000, Yamaguchi et al., 2000, Shimizu et al., 2002a, Shimizu et al., 2002b). Because MIF is so ubiquitously expressed, and is also expressed at early times in regions of the body that undergo development before the immune system develops, it may have additional role(s) beyond those in the immune system (Kobayashi et al., 1999, Suzuki et al., 2004). Kobayashi et al. demonstrated the spatiotemporal expression of MIF during murine embryogenesis (Kobayashi et al., 1999). MIF mRNA expression was apparent in the neural folds at E8. By E8.5, its expression was detected in many tissues including the somites. Somitogenesis is considered to commence at E8 and continues to E14, following a rostral to caudal gradient of differentiation. An increase in the number of somites was observed along with more prominent MIF expression in the caudal region. MIF expression was also observed in muscle cells, including cardiac muscle, both at the mRNA and protein levels at E9.5, and in smooth muscle of the arteries and in skeletal muscles at the protein level at E16.5. In addition, the authors detected the expression of MIF mRNA in neural tissues, including the forebrain, midbrain, hindbrain, neural tube, in cranial ganglia, and dorsal root ganglia during all embryonic stages. The expression of MIF was detected in optic and otic vesicles/otocysts at E9.5 (Figure 1.5) (Kobayashi et al., 1999).

b. Role of MIF in neurulation: Based on the observations that MIF is expressed in many tissues during murine embryogenesis including neuronal cell types (Kobayashi et al., 1999), Suzuki et al. speculated that MIF plays a critical role in development, particularly in axis formation and neural development (Suzuki et al., 2004). These authors detected the expression of MIF in the developing central nervous system in the embryos of the African clawed frog *Xenopus*. They conducted both loss- and gain-of- function experiments to determine the role of MIF in neurulation using the same model system. They observed that an injection of MIF MOs into the one to eight cell stage frog embryo resulted in complete failure of neural axis formation and neural development (Figure 1.5) (Suzuki et al., 2004). Co-injection of MIF capped RNA with the MIF MOs showed at least partial phenotypic rescue, indicating that MIF appears to be essential for neurulation in Xenopus embryogenesis. Investigators in the Barald lab have performed similar studies in the zebrafish, which, due to a gene duplication, has 2 mif genes and two mif receptors (Shen et al, in preparation). Although blockage of the 2 zebrafish mif genes at the 1-8 cell stage in the zebrafish with either start-site MOs or splice junction MOs alone or in combination, did not block neurulation in the zebrafish, the size of both the CNS and the inner ear was greatly reduced (Shen et al, submitted).

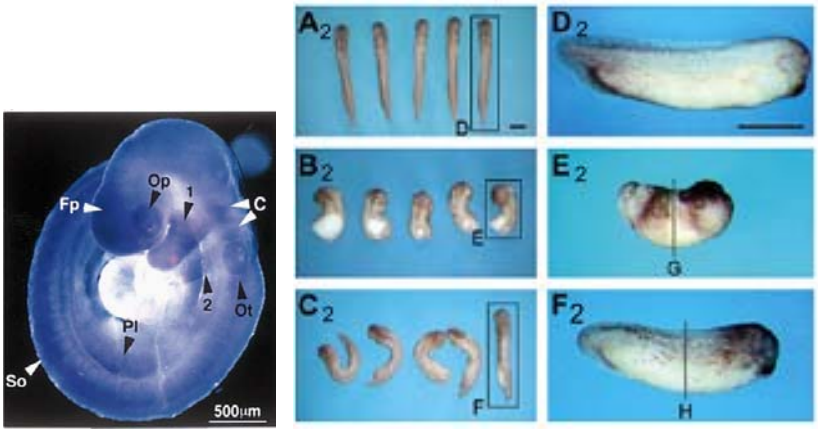


Figure 1.5: MIF mRNA expression in the embryonic mouse (left) and Xenopus (right). MIF mRNA in the otic vesicle/otocyst in the 9.5 day mouse embryo (left) (Kobayashi et al., 1999). The expression of MIF mRNA and the role of MIF in neurulation are illustrated in developing Xenopus embryos (right) (Suzuki et al., 2004). MOs were injected into the 1-8 cell frog embryo to block splicing. Uninjected control (A and D). Injection of MIF splice junction-blocking MOs (MIF MOs) block neurulation (B and E). Co-injection of MIF capped RNA with the MIF MOs partially rescues phenotype (C and F).

c. Role of MIF in inner ear development: Our laboratory has demonstrated a role of MIF inner ear development using zebrafish as model system (Thompson et al., in preparation; Shen et al., in preparation). We have recently cloned two zebrafish *MIF* genes, *mif* and *mif-like*. The sequences and expression patterns of both genes are highly similar to both the Xenopus and the mammalian *MIF* sequences (and they are also homologous to each other) and their expression patterns in the fish resemble those in the mammal and particularly resemble those in Xenopus. Zebrafish *mif* has higher homology to the mammalian MIF gene, while *mif-like* has higher homology to a mammalian DDT gene. Both zebrafish *mif* and *mif-like* contain all of the conserved amino acid residues that

are found among all *MIF* family genes in different species. Shen et al. detected zebrafish *mif* expression in the neural keel, the developing eye, brain and inner ear as shown in Figure 1.6 (above). Shen and colleagues then injected 3 types of antisense morpholino oligonucleotides (MOs): an antisense morpholino oligonucleotide (MO) that was complimentary to the start codon of zebrafish mif mRNA and two *mif-like* MOs to splice junctions. These injections were done at the one-cell stage in zebrafish, since SAG neurogenesis takes place before 18 hours post fertilization (hpf) and MO effects last for 72 hours. Shen et al. (in preparation) observed that the combination of these three MOs significantly reduced the number of the SAG neurons as well as other head ganglia, as well as reducing size of the head, as compared to the effect of the control MO (Figure 1.6, middle on the bottom), while co-injection of *mif* MO or MOs with capped *mif* RNAs rescued neuronal development (Figure 1.6, right on the bottom), indicating that the zebrafish *mif* family genes are as important for neurogenesis in the fish as they are in the frog.

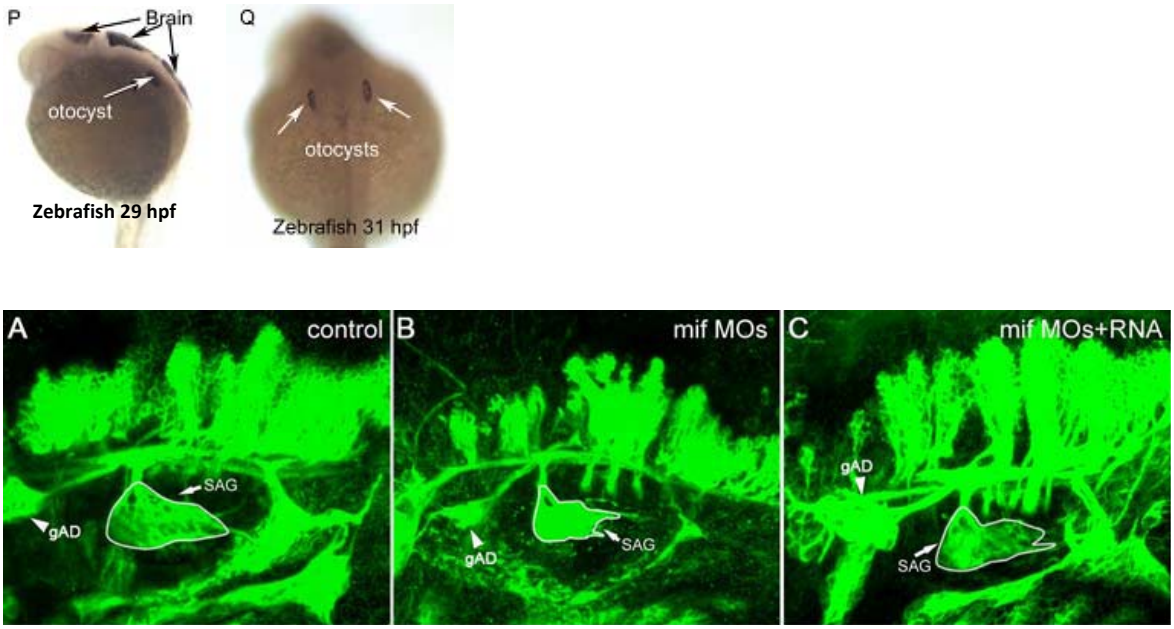


Figure 1.6: **Above:** Zebrafish *mif* expression in the neural keel, the developing eyes, brain and inner ear of the zebrafish at 29 hpf and 31 hpf. **Bottom**: ZN5, an antibody that stains neurons in the zebrafish, was used in the 48 hpf inner ear. (A) control; (B) *mif* morphant embryo in which MOs to both zebrafish *mif* genes were administered at the 1 cell stage; (C) *mif* morphant which was injected concomitantly with the 2 *mif* MOs and with capped *mif* RNAs. The white line outlines the SAGs. The SAG is considerably smaller in the MOs-treated embryo. Note that other cranial ganglia (the tree-like green projections above the SAG, were also smaller in the *mif* morphants (B) than the control (A) or the "rescued" embryo (C). gAD: anterodorsal LL ganglia. Scale bar: 25 μm. Control embryos (n=30), *mif* MOs (n=14), *mif* MOs+RNA (n=12) (from Shen et al., in preparation).

Role of MIF in Regeneration:

Tissue repair and wound healing are both continuously ongoing in the body and complex processes (Shimizu, 2005, Velnar et al., 2009). They involve blood coagulation and hemostasis followed by inflammation, proliferation and wound remodeling with concomitant scar tissue formation (Velnar et al., 2009). Recent studies demonstrate that MIF may play an important role in the wound healing

process. Thus, MIF has been a major target of research in association with wound healing (Abe et al., 2000, Dewor et al., 2007).

a. Wound healing in the skin: The expression of MIF was detected in the human epidermis, particularly in the basal cell layers (Shimizu et al., 1996). Further studies identified a pathophysiological function for MIF in the skin (Abe et al., 2000, Zhao et al., 2005). Abe et al. detected the temporal expression of MIF both at mRNA and protein levels following an excision wound by a punch biopsy instrument. Maximum expression of MIF mRNA was observed 3 and 24 hours post-injury, while 12 hours post-injury maximum protein levels were seen in the whole epidermal lesion of the wound tissue. In addition, they demonstrated that MIF has a chemotactic effect on freshly prepared keratinocytes from rat skin. Fibroblasts have been implicated as having a critical role in the wound healing process in the skin and keratinocytes, fibroblasts that synthesize extracellular martrix components, help to remodel the damaged tissue (Abe et al., 2000). An effect of MIF on the wound healing process was further examined in mice by the same authors. They introduced penetrating wounds through both epidermis and dermis using a punch biopsy instrument, followed by administration of either polyclonal anti-rat MIF antibodies that cross react with mouse MIF or non-immune rabbit IgG every 2 days until wound healing was completed. A delay in the healing process was seen in the mice injected with anti-MIF antibodies (which took 11-15 days for complete healing of the wound to be observed) compared to the control mice (which took 5-9 days for complete healing), suggesting that MIF also contributes to the wound healing process in skin tissues.

Additional *in vivo* studies conducted by Zhao et al. support Abe and colleagues' work, in which MIF also plays an important role in skin wound healing (Zhao et al., 2005). In this study, the authors compared the time required for the complete healing of skin lesions in MIF KO mice with the time required in WT mice after the introduction of wounds to the skin in the same manner as Abe et al. (Abe et al., 2000). Healing was significantly delayed in MIF KO mice (15 days on average) compared to WT mice (10 days on average). They further examined whether exogenous MIF could accelerate the healing process by administrating various concentrations (1 to 6 μg/500 μl) of rMIF-impregnated hydrogel microbeads around the skin wounds in MIF KO mice. Six days post-MIF microbead administration, more than 80% wound recovery was seen in the mice that received 6 μg/500 μl of rMIF, while about 60% recovery was observed in the mice that did not receive any rMIF. The differences were considered to be significant. Their work provides further evidence that MIF plays an essential role in skin wound healing.

Given that fibroblast migration plays an important role in wound healing and that MIF has also been demonstrated to promote the migration of primary human neutrophils through its association with G-protein coupled receptors (Bernhagen et al., 2007), Dewor et al. further examined the role of MIF during the wound healing processes in scratch-wounded monolayers, or scrape wounds introduced into monolayers of cells *in vitro* (Dewor et al., 2007). For this investigation, the authors used MEFs or human foreskin dermal fibroblasts (HFDFs). MEFs were first isolated from mice genetically deficient for MIF ($MIF^{-/-}$

MEFs) and from WT mice ($MIF^{+/+}$ MEFs). These cells were grown in monolayers and then scraped with a rubber policeman when they reached cell confluency, followed by an immediate addition of 50 ng/ml rMIF. Two hours post-rMIF administration, the authors observed that both $MIF^{-/-}$ MEFs and $MIF^{+/+}$ MEFs treated with exogenous MIF increased their motility significantly when compared to those without rMIF. In contrast, prolonged exposure (24 hours) of the cells to the same concentration of rMIF showed a slight inhibitory effect in migration compared to the cells without rMIF. The same experiments were conducted with HFDFs in the presence of mitomycin C, a proliferation blocking agent, and the same results were obtained as with MEFs, in which transient treatment (2 hours) of HFDFs with 50 ng/ml rMIF promoted migration, whereas prolonged MIF treatment had an inhibitory effect in HFDFs. Immunostaining analysis of HFDFs with DAPI and anti-Ki67 showed that the rMIF-stimulated wound closure response in HFDFs was predicated upon its proliferative effect. Besides CD74 (see chapter 3), the best known receptor for MIF, two additional receptors, CXCR2 and CXCR4, were recently discovered; MIF acts as a non-cognate ligand for these receptors (Bernhagen et al., 2007), which will be discussed in more detail in the following section. Binding of MIF to CXCR2 and CXCR4 promotes the chemotactic migration of leukocytes through a G-protein coupled receptor (GPCR)-mediated receptor pathway, in which both CD74 and calcium signaling are also involved (Bernhagen et al., 2007). The authors detected the expression of both CD74 and CXCR4, but not CXCR2, in the fibroblasts in their study. They speculated that MIF-induced wound closure effects may be mediated through

calcium signaling. They demonstrated that treatment of $MIF^{-/-}$ MEFs with rMIF induced a rapid calcium transient influx within a few seconds, while chronic exposure (2 hours) of the cells to rMIF down-regulated the calcium transient influx. Additional studies have shown that CXCR4 also interacts with its cognate ligand, stromal cell-derived factor-1 (SDF-1); these interactions are implicated in both fibroblast migration and in dermal wound healing responses (Kucia et al., 2004, Avniel et al., 2006), although there is no information yet available for the actual mechanism or interactions of MIF/CXCR4 in fibroblast migration and wound healing (Dewor et al., 2007). Further studies need to be done in order to determine the precise contributions of these MIF receptors in MIF-mediated wound closure responses as well as in the effects of MIF on SAG neurite outgrowth and survival described in these studies (see chapters 2 and 3).

b. Nerve regeneration: The processes of both nerve degeneration and regeneration seen after peripheral nerve injury (Nishio et al., 2002) are complex. During the process of degeneration, both the axon and its myelin sheath disintegrate and are digested by Schwann cells (and possibly macrophages drawn to the site of injury by release of MIF by SC) during a phagocytotic process, followed by extensive Schwann cell mitosis in the distal stump after peripheral nerve injury, indicating that axonal re-growth is dependent on the survival and proliferation of Schwann cells (Nishio et al., 1999, Nishio et al., 2002). Macrophages also play a pivotal role in the nerve degeneration-regeneration processes by producing cytokines, stimulating Schwann cell proliferation and phagocytosis. MIF has been considered to play an essential

role to concentrate macrophages at these inflammatory sites (Bloom and Bennett, 1966, David, 1966).

Nishio et al. detected an expression of MIF in the rat sciatic nerve at both the mRNA and protein levels (Nishio et al., 1999). Immunohistochemical analysis demonstrated MIF expression in Schwann cells migrating into neuronal fibers, where the SC are also sources of MIF protein. Further, the authors observed an elevation of MIF mRNA in both proximal and distal nerve segments 12 hours post-nerve transection that remained elevated for 7 days (Nishio et al., 1999). Additional studies demonstrated that MIF plays an essential role in rat peripheral nerve regeneration (Nishio et al., 2002). The authors conducted functional experiments with rat sciatic nerve. Rats were treated either with anti-MIF antibody or with non-immune rabbit IgG following sciatic nerve transection. A significantly shorter length of the regenerating nerve was observed in the anti-MIF treated nerves compared to the controls (Nishio et al., 2002). Immunostaining demonstrated that this was apparently due to a pro-apoptotic effect in nerves treated with anti-MIF antibody. The authors observed a decrease in the number of Ki-67 positive (a measure of cell proliferation) cells along with the upregulation of proapoptotic genes, such as p53 and Fas, in the nerves treated with anti-MIF antibody compared to the untreated controls.

Mechanisms of MIF

MIF was first identified in the early 1960s, in the cells and critical cellular processes in the immune system (Bloom and Bennett, 1966, David, 1966). However, it took some time to identify a possible receptor for MIF and to obtain information concerning the intracellular signaling cascades involved when MIF concentrations increase (Bach et al., 2009). Expression cloning and functional analysis from Leng et al. contributed to the identification of CD74 as a MIF receptor (Leng et al., 2003).

CD74, also called the MHC class II associated invariant chain (Ii), is a type II transmembrane glycoprotein. In normal tissues, CD74 is expressed on HLA class II positive cells, such as B cells, monocytes, macrophages and thymic epithelial cells. Under inflammatory conditions, its expression is observed on endothelial as well as some epithelial cells (Stein et al., 2007). CD74 is been best known as an enzyme transporter, shuttling enzymes from the Golgi apparatus to the endoplasmic reticulum (Bach et al., 2009). Given that CD74 is also abundant on the cell surface, it appears very likely that it also serves as a receptor for MIF (Bach et al., 2009). Later studies found that the complex of MIF and CD74 binds to another cell-surface receptor, CD44, and that this binding is required to activate ERK½-MAPK signaling pathway (Shi et al., 2006). This is mediated by Src kinase (Shi et al., 2006) and results in phosphorylation of the retinoblastoma (Rb) gene and subsequent cell proliferation as shown in Figure 1.7 (Bach et al., 2009).

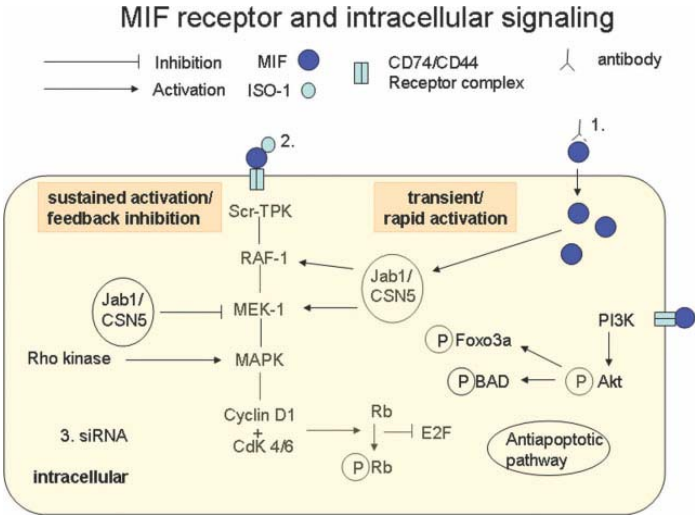


Figure 1.7: Intracellular mechanisms of MIF. MIF action is mediated in part by the CD74/CD44 receptor complex and leads to activation of ERK½-MAPK pathway. This is mediated by activation of Src tyrosine kinase and results in phosphorylation of the Rb gene and subsequently affects cell proliferation. In addition, MIF interacts directly with the c-Jun activation domain–binding protein-1 (Jab1). Jab1 is necessary for ERK activation, while excess Jab1 expression inhibits its activation (Bach et al., 2009).

In addition to interactions with CD74 and CD44, MIF has been shown to be a noncognate ligand for CXCR2 and CXCR4 by Berhagen and colleagues (Bernhagen et al., 2007). The CXCR receptors are potential additional functional receptors for MIF. Shen et al, in our laboratory, have also noted a potential interaction between the two mif receptors in the zebrafish with CXCR4 and with CXCR2. In this context, MIF competes with the cognate ligands for these receptors, CXCL 1 and 8 for CXCR2 (Bernhagen et al., 2007, Schober et al., 2008, Beswick and Reyes, 2009) and CXCR12 for CXCR4 (Schober et al., 2008). CXCR2 is commonly expressed on macrophages and functions in recruiting leukocytes to sites of infection (Bernhagen et al., 2007, Beswick and Reyes,

2009). Besides its expression in the immune system, CXCR2 expression has also been detected in the nervous system (Horuk et al., 1997, Giovannelli et al., 1998, Luan et al., 2001, Valles et al., 2006). Developmental studies have shown that CXCR2 is expressed throughout the mouse brain during early development (Luan et al., 2001) and $CXCR2^{-/-}$ mice exhibited reduced numbers in oligodendrocyte lineages, myelination, and white matter in the vertebrate CNS (Padovani-Claudio et al., 2006). CXCR4 is a G-protein coupled receptor for SDF-1/CXCL12 (Ganju et al., 1998, Vera et al., 2008), which is functionally expressed on the cell surface of various types of cancer cells, and also plays a role in cell proliferation and migration of these cells (Burger and Kipps, 2006) as previously discussed. Colocalization and coimmunoprecipitation studies demonstrated that CXCR2 is colocalized and physically interacts with CD74 (Bernhagen et al., 2007), while a different study reported that CXCR4 is associated with MIF in the rat bladder in the urothelium and this association increases in experimentally induced cystitis (Vera et al., 2008). In the developing mouse CNS, CXCR4 expression is detected as early as E8.5 and its expression is also found in adulthood (McGrath et al., 1999, Tissir et al., 2004, Lieberam et al., 2005). Abnormal development of the cerebellum (Zou et al., 1998), the hippocampal dentate gyrus (Lu et al., 2002) and uncontrolled initial motor axon trajectories (Lieberam et al., 2005) have been reported in CXCR4 KO mice.

Besides MIF's binding to those specific receptors, Kleemann and colleagues discovered an unconventional cellular function to account for the direct interaction of MIF with a cytoplasmic protein, Jun activation domain-binding

protein 1, Jab1, when the extracellular MIF concentrations are high (Kleemann et al., 2000). In this case, MIF is taken up into target cells by endocytosis (Kleemann et al., 2000). Jab1 functions in the activation of activation protein 1 (AP-1), a transcription factor that activates pro-inflammatory genes, and in promoting the degradation of $p27^{kip1}$, a protein that controls the cell cycle (Baugh and Bucala, 2002a). The physical interaction of MIF with Jab1 modulates both the roles of Jab1 in the AP-1 pathway and in cell cycle progression (Kleemann et al., 2000, Lue et al., 2006). Further studies demonstrated that the role of MIF in ERK signaling activation is a dose-dependent (Lue et al., 2006). MIF can activate ERK phosphorylation at lower concentrations in the range of 50-100 ng/ml in fibroblasts, which could physiologically occur in the inflammatory context, while exceedingly higher concentrations in the upper ng/ml to µg/ml range of MIF act as inhibitory in these cells (Lue et al., 2006). According to the authors, MIF would act to impair a previously initiated signaling process and might possess a "switch-off" mechanism when MIF is present at high concentrations (Lue et al., 2006). In addition, the authors indicated that this feedback response may involve MIF endocytosis as well as a Jab1 mediated process based on their results that application of high concentrations of MIF to the fibroblasts for several hours could lead to increased p27 levels and cell cycle arrest. Further, they demonstrated that Jab1, in fact, can exert a dual effect on MIF-induced ERK signaling (Lue et al., 2006). They found that both the overexpression of Jab1 and a high concentrations of exogenous MIF led to an inhibition of ERK phosphorylation, while knockdown of Jab1 with RNAi significantly inhibited MIF promotion of

ERK1/2 phosphorylation, indicating that Jab1 also appears to be necessary for the effects of MIF on transient ERK signaling activation (Figure 1.7) (Lue et al., 2006). Our laboratory is presently investigating the possible role of Jab1 in inner ear development and cell differentiation (Linn et al, in preparation) and has found that Jab1 is expressed in the inner ears of developing zebrafish as well as in the developing nervous system (Linn et al, in preparation). Furthermore, Jab1 was found in ODF in proteomic screen and was also found to interact with MIF in a yeast 2-hybrid screen for MIF interacting proteins (Thompson, Beck, Flynn and Barald, manuscript in preparation).

All these intriguing roles of MIF in neurulation, neuronal development, pathfinding and cell migration and the discovery that MIF is a major component of the otocyst-generated ODF, prompted us to determine the role of MIF and its receptors and interacting proteins in inner ear development. The following chapter details the role of MIF in the development of the mouse and chick inner ears.

References

Abe R, Shimizu T, Ohkawara A, Nishihira J (Enhancement of macrophage migration inhibitory factor (MIF) expression in injured epidermis and cultured fibroblasts. Biochim Biophys Acta 1500:1-9.2000).

Aderem A, Underhill DM (Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 17:593-623.1999).

Agterberg MJ, Versnel H, de Groot JC, Smoorenburg GF, Albers FW, Klis SF (Morphological changes in spiral ganglion cells after intracochlear application of brain-derived neurotrophic factor in deafened guinea pigs. Hear Res 244:25-34.2008).

Al-Abed Y, Dabideen D, Aljabari B, Valster A, Messmer D, Ochani M, Tanovic M, Ochani K, Bacher M, Nicoletti F, Metz C, Pavlov VA, Miller EJ, Tracey KJ (ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis. J Biol Chem 280:36541-36544.2005).

Aletsee C, Brors D, Mlynski R, Ryan AF, Dazert S (Branching of spiral ganglion neurites is induced by focal application of fibroblast growth factor-1. Laryngoscope 113:791-796.2003).

Aletsee C, Volter C, Brors D, Ryan AF, Dazert S ([Effect of fibroblast growth factor-1 (FGF-1) on spiral ganglion cells of the mammalian cochlea]. HNO 48:457-461.2000).

Altschuler RA, Cho Y, Ylikoski J, Pirvola U, Magal E, Miller JM (Rescue and regrowth of sensory nerves following deafferentation by neurotrophic factors. Ann N Y Acad Sci 884:305-311.1999).

Altschuler RA, O'Shea KS, Miller JM (Stem cell transplantation for auditory nerve replacement. Hear Res 242:110-116.2008).

Apte RS, Niederkorn JY (Isolation and characterization of a unique natural killer cell inhibitory factor present in the anterior chamber of the eye. J Immunol 156:2667-2673.1996).

Apte RS, Sinha D, Mayhew E, Wistow GJ, Niederkorn JY (Cutting edge: role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege. J Immunol 160:5693-5696.1998).

Ard MD, Morest DK, Hauger SH (Trophic interactions between the cochleovestibular ganglion of the chick embryo and its synaptic targets in culture. Neuroscience 16:151-170.1985).

Avniel S, Arik Z, Maly A, Sagie A, Basst HB, Yahana MD, Weiss ID, Pal B, Wald O, Ad-El D, Fujii N, Arenzana-Seisdedos F, Jung S, Galun E, Gur E, Peled A (Involvement of the CXCL12/CXCR4 pathway in the recovery of skin following burns. J Invest Dermatol 126:468-476.2006).

Bach JP, Deuster O, Balzer-Geldsetzer M, Meyer B, Dodel R, Bacher M (The role of macrophage inhibitory factor in tumorigenesis and central nervous system tumors. Cancer 115:2031-2040.2009).

Bacher M, Metz CN, Calandra T, Mayer K, Chesney J, Lohoff M, Gemsa D, Donnelly T, Bucala R (An essential regulatory role for macrophage

migration inhibitory factor in T-cell activation. Proc Natl Acad Sci U S A 93:7849-7854.1996).
Bajetto A, Bonavia R, Barbero S, Florio T, Schettini G (Chemokines and their receptors in the central nervous system. Front Neuroendocrinol 22:147-184.2001).
Bajetto A, Bonavia R, Barbero S, Schettini G (Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. J Neurochem 82:1311-1329.2002).
Balak KJ, Corwin JT, Jones JE (Regenerated hair cells can originate from supporting cell progeny: evidence from phototoxicity and laser ablation experiments in the lateral line system. J Neurosci 10:2502-2512.1990).
Barald KF, Kelley MW (From placode to polarization: new tunes in inner ear development. Development 131:4119-4130.2004).
Barald KF, Lindberg KH, Hardiman K, Kavka AI, Lewis JE, Victor JC, Gardner CA, Poniatowski A (Immortalized cell lines from embryonic avian and murine otocysts: tools for molecular studies of the developing inner ear. Int J Dev Neurosci 15:523-540.1997).
Baugh JA, Bucala R (Macrophage migration inhibitory factor. Crit Care Med 30:S27-S35.2002a).
Baugh JA, Bucala R (Macrophage migration inhibitory factor. Crit Care Med 30:S27-35.2002b).
Beisel K, Hansen L, Soukup G, Fritzsch B (Regenerating cochlear hair cells: quo vadis stem cell. Cell Tissue Res 333:373-379.2008).
Belmadani A, Tran PB, Ren D, Assimacopoulos S, Grove EA, Miller RJ (The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J Neurosci 25:3995-4003.2005).
Bermingham-McDonogh O, Stone JS, Reh TA, Rubel EW (FGFR3 expression during development and regeneration of the chick inner ear sensory epithelia. Dev Biol 238:247-259.2001).
Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R (MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365:756-759.1993).
Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C (MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med 13:587-596.2007).
Beswick EJ, Reyes VE (CD74 in antigen presentation, inflammation, and cancers of the gastrointestinal tract. World J Gastroenterol 15:2855-2861.2009).
Bhave SA, Stone JS, Rubel EW, Coltrera MD (Cell cycle progression in gentamicin-damaged avian cochleas. J Neurosci 15:4618-4628.1995).
Bianchi LM, Cohan CS (Developmental regulation of a neurite-promoting factor influencing statoacoustic neurons. Brain Res Dev Brain Res 64:167-174.1991).

Bianchi LM, Cohan CS (Effects of the neurotrophins and CNTF on developing statoacoustic neurons: comparison with an otocyst-derived factor. Dev Biol 159:353-365.1993).
Bianchi LM, Daruwalla Z, Roth TM, Attia NP, Lukacs NW, Richards AL, White IO, Allen SJ, Barald KF (Immortalized mouse inner ear cell lines demonstrate a role for chemokines in promoting the growth of developing statoacoustic ganglion neurons. J Assoc Res Otolaryngol 6:355-367.2005).
Bianchi LM, Dolnick R, Medd A, Cohan CS (Developmental changes in growth factors released by the embryonic inner ear. Exp Neurol 150:98-106.1998).
Bichler E, Spoendlin H, Rauchegger H (Degeneration of cochlear neurons after amikacin intoxication in the rat. Arch Otorhinolaryngol 237:201-208.1983).
Bloom BR, Bennett B (Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science 153:80-82.1966).
Bolin LM, Murray R, Lukacs NW, Strieter RM, Kunkel SL, Schall TJ, Bacon KB (Primary sensory neurons migrate in response to the chemokine RANTES. J Neuroimmunol 81:49-57.1998).
Brignull HR, Raible DW, Stone JS (Feathers and fins: non-mammalian models for hair cell regeneration. Brain Res 1277:12-23.2009).
Brumwell CL, Hossain WA, Morest DK, Bernd P (Role for basic fibroblast growth factor (FGF-2) in tyrosine kinase (TrkB) expression in the early development and innervation of the auditory receptor: in vitro and in situ studies. Exp Neurol 162:121-145.2000).
Burger-Kentischer A, Gobel H, Kleemann R, Zernecke A, Bucala R, Leng L, Finkelmeier D, Geiger G, Schaefer HE, Schober A, Weber C, Brunner H, Rutten H, Ihling C, Bernhagen J (Reduction of the aortic inflammatory response in spontaneous atherosclerosis by blockade of macrophage migration inhibitory factor (MIF). Atherosclerosis 184:28-38.2006).
Burger-Kentischer A, Goebel H, Seiler R, Fraedrich G, Schaefer HE, Dimmeler S, Kleemann R, Bernhagen J, Ihling C (Expression of macrophage migration inhibitory factor in different stages of human atherosclerosis. Circulation 105:1561-1566.2002).
Burger JA, Kipps TJ (CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107:1761-1767.2006).
Calandra T (Macrophage migration inhibitory factor and host innate immune responses to microbes. Scand J Infect Dis 35:573-576.2003).
Calandra T, Bernhagen J, Mitchell RA, Bucala R (The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. J Exp Med 179:1895-1902.1994).
Calandra T, Echtenacher B, Roy DL, Pugin J, Metz CN, Hultner L, Heumann D, Mannel D, Bucala R, Glauser MP (Protection from septic shock by neutralization of macrophage migration inhibitory factor. Nat Med 6:164-170.2000).
Calandra T, Roger T (Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 3:791-800.2003).

Camarero G, Avendano C, Fernandez-Moreno C, Villar A, Contreras J, de Pablo F, Pichel JG, Varela-Nieto I (Delayed inner ear maturation and neuronal loss in postnatal Igf-1-deficient mice. J Neurosci 21:7630-7641.2001).
Camarero G, Leon Y, Gorospe I, De Pablo F, Alsina B, Giraldez F, Varela-Nieto I (Insulin-like growth factor 1 is required for survival of transit-amplifying neuroblasts and differentiation of otic neurons. Dev Biol 262:242-253.2003).
Chalasani SH, Baribaud F, Coughlan CM, Sunshine MJ, Lee VM, Doms RW, Littman DR, Raper JA (The chemokine stromal cell-derived factor-1 promotes the survival of embryonic retinal ganglion cells. J Neurosci 23:4601-4612.2003a).
Chalasani SH, Sabelko KA, Sunshine MJ, Littman DR, Raper JA (A chemokine, SDF-1, reduces the effectiveness of multiple axonal repellents and is required for normal axon pathfinding. J Neurosci 23:1360-1371.2003b).
Chavez-Galan L, Arenas-Del Angel MC, Zenteno E, Chavez R, Lascurain R (Cell death mechanisms induced by cytotoxic lymphocytes. Cell Mol Immunol 6:15-25.2009).
Cheng AG, Cunningham LL, Rubel EW (Mechanisms of hair cell death and protection. Curr Opin Otolaryngol Head Neck Surg 13:343-348.2005).
Cooke G, Armstrong ME, Donnelly SC (Macrophage migration inhibitory factor (MIF), enzymatic activity and the inflammatory response. Biofactors 35:165-168.2009).
Corey DP, Hudspeth AJ (Analysis of the microphonic potential of the bullfrog's sacculus. J Neurosci 3:942-961.1983a).
Corey DP, Hudspeth AJ (Kinetics of the receptor current in bullfrog saccular hair cells. J Neurosci 3:962-976.1983b).
Corwin JT, Cotanche DA (Regeneration of sensory hair cells after acoustic trauma. Science 240:1772-1774.1988).
Corwin JT, Jones JE, Katayama A, Kelley MW, Warchol ME (Hair cell regeneration: the identities of progenitor cells, potential triggers and instructive cues. Ciba Found Symp 160:103-120; discussion 120-130.1991).
Coughlan CM, McManus CM, Sharron M, Gao Z, Murphy D, Jaffer S, Choe W, Chen W, Hesselgesser J, Gaylord H, Kalyuzhny A, Lee VM, Wolf B, Doms RW, Kolson DL (Expression of multiple functional chemokine receptors and monocyte chemoattractant protein-1 in human neurons. Neuroscience 97:591-600.2000).
Cunha FQ, Weiser WY, David JR, Moss DW, Moncada S, Liew FY (Recombinant migration inhibitory factor induces nitric oxide synthase in murine macrophages. J Immunol 150:1908-1912.1993).
David JR (Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. Proc Natl Acad Sci U S A 56:72-77.1966).

Dazert S, Kim D, Luo L, Aletsee C, Garfunkel S, Maciag T, Baird A, Ryan AF (Focal delivery of fibroblast growth factor-1 by transfected cells induces spiral ganglion neurite targeting in vitro. J Cell Physiol 177:123-129.1998).
Denk W, Holt JR, Shepherd GM, Corey DP (Calcium imaging of single stereocilia in hair cells: localization of transduction channels at both ends of tip links. Neuron 15:1311-1321.1995).
Desai M, Pratt LA, Lentzner H, Robinson KN (Trends in vision and hearing among older Americans. Aging Trends 1-8.2001).
Dewor M, Steffens G, Krohn R, Weber C, Baron J, Bernhagen J (Macrophage migration inhibitory factor (MIF) promotes fibroblast migration in scratch-wounded monolayers in vitro. FEBS Lett 581:4734-4742.2007).
Dinh CT, Van De Water TR (Blocking pro-cell-death signal pathways to conserve hearing. Audiol Neurootol 14:383-392.2009).
Dror AA, Avraham KB (Hearing loss: mechanisms revealed by genetics and cell biology. Annu Rev Genet 43:411-437.2009).
El-Turk F, Cascella M, Ouertatani-Sakouhi H, Narayanan RL, Leng L, Bucala R, Zweckstetter M, Rothlisberger U, Lashuel HA (The conformational flexibility of the carboxy terminal residues 105-114 is a key modulator of the catalytic activity and stability of macrophage migration inhibitory factor. Biochemistry 47:10740-10756.2008).
Fekete DM, Campero AM (Axon guidance in the inner ear. Int J Dev Biol 51:549-556.2007).
Fekete DM, Muthukumar S, Karagogeos D (Hair cells and supporting cells share a common progenitor in the avian inner ear. J Neurosci 18:7811-7821.1998).
Fingerle-Rowson G, Petrenko O, Metz CN, Forsthuber TG, Mitchell R, Huss R, Moll U, Muller W, Bucala R (The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. Proc Natl Acad Sci U S A 100:9354-9359.2003).
Frago LM, Camerero G, Canon S, Paneda C, Sanz C, Leon Y, Giraldez F, Varela-Nieto I (Role of diffusible and transcription factors in inner ear development: implications in regeneration. Histol Histopathol 15:657-666.2000).
Frago LM, Canon S, de la Rosa EJ, Leon Y, Varela-Nieto I (Programmed cell death in the developing inner ear is balanced by nerve growth factor and insulin-like growth factor I. J Cell Sci 116:475-486.2003).
Friedman LM, Avraham KB (MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mamm Genome 20:581-603.2009).
Fritzsch B, Farinas I, Reichardt LF (Lack of neurotrophin 3 causes losses of both classes of spiral ganglion neurons in the cochlea in a region-specific fashion. J Neurosci 17:6213-6225.1997a).
Fritzsch B, Silos-Santiago I, Bianchi LM, Farinas I (The role of neurotrophic factors in regulating the development of inner ear innervation. Trends Neurosci 20:159-164.1997b).

Fritzsch B, Silos-Santiago II, Bianchi LM, Farinas II (Effects of neurotrophin and neurotrophin receptor disruption on the afferent inner ear innervation. Semin Cell Dev Biol 8:277-284.1997c).
Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, Newman W, Groopman JE (The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. J Biol Chem 273:23169-23175.1998).
Geldhoff M, Mook-Kanamori BB, van de Beek D (Macrophage migration inhibitory factor, infection, the brain, and corticosteroids. Crit Care 13:170.2009).
Germiller JA, Smiley EC, Ellis AD, Hoff JS, Deshmukh I, Allen SJ, Barald KF (Molecular characterization of conditionally immortalized cell lines derived from mouse early embryonic inner ear. Dev Dyn 231:815-827.2004).
Gestwa G, Wiechers B, Zimmermann U, Praetorius M, Rohbock K, Kopschall I, Zenner HP, Knipper M (Differential expression of trkB.T1 and trkB.T2, truncated trkC, and p75(NGFR) in the cochlea prior to hearing function. J Comp Neurol 414:33-49.1999).
Gillespie LN (Regulation of axonal growth and guidance by the neurotrophin family of neurotrophic factors. Clin Exp Pharmacol Physiol 30:724-733.2003).
Gillespie LN, Shepherd RK (Clinical application of neurotrophic factors: the potential for primary auditory neuron protection. Eur J Neurosci 22:2123-2133.2005).
Gillespie PG (Myosin I and adaptation of mechanical transduction by the inner ear. Philos Trans R Soc Lond B Biol Sci 359:1945-1951.2004).
Giovannelli A, Limatola C, Ragozzino D, Mileo AM, Ruggieri A, Ciotti MT, Mercanti D, Santoni A, Eusebi F (CXC chemokines interleukin-8 (IL-8) and growth-related gene product alpha (GROalpha) modulate Purkinje neuron activity in mouse cerebellum. J Neuroimmunol 92:122-132.1998).
Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez AC, Siegelman MH, Cybulsky M, Gutierrez-Ramos JC (Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. J Clin Invest 98:2332-2345.1996).
Grillet N, Kazmierczak P, Xiong W, Schwander M, Reynolds A, Sakaguchi H, Tokita J, Kachar B, Muller U (The mechanotransduction machinery of hair cells. Sci Signal 2:pt5.2009).
Hansson GK, Libby P (The immune response in atherosclerosis: a double-edged sword. Nat Rev Immunol 6:508-519.2006).
Hemond SG, Morest DK (Ganglion formation from the otic placode and the otic crest in the chick embryo: mitosis, migration, and the basal lamina. Anat Embryol (Berl) 184:1-13.1991).
Hoi AY, Hickey MJ, Hall P, Yamana J, O'Sullivan KM, Santos LL, James WG, Kitching AR, Morand EF (Macrophage migration inhibitory factor

deficiency attenuates macrophage recruitment, glomerulonephritis, and lethality in MRL/lpr mice. J Immunol 177:5687-5696.2006).
Hoi AY, Morand EF, Leech M (Is macrophage migration inhibitory factor a therapeutic target in systemic lupus erythematosus? Immunol Cell Biol 81:367-373.2003).
Holley MC, Lawlor PW (Production of conditionally immortalised cell lines from a transgenic mouse. Audiol Neurootol 2:25-35.1997).
Honda A, Abe R, Yoshihisa Y, Makino T, Matsunaga K, Nishihira J, Shimizu H, Shimizu T (Deficient deletion of apoptotic cells by macrophage migration inhibitory factor (MIF) overexpression accelerates photocarcinogenesis. Carcinogenesis 30:1597-1605.2009).
Horuk R, Martin AW, Wang Z, Schweitzer L, Gerassimides A, Guo H, Lu Z, Hesselgesser J, Perez HD, Kim J, Parker J, Hadley TJ, Peiper SC (Expression of chemokine receptors by subsets of neurons in the central nervous system. J Immunol 158:2882-2890.1997).
Hossain WA, Morest DK (Fibroblast growth factors (FGF-1, FGF-2) promote migration and neurite growth of mouse cochlear ganglion cells in vitro: immunohistochemistry and antibody perturbation. J Neurosci Res 62:40-55.2000).
Hossain WA, Rutledge A, Morest DK (Critical periods of basic fibroblast growth factor and brain-derived neurotrophic factor in the development of the chicken cochleovestibular ganglion in vitro. Exp Neurol 147:437-451.1997).
Hossain WA, Zhou X, Rutledge A, Baier C, Morest DK (Basic fibroblast growth factor affects neuronal migration and differentiation in normotypic cell cultures from the cochleovestibular ganglion of the chick embryo. Exp Neurol 138:121-143.1996).
Huang EJ, Reichardt LF (Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609-642.2003).
Hudson JD, Shoaibi MA, Maestro R, Carnero A, Hannon GJ, Beach DH (A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med 190:1375-1382.1999).
Iguchi F, Nakagawa T, Tateya I, Kim TS, Endo T, Taniguchi Z, Naito Y, Ito J (Trophic support of mouse inner ear by neural stem cell transplantation. Neuroreport 14:77-80.2003).
Jones JE, Corwin JT (Regeneration of sensory cells after laser ablation in the lateral line system: hair cell lineage and macrophage behavior revealed by time-lapse video microscopy. J Neurosci 16:649-662.1996).
Kalinec F, Kalinec G, Boukhvalova M, Kachar B (Establishment and characterization of conditionally immortalized organ of corti cell lines. Cell Biol Int 23:175-184.1999).
Kaplan DR, Miller FD (Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10:381-391.2000).
Kelley MW (Regulation of cell fate in the sensory epithelia of the inner ear. Nat Rev Neurosci 7:837-849.2006).

Kleemann R, Hausser A, Geiger G, Mischke R, Burger-Kentischer A, Flieger O, Johannes FJ, Roger T, Calandra T, Kapurniotu A, Grell M, Finkelmeier D, Brunner H, Bernhagen J (Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. Nature 408:211-216.2000).

Kleemann R, Kapurniotu A, Frank RW, Gessner A, Mischke R, Flieger O, Juttner S, Brunner H, Bernhagen J (Disulfide analysis reveals a role for macrophage migration inhibitory factor (MIF) as thiol-protein oxidoreductase. J Mol Biol 280:85-102.1998).

Kleemann R, Zadelaar S, Kooistra T (Cytokines and atherosclerosis: a comprehensive review of studies in mice. Cardiovasc Res 79:360-376.2008).

Kobayashi S, Satomura K, Levsky JM, Sreenath T, Wistow GJ, Semba I, Shum L, Slavkin HC, Kulkarni AB (Expression pattern of macrophage migration inhibitory factor during embryogenesis. Mech Dev 84:153-156.1999).

Koda M, Hashimoto M, Murakami M, Yoshinaga K, Ikeda O, Yamazaki M, Koshizuka S, Kamada T, Moriya H, Shirasawa H, Sakao S, Ino H (Adenovirus vector-mediated in vivo gene transfer of brain-derived neurotrophic factor (BDNF) promotes rubrospinal axonal regeneration and functional recovery after complete transection of the adult rat spinal cord. J Neurotrauma 21:329-337.2004).

Kong WJ, Yin ZD, Fan GR, Li D, Huang X (Time sequence of auditory nerve and spiral ganglion cell degeneration following chronic kanamycin-induced deafness in the guinea pig. Brain Res 1331:28-38).

Kricos PB (Hearing assistive technology considerations for older individuals with dual sensory loss. Trends Amplif 11:273-279.2007).

Kucia M, Jankowski K, Reca R, Wysoczynski M, Bandura L, Allendorf DJ, Zhang J, Ratajczak J, Ratajczak MZ (CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. J Mol Histol 35:233-245.2004).

Lee CC, Sherman SM (Topography and physiology of ascending streams in the auditory tectothalamic pathway. Proc Natl Acad Sci U S A 107:372-377).

Lee CC, Sherman SM (Topography and physiology of ascending streams in the auditory tectothalamic pathway. Proc Natl Acad Sci U S A 107:372-377.2010).

Lee FS, Kim AH, Khursigara G, Chao MV (The uniqueness of being a neurotrophin receptor. Curr Opin Neurobiol 11:281-286.2001a).

Lee R, Kermani P, Teng KK, Hempstead BL (Regulation of cell survival by secreted proneurotrophins. Science 294:1945-1948.2001b).

Leech M, Lacey D, Xue JR, Santos L, Hutchinson P, Wolvetang E, David JR, Bucala R, Morand EF (Regulation of p53 by macrophage migration inhibitory factor in inflammatory arthritis. Arthritis Rheum 48:1881-1889.2003).

Leech M, Metz C, Hall P, Hutchinson P, Gianis K, Smith M, Weedon H, Holdsworth SR, Bucala R, Morand EF (Macrophage migration inhibitory

factor in rheumatoid arthritis: evidence of proinflammatory function and regulation by glucocorticoids. Arthritis Rheum 42:1601-1608.1999).
Leech M, Xue JR, Dacumos A, Hall P, Santos L, Yang Y, Li M, Kitching AR, Morand EF (The tumour suppressor gene p53 modulates the severity of antigen-induced arthritis and the systemic immune response. Clin Exp Immunol 152:345-353.2008).
Lefebvre PP, Leprince P, Weber T, Rigo JM, Delree P, Moonen G (Neuronotrophic effect of developing otic vesicle on cochleo-vestibular neurons: evidence for nerve growth factor involvement. Brain Res 507:254-260.1990).
Leng L, Bucala R (Insight into the biology of macrophage migration inhibitory factor (MIF) revealed by the cloning of its cell surface receptor. Cell Res 16:162-168.2006).
Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery T, Chen Y, Mitchell RA, Bucala R (MIF signal transduction initiated by binding to CD74. J Exp Med 197:1467-1476.2003).
Leon Y, Sanz C, Giraldez F, Varela-Nieto I (Induction of cell growth by insulin and insulin-like growth factor-I is associated with Jun expression in the otic vesicle. J Comp Neurol 398:323-332.1998).
Leon Y, Vazquez E, Sanz C, Vega JA, Mato JM, Giraldez F, Represa J, Varela-Nieto I (Insulin-like growth factor-I regulates cell proliferation in the developing inner ear, activating glycosyl-phosphatidylinositol hydrolysis and Fos expression. Endocrinology 136:3494-3503.1995).
Levine AJ (p53, the cellular gatekeeper for growth and division. Cell 88:323-331.1997).
Liao H, Bucala R, Mitchell RA (Adhesion-dependent signaling by macrophage migration inhibitory factor (MIF). J Biol Chem 278:76-81.2003).
Lieberam I, Agalliu D, Nagasawa T, Ericson J, Jessell TM (A Cxcl12-CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. Neuron 47:667-679.2005).
Lin SG, Yu XY, Chen YX, Huang XR, Metz C, Bucala R, Lau CP, Lan HY (De novo expression of macrophage migration inhibitory factor in atherogenesis in rabbits. Circ Res 87:1202-1208.2000).
Littlewood Evans A, Muller U (Stereocilia defects in the sensory hair cells of the inner ear in mice deficient in integrin alpha8beta1. Nat Genet 24:424-428.2000).
Lombarte A, Yan HY, Popper AN, Chang JS, Platt C (Damage and regeneration of hair cell ciliary bundles in a fish ear following treatment with gentamicin. Hear Res 64:166-174.1993).
Lu M, Grove EA, Miller RJ (Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. Proc Natl Acad Sci U S A 99:7090-7095.2002).
Luan J, Furuta Y, Du J, Richmond A (Developmental expression of two CXC chemokines, MIP-2 and KC, and their receptors. Cytokine 14:253-263.2001).

Lue H, Kapurniotu A, Fingerle-Rowson G, Roger T, Leng L, Thiele M, Calandra T, Bucala R, Bernhagen J (Rapid and transient activation of the ERK MAPK signalling pathway by macrophage migration inhibitory factor (MIF) and dependence on JAB1/CSN5 and Src kinase activity. Cell Signal 18:688-703.2006).

Lue H, Thiele M, Franz J, Dahl E, Speckgens S, Leng L, Fingerle-Rowson G, Bucala R, Luscher B, Bernhagen J (Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. Oncogene 26:5046-5059.2007).

Luo L, Koutnouyan H, Baird A, Ryan AF (Acidic and basic FGF mRNA expression in the adult and developing rat cochlea. Hear Res 69:182-193.1993).

Ma EY, Rubel EW, Raible DW (Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. J Neurosci 28:2261-2273.2008).

Malgrange B, Lefebvre P, Van de Water TR, Staecker H, Moonen G (Effects of neurotrophins on early auditory neurones in cell culture. Neuroreport 7:913-917.1996).

Mangge H, Hubmann H, Pilz S, Schauenstein K, Renner W, Marz W (Beyond cholesterol--inflammatory cytokines, the key mediators in atherosclerosis. Clin Chem Lab Med 42:467-474.2004).

Mantovani A, Bonecchi R, Martinez FO, Galliera E, Perrier P, Allavena P, Locati M (Tuning of innate immunity and polarized responses by decoy receptors. Int Arch Allergy Immunol 132:109-115.2003).

Martinez-Monedero R, Corrales CE, Cuajungco MP, Heller S, Edge AS (Reinnervation of hair cells by auditory neurons after selective removal of spiral ganglion neurons. J Neurobiol 66:319-331.2006).

Martinez-Monedero R, Oshima K, Heller S, Edge AS (The potential role of endogenous stem cells in regeneration of the inner ear. Hear Res 227:48-52.2007).

Maruyama J, Miller JM, Ulfendahl M (Glial cell line-derived neurotrophic factor and antioxidants preserve the electrical responsiveness of the spiral ganglion neurons after experimentally induced deafness. Neurobiol Dis 29:14-21.2008).

Marzella PL, Clark GM, Shepherd RK, Bartlett PF, Kilpatrick TJ (Synergy between TGF-beta 3 and NT-3 to promote the survival of spiral ganglia neurones in vitro. Neurosci Lett 240:77-80.1998).

Marzella PL, Gillespie LN, Clark GM, Bartlett PF, Kilpatrick TJ (The neurotrophins act synergistically with LIF and members of the TGF-beta superfamily to promote the survival of spiral ganglia neurons in vitro. Hear Res 138:73-80.1999).

Matsui JI, Cotanche DA (Sensory hair cell death and regeneration: two halves of the same equation. Curr Opin Otolaryngol Head Neck Surg 12:418-425.2004).

McCullar JS, Oesterle EC (Cellular targets of estrogen signaling in regeneration of inner ear sensory epithelia. Hear Res 252:61-70.2009).
McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J (Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol 213:442-456.1999).
Meng W, Sawasdikosol S, Burakoff SJ, Eck MJ (Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. Nature 398:84-90.1999).
Mikulowska A, Metz CN, Bucala R, Holmdahl R (Macrophage migration inhibitory factor is involved in the pathogenesis of collagen type II-induced arthritis in mice. J Immunol 158:5514-5517.1997).
Miller AL, Morris DJ, Pfingst BE (Effects of time after deafening and implantation on guinea pig electrical detection thresholds. Hear Res 144:175-186.2000).
Miller JM, Le Prell CG, Prieskorn DM, Wys NL, Altschuler RA (Delayed neurotrophin treatment following deafness rescues spiral ganglion cells from death and promotes regrowth of auditory nerve peripheral processes: effects of brain-derived neurotrophic factor and fibroblast growth factor. J Neurosci Res 85:1959-1969.2007).
Miller JM, Miller AL, Yamagata T, Bredberg G, Altschuler RA (Protection and regrowth of the auditory nerve after deafness: neurotrophins, antioxidants and depolarization are effective in vivo. Audiol Neurootol 7:175-179.2002).
Mitchell RA, Bucala R (Tumor growth-promoting properties of macrophage migration inhibitory factor (MIF). Semin Cancer Biol 10:359-366.2000).
Mitchell RA, Metz CN, Peng T, Bucala R (Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. J Biol Chem 274:18100-18106.1999).
Molea D, Stone JS, Rubel EW (Class III beta-tubulin expression in sensory and nonsensory regions of the developing avian inner ear. J Comp Neurol 406:183-198.1999).
Morrison D, Schindler RA, Wersall J (A quantitative analysis of the afferent innervation of the organ of corti in guinea pig. Acta Otolaryngol 79:11-23.1975).
Nemajerova A, Moll UM, Petrenko O, Fingerle-Rowson G (Macrophage migration inhibitory factor coordinates DNA damage response with the proteasomal control of the cell cycle. Cell Cycle 6:1030-1034.2007).
Nilsson J, Hansson GK (Autoimmunity in atherosclerosis: a protective response losing control? J Intern Med 263:464-478.2008).
Nishihira J, Ishibashi T, Fukushima T, Sun B, Sato Y, Todo S (Macrophage migration inhibitory factor (MIF): Its potential role in tumor growth and tumor-associated angiogenesis. Ann N Y Acad Sci 995:171-182.2003).
Nishio Y, Minami A, Kato H, Kaneda K, Nishihira J (Identification of macrophage migration inhibitory factor (MIF) in rat peripheral nerves: its possible

involvement in nerve regeneration. Biochim Biophys Acta 1453:74-82.1999).
Nishio Y, Nishihira J, Ishibashi T, Kato H, Minami A (Role of macrophage migration inhibitory factor (MIF) in peripheral nerve regeneration: anti-MIF antibody induces delay of nerve regeneration and the apoptosis of Schwann cells. Mol Med 8:509-520.2002).
Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM (Sortilin is essential for proNGF-induced neuronal cell death. Nature 427:843-848.2004).
Ohyama T, Groves AK, Martin K (The first steps towards hearing: mechanisms of otic placode induction. Int J Dev Biol 51:463-472.2007).
Onodera S, Kaneda K, Mizue Y, Koyama Y, Fujinaga M, Nishihira J (Macrophage migration inhibitory factor up-regulates expression of matrix metalloproteinases in synovial fibroblasts of rheumatoid arthritis. J Biol Chem 275:444-450.2000).
Onodera S, Nishihira J, Koyama Y, Majima T, Aoki Y, Ichiyama H, Ishibashi T, Minami A (Macrophage migration inhibitory factor up-regulates the expression of interleukin-8 messenger RNA in synovial fibroblasts of rheumatoid arthritis patients: common transcriptional regulatory mechanism between interleukin-8 and interleukin-1beta. Arthritis Rheum 50:1437-1447.2004).
Onodera S, Tanji H, Suzuki K, Kaneda K, Mizue Y, Sagawa A, Nishihira J (High expression of macrophage migration inhibitory factor in the synovial tissues of rheumatoid joints. Cytokine 11:163-167.1999).
Ota CY, Kimura RS (Ultrastructural study of the human spiral ganglion. Acta Otolaryngol 89:53-62.1980).
Otte J, Schunknecht HF, Kerr AG (Ganglion cell populations in normal and pathological human cochleae. Implications for cochlear implantation. Laryngoscope 88:1231-1246.1978).
Padovani-Claudio DA, Liu L, Ransohoff RM, Miller RH (Alterations in the oligodendrocyte lineage, myelin, and white matter in adult mice lacking the chemokine receptor CXCR2. Glia 54:471-483.2006).
Pfingst BE, Rai DT (Effects of level on nonspectral frequency difference limens for electrical and acoustic stimuli. Hear Res 50:43-56.1990).
Pichora-Fuller MK, Singh G (Effects of age on auditory and cognitive processing: implications for hearing aid fitting and audiologic rehabilitation. Trends Amplif 10:29-59.2006).
Pickles JO, Corey DP (Mechanoelectrical transduction by hair cells. Trends Neurosci 15:254-259.1992).
Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B (Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085-2088.1998).

Powell-Braxton L, Hollingshead P, Giltinan D, Pitts-Meek S, Stewart T (Inactivation of the IGF-I gene in mice results in perinatal lethality. Ann N Y Acad Sci 692:300-301.1993a).
Powell-Braxton L, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, Gillett N, Stewart TA (IGF-I is required for normal embryonic growth in mice. Genes Dev 7:2609-2617.1993b).
Puligilla C, Dabdoub A, Brenowitz SD, Kelley MW (Sox2 induces neuronal formation in the developing mammalian cochlea. J Neurosci 30:714-722).
Qun LX, Pirvola U, Saarma M, Ylikoski J (Neurotrophic factors in the auditory periphery. Ann N Y Acad Sci 884:292-304.1999).
Raphael Y, Altschuler RA (Structure and innervation of the cochlea. Brain Res Bull 60:397-422.2003).
Raphael Y, Kim YH, Osumi Y, Izumikawa M (Non-sensory cells in the deafened organ of Corti: approaches for repair. Int J Dev Biol 51:649-654.2007).
Repp AC, Mayhew ES, Apte S, Niederkorn JY (Human uveal melanoma cells produce macrophage migration-inhibitory factor to prevent lysis by NK cells. J Immunol 165:710-715.2000).
Riccomagno MM, Martinu L, Mulheisen M, Wu DK, Epstein DJ (Specification of the mammalian cochlea is dependent on Sonic hedgehog. Genes Dev 16:2365-2378.2002).
Richardson GP, Lukashkin AN, Russell IJ (The tectorial membrane: one slice of a complex cochlear sandwich. Curr Opin Otolaryngol Head Neck Surg 16:458-464.2008).
Rivolta MN, Holley MC (Asymmetric segregation of mitochondria and mortalin correlates with the multi-lineage potential of inner ear sensory cell progenitors in vitro. Brain Res Dev Brain Res 133:49-56.2002).
Robertson D, Sellick PM, Patuzzi R (The continuing search for outer hair cell afferents in the guinea pig spiral ganglion. Hear Res 136:151-158.1999).
Roehm PC, Hansen MR (Strategies to preserve or regenerate spiral ganglion neurons. Curr Opin Otolaryngol Head Neck Surg 13:294-300.2005).
Roger T, David J, Glauser MP, Calandra T (MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature 414:920-924.2001).
Rosengren E, Bucala R, Aman P, Jacobsson L, Odh G, Metz CN, Rorsman H (The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. Mol Med 2:143-149.1996).
Rosowski JJ, Peake WT, Lynch TJ, 3rd, Leong R, Weiss TF (A model for signal transmission in an ear having hair cells with free-standing stereocilia. II. Macromechanical stage. Hear Res 20:139-155.1985).
Roux PP, Colicos MA, Barker PA, Kennedy TE (p75 neurotrophin receptor expression is induced in apoptotic neurons after seizure. J Neurosci 19:6887-6896.1999).
Rovensky J, Svejcar J, Pekarek J, Zitnan D, Hajzok O, Cebecauer L (Correlation between the results of the migration inhibitory factor production test with

DNA and the severity of the disease in the systemic lupus erythematosus patients. Z Immunitatsforsch Exp Klin Immunol 150:24-30.1975).
Rusznak Z, Szucs G (Spiral ganglion neurones: an overview of morphology, firing behaviour, ionic channels and function. Pflugers Arch 457:1303-1325.2009).
Ryals BM, Rubel EW (Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240:1774-1776.1988).
Sampey AV, Hall PH, Mitchell RA, Metz CN, Morand EF (Regulation of synoviocyte phospholipase A2 and cyclooxygenase 2 by macrophage migration inhibitory factor. Arthritis Rheum 44:1273-1280.2001).
Santos L, Hall P, Metz C, Bucala R, Morand EF (Role of macrophage migration inhibitory factor (MIF) in murine antigen-induced arthritis: interaction with glucocorticoids. Clin Exp Immunol 123:309-314.2001).
Santos LL, Dacumos A, Yamana J, Sharma L, Morand EF (Reduced arthritis in MIF deficient mice is associated with reduced T cell activation: down-regulation of ERK MAP kinase phosphorylation. Clin Exp Immunol 152:372-380.2008).
Santos LL, Lacey D, Yang Y, Leech M, Morand EF (Activation of synovial cell p38 MAP kinase by macrophage migration inhibitory factor. J Rheumatol 31:1038-1043.2004).
Santos LL, Morand EF (Macrophage migration inhibitory factor: a key cytokine in RA, SLE and atherosclerosis. Clin Chim Acta 399:1-7.2009).
Sanz C, Leon Y, Canon S, Alvarez L, Giraldez F, Varela-Nieto I (Pattern of expression of the jun family of transcription factors during the early development of the inner ear: implications in apoptosis. J Cell Sci 112 (Pt 22):3967-3974.1999).
Scheper V, Paasche G, Miller JM, Warnecke A, Berkingali N, Lenarz T, Stover T (Effects of delayed treatment with combined GDNF and continuous electrical stimulation on spiral ganglion cell survival in deafened guinea pigs. J Neurosci Res 87:1389-1399.2009).
Schimmang T, Tan J, Muller M, Zimmermann U, Rohbock K, Kopschall I, Limberger A, Minichiello L, Knipper M (Lack of Bdnf and TrkB signalling in the postnatal cochlea leads to a spatial reshaping of innervation along the tonotopic axis and hearing loss. Development 130:4741-4750.2003).
Schober A, Bernhagen J, Weber C (Chemokine-like functions of MIF in atherosclerosis. J Mol Med 86:761-770.2008).
Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ (Structure and function of lipopolysaccharide binding protein. Science 249:1429-1431.1990).
Serin GM, Derinsu U, Sari M, Gergin O, Ciprut A, Akdas F, Batman C (Cochlear implantation in patients with bilateral cochlear trauma. Am J Otolaryngol.2009).
Shepherd RK, Javel E (Electrical stimulation of the auditory nerve. I. Correlation of physiological responses with cochlear status. Hear Res 108:112-144.1997).

Shi X, Leng L, Wang T, Wang W, Du X, Li J, McDonald C, Chen Z, Murphy JW, Lolis E, Noble P, Knudson W, Bucala R (CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. Immunity 25:595-606.2006).
Shibata SB, Cortez SR, Beyer LA, Wiler JA, Di Polo A, Pfingst BE, Raphael Y (Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae. Exp Neurol 223:464-472.2010).
Shibata SB, Raphael Y (Future approaches for inner ear protection and repair. J Commun Disord 43:295-310).
Shimizu T (Role of macrophage migration inhibitory factor (MIF) in the skin. J Dermatol Sci 37:65-73.2005).
Shimizu T, Abe R, Nakamura H, Ohkawara A, Suzuki M, Nishihira J (High expression of macrophage migration inhibitory factor in human melanoma cells and its role in tumor cell growth and angiogenesis. Biochem Biophys Res Commun 264:751-758.1999).
Shimizu T, Mizue Y, Abe R, Watanabe H, Shimizu H (Increased macrophage migration inhibitory factor (MIF) in the sera of patients with extensive alopecia areata. J Invest Dermatol 118:555-557.2002a).
Shimizu T, Nishihira J, Mizue Y, Nakamura H, Abe R, Watanabe H, Ishibashi T, Shimizu H (Histochemical analysis of macrophage migration inhibitory factor in psoriasis vulgaris. Histochem Cell Biol 118:251-257.2002b).
Shimizu T, Ohkawara A, Nishihira J, Sakamoto W (Identification of macrophage migration inhibitory factor (MIF) in human skin and its immmunohistochemical localization. FEBS Lett 381:199-202.1996).
Siemion IZ, Kluczyk A, Cebrat M (The peptide molecular links between the central nervous and the immune systems. Amino Acids 29:161-176.2005).
Spicer SS, Schulte BA (Differences along the place-frequency map in the structure of supporting cells in the gerbil cochlea. Hear Res 79:161-177.1994a).
Spicer SS, Schulte BA (Ultrastructural differentiation of the first Hensen cell in the gerbil cochlea as a distinct cell type. Anat Rec 240:149-156.1994b).
Spoendlin H (Innervation patterns in the organ of corti of the cat. Acta Otolaryngol 67:239-254.1969).
Spoendlin H (Degeneration behaviour of the cochlear nerve. Arch Klin Exp Ohren Nasen Kehlkopfheilkd 200:275-291.1971).
Spoendlin H (Innervation densities of the cochlea. Acta Otolaryngol 73:235-248.1972).
Spoendlin H (Retrograde degeneration of the cochlear nerve. Acta Otolaryngol 79:266-275.1975).
Spoendlin H (Differentiation of cochlear afferent neurons. Acta Otolaryngol 91:451-456.1981).
Spoendlin H (Anatomy of cochlear innervation. Am J Otolaryngol 6:453-467.1985).
Spoendlin H, Schrott A (The spiral ganglion and the innervation of the human organ of Corti. Acta Otolaryngol 105:403-410.1988).

Staecker H, Galinovic-Schwartz V, Liu W, Lefebvre P, Kopke R, Malgrange B, Moonen G, Van De Water TR (The role of the neurotrophins in maturation and maintenance of postnatal auditory innervation. Am J Otol 17:486-492.1996a).
Staecker H, Van De Water TR, Lefebvre PP, Liu W, Moghadassi M, Galinovic-Schwartz V, Malgrange B, Moonen G (NGF, BDNF and NT-3 play unique roles in the in vitro development and patterning of innervation of the mammalian inner ear. Brain Res Dev Brain Res 92:49-60.1996b).
Stein R, Mattes MJ, Cardillo TM, Hansen HJ, Chang CH, Burton J, Govindan S, Goldenberg DM (CD74: a new candidate target for the immunotherapy of B-cell neoplasms. Clin Cancer Res 13:5556s-5563s.2007).
Stone JS, Cotanche DA (Hair cell regeneration in the avian auditory epithelium. Int J Dev Biol 51:633-647.2007).
Stone JS, Leano SG, Baker LP, Rubel EW (Hair cell differentiation in chick cochlear epithelium after aminoglycoside toxicity: in vivo and in vitro observations. J Neurosci 16:6157-6174.1996).
Stone JS, Rubel EW (Delta1 expression during avian hair cell regeneration. Development 126:961-973.1999).
Stone JS, Rubel EW (Cellular studies of auditory hair cell regeneration in birds. Proc Natl Acad Sci U S A 97:11714-11721.2000a).
Stone JS, Rubel EW (Temporal, spatial, and morphologic features of hair cell regeneration in the avian basilar papilla. J Comp Neurol 417:1-16.2000b).
Stosic-Grujicic S, Stojanovic I, Nicoletti F (MIF in autoimmunity and novel therapeutic approaches. Autoimmun Rev 8:244-249.2009).
Sugimoto H, Taniguchi M, Nakagawa A, Tanaka I, Suzuki M, Nishihira J (Crystallization and preliminary X-ray analysis of human D-dopachrome tautomerase. J Struct Biol 120:105-108.1997).
Sun HW, Swope M, Cinquina C, Bedarkar S, Bernhagen J, Bucala R, Lolis E (The subunit structure of human macrophage migration inhibitory factor: evidence for a trimer. Protein Eng 9:631-635.1996).
Suzuki M, Takamura Y, Maeno M, Tochinai S, Iyaguchi D, Tanaka I, Nishihira J, Ishibashi T (Xenopus laevis macrophage migration inhibitory factor is essential for axis formation and neural development. J Biol Chem 279:21406-21414.2004).
Takahashi N, Nishihira J, Sato Y, Kondo M, Ogawa H, Ohshima T, Une Y, Todo S (Involvement of macrophage migration inhibitory factor (MIF) in the mechanism of tumor cell growth. Mol Med 4:707-714.1998).
Tan J, Shepherd RK (Aminoglycoside-induced degeneration of adult spiral ganglion neurons involves differential modulation of tyrosine kinase B and p75 neurotrophin receptor signaling. Am J Pathol 169:528-543.2006).
Tessarollo L, Coppola V, Fritzsch B (NT-3 replacement with brain-derived neurotrophic factor redirects vestibular nerve fibers to the cochlea. J Neurosci 24:2575-2584.2004).

Thompson DL, Gerlach-Bank LM, Barald KF, Koenig RJ (Retinoic acid repression of bone morphogenetic protein 4 in inner ear development. Mol Cell Biol 23:2277-2286.2003).
Tissir F, Wang CE, Goffinet AM (Expression of the chemokine receptor Cxcr4 mRNA during mouse brain development. Brain Res Dev Brain Res 149:63-71.2004).
Valles A, Grijpink-Ongering L, de Bree FM, Tuinstra T, Ronken E (Differential regulation of the CXCR2 chemokine network in rat brain trauma: implications for neuroimmune interactions and neuronal survival. Neurobiol Dis 22:312-322.2006).
Varela-Nieto I, Morales-Garcia JA, Vigil P, Diaz-Casares A, Gorospe I, Sanchez-Galiano S, Canon S, Camarero G, Contreras J, Cediel R, Leon Y (Trophic effects of insulin-like growth factor-I (IGF-I) in the inner ear. Hear Res 196:19-25.2004).
Velnar T, Bailey T, Smrkolj V (The wound healing process: an overview of the cellular and molecular mechanisms. J Int Med Res 37:1528-1542.2009).
Vera PL, Iczkowski KA, Wang X, Meyer-Siegler KL (Cyclophosphamide-induced cystitis increases bladder CXCR4 expression and CXCR4-macrophage migration inhibitory factor association. PLoS One 3:e3898.2008).
Vogelstein B, Lane D, Levine AJ (Surfing the p53 network. Nature 408:307-310.2000).
Vollrath MA, Kwan KY, Corey DP (The micromachinery of mechanotransduction in hair cells. Annu Rev Neurosci 30:339-365.2007).
Warchol ME, Corwin JT (Regenerative proliferation in organ cultures of the avian cochlea: identification of the initial progenitors and determination of the latency of the proliferative response. J Neurosci 16:5466-5477.1996).
Webster M, Webster DB (Spiral ganglion neuron loss following organ of Corti loss: a quantitative study. Brain Res 212:17-30.1981).
Weiser WY, Temple PA, Witek-Giannotti JS, Remold HG, Clark SC, David JR (Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. Proc Natl Acad Sci U S A 86:7522-7526.1989).
Whitcomb DC (Inflammation and Cancer V. Chronic pancreatitis and pancreatic cancer. Am J Physiol Gastrointest Liver Physiol 287:G315-319.2004).
Wiechers B, Gestwa G, Mack A, Carroll P, Zenner HP, Knipper M (A changing pattern of brain-derived neurotrophic factor expression correlates with the rearrangement of fibers during cochlear development of rats and mice. J Neurosci 19:3033-3042.1999).
Wiersinga WJ, Calandra T, Kager LM, van der Windt GJ, Roger T, le Roy D, Florquin S, Peacock SJ, Sweep FC, van der Poll T (Expression and function of macrophage migration inhibitory factor (MIF) in melioidosis. PLoS Negl Trop Dis 4:e605).
Wilson BS, Dorman MF (Cochlear implants: a remarkable past and a brilliant future. Hear Res 242:3-21.2008).
Winner M, Meier J, Zierow S, Rendon BE, Crichlow GV, Riggs R, Bucala R, Leng L, Smith N, Lolis E, Trent JO, Mitchell RA (A novel, macrophage migration

inhibitory factor suicide substrate inhibits motility and growth of lung cancer cells. Cancer Res 68:7253-7257.2008).
Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC (CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249:1431-1433.1990).
Wrona D (Neural-immune interactions: an integrative view of the bidirectional relationship between the brain and immune systems. J Neuroimmunol 172:38-58.2006).
Wyatt S, Ensor L, Begbie J, Ernfors P, Reichardt LF, Latchman DS (NT-3 regulates expression of Brn3a but not Brn3b in developing mouse trigeminal sensory neurons. Brain Res Mol Brain Res 55:254-264.1998).
Yamaguchi E, Nishihira J, Shimizu T, Takahashi T, Kitashiro N, Hizawa N, Kamishima K, Kawakami Y (Macrophage migration inhibitory factor (MIF) in bronchial asthma. Clin Exp Allergy 30:1244-1249.2000).
Ylikoski J, Pirvola U, Virkkala J, Suvanto P, Liang XQ, Magal E, Altschuler R, Miller JM, Saarma M (Guinea pig auditory neurons are protected by glial cell line-derived growth factor from degeneration after noise trauma. Hear Res 124:17-26.1998).
Zernecke A, Bernhagen J, Weber C (Macrophage migration inhibitory factor in cardiovascular disease. Circulation 117:1594-1602.2008).
Zhao Y, Shimizu T, Nishihira J, Koyama Y, Kushibiki T, Honda A, Watanabe H, Abe R, Tabata Y, Shimizu H (Tissue regeneration using macrophage migration inhibitory factor-impregnated gelatin microbeads in cutaneous wounds. Am J Pathol 167:1519-1529.2005).
Zheng JL, Gao WQ (Differential damage to auditory neurons and hair cells by ototoxins and neuroprotection by specific neurotrophins in rat cochlear organotypic cultures. Eur J Neurosci 8:1897-1905.1996).
Zheng JL, Stewart RR, Gao WQ (Neurotrophin-4/5, brain-derived neurotrophic factor, and neurotrophin-3 promote survival of cultured vestibular ganglion neurons and protect them against neurotoxicity of ototoxins. J Neurobiol 28:330-340.1995).
Zheng W, Huang L, Wei ZB, Silvius D, Tang B, Xu PX (The role of Six1 in mammalian auditory system development. Development 130:3989-4000.2003).
Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR (Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393:595-599.1998).

Chapter II

The cytokine, Macrophage Migration Inhibitory Factor (MIF), acts as a neurotrophin for neurons in the developing inner ear

Abstract

We have discovered that a key signaling factor in early inner ear neuronal development is an immune system “inflammatory” cytokine, macrophage migration inhibitory factor (MIF). As this study demonstrates, MIF acts as a neurotrophic factor at the earliest stages of inner ear development. MIF is a major bioactive component of the embryonic otocyst derived factor (ODF), which is produced by the otocyst (the primitive inner ear) for a brief time during its early development. ODF promotes both neurite outgrowth in and neuronal survival of embryonic statoacoustic ganglion (SAG) neurons. In the present study, MIF protein and bioactivity were detected in ODF generated from both the mouse and chick inner ear and in ODF generated from Immortalized Otocyst (IMO) cell lines derived from the 9.5 day Immortomouse otocyst. IMO ODF mimicked the activity of otocyst-generated ODF in SAG neuronal cultures. MIF protein also elicited a neuron-like morphology from mouse embryonic stem cells (mESC). MIF was expressed in circumscribed regions of the developing otocyst, and later in

supporting cells (SC) of the mature inner ear, which underlie the sensory hair cells (HC). The MIF receptor, CD74, was detected on developing SAG neurons and on the mature form of these neurons, the spiral ganglion neurons (SGNs) that innervate the HC of the inner ear. Evidence demonstrating a requirement for MIF in normal inner ear development was also found in MIF knock-out (KO) mice. These mice are hearing-impaired with a corresponding reduction in SGN density as well as some SC and HC loss in the high frequency region of the cochlea (48kHz). Neurites from spiral ganglion (SG) explants extend directionally in culture toward an excised wild-type (WT) Organ of Corti (OC), but not toward the isolated MIF KO OC, further demonstrating a role for this cytokine in neural development. Together, these data demonstrate that MIF is a cytokine protein necessary for normal formation and innervation of the inner ear.

Introduction

New links between the neural and immune systems continue to be discovered, and in recent years, it has been demonstrated that the neural and immune systems interact to direct normal organismal development and function (Armstrong et al., 2003, Siemion et al., 2005, Wrona, 2006). Our labs have been investigating the neurotrophic role of immune system chemokines and cytokines in the earliest stages of neuronal innervation (Bianchi et al., 2005), using the ear as a model system (Bianchi and Cohan, 1991, Barald et al., 1997, Germiller et al., 2004, Bianchi et al., 2005). In this report, we demonstrate that the cytokine

macrophage migration inhibitory factor (MIF) acts as a neurotrophic factor for the developing inner ear.

Previous studies indicated that cells in the otocyst secrete a factor or factors that support(s) the outgrowth and survival of the statoacoustic ganglion (SAG) neurons that innervate inner ear sensory cells (Bianchi and Cohan, 1991, 1993). The stages at which otocyst derived factor (ODF) is released correspond to the period of initial neurite outgrowth from the SAG [Embryonic day (E)4-6 in the chick, E11-14 in the mouse and rat (Bianchi and Cohan, 1991, 1993, Bianchi et al., 1998, Bianchi et al., 2000)]. Because so little ODF can be obtained from embryonic otocysts, we developed immortalized otocyst cell lines from the embryonic day 9.5 Immortomouse otocyst (IMO) cells (Barald et al., 1997), which secrete a functionally bioactive ODF equivalent (Thompson et al., 2003, Germiller et al., 2004, Bianchi et al., 2005). This system initially allowed us to identify the cytokine monocyte chemotactic protein-1 (MCP-1) as an active, but not the sole, component of ODF (Bianchi et al., 2005). We now demonstrate that MIF is the major bioactive component of ODF and plays a vital neurotrophic role in normal early inner ear development and innervation, promoting both the directional neurite outgrowth of statoacoustic ganglion neurons and neuronal survival. It is both necessary and sufficient for these functions. MIF is expressed in and released by the early stage inner ear (otocyst) in both the mouse and chick. MIF also promotes the development of embryonic stem cells into neurons.

The absence of MIF in MIF knock-out (KO) mice caused hearing loss as well as structural abnormalities in the cochlea and loss of supporting cells (SC), hair cells (HC) and spiral ganglion neurons (SGNs). Hearing impairment, HC and SGN loss are also seen in MCP1 KO mice, although MCP1 alone did not promote directional neurite outgrowth from excised SAG, although it contributes to the survival of dissociated SAG (Bianchi et al., 2005). Together, these data demonstrate that the chemokines MIF and MCP1 play critical roles in cochlear development and innervation, directly demonstrating another important connection between the immune and neural systems.

Materials and experimental methods

Cell cultures and conditioned medium (CM) collection: Immortomouse otocyst (IMO) cells which had been generated from the H2kbtsA58 transgenic mouse as previously described (Barald et al., 1997, Thompson et al., 2003, Germiller et al., 2004) were cultured as described in those reports. A subcloned cell line from one of these IMO cell lines, called 2B1DT cells (Germiller et al., 2004), was initially grown at 32°C in Chick embryo fibroblast (CEF) medium containing 4 % fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1 unit of γ-interferon (Chemicon International, Inc., Temecula, CA) on 100 mm Primaria culture plates (Fisher Scientific, Pittsburgh, PA) to allow the cells to proliferate. When cells reached approximately 70 % confluency, the medium was replaced with serum-free CEF medium with γ-interferon and the cells were

incubated an additional 5 days at 32°C. During the incubation and CM collection, 1 unit of γ-interferon was replenished every other day in these cultures. CM was collected on the fifth day in a siliconized falcon test tube (Fisher Scientific, Pittsburgh, PA) to minimize loss of active components that might stick to the surface of the tube. The CM was stored at 4°C until use. CM without γ-interferon was similarly collected in parallel experiments.

The D3 (NF11/1) mouse embryonic stem (mES) cell line (Doetschman et al., 1985) was also cultured as described by Roth et al. (Roth et al., 2007) to assess an effect of MIF bioactivity on differentiation. In brief, D3 cells in ES proliferation medium [81% Dulbecco's Modified Eagle (DME) medium without phenol red, 1% L-Glut, 1% Pen/Strep, 1% nonessential amino acids (Gibco, Carlsbad, CA), 15% FBS (Atlanta Biological, Nor-cross, GA), 1% sodium pyruvate (2% stock), 7 μL/L β-mercaptoethanol (Sigma, St. Louis, MO), 1,000 U/mL ESGRO (Chemicon, Temecula, CA] were plated onto Primaria (Falcon) plates (Fisher Scientific, Pittsburgh, PA), incubated at 37°C and allowed to attach overnight. The following day, cells were washed with phosphate buffered saline (PBS) once and placed in neuronal differentiation medium [95%F-12, 1% Pen/Strep, 1% N2, 2% B27 (Gibco), 2% sodium pyruvate (2% stock)]. Test media consisted of either 1 μg/L ciliary neurotrophic factor (CNTF) (R & D, Minneapolis, MN), 1 μg/L nerve growth factor (NGF) (Chemicon, Temecula, CA)], or various concentrations (1 to 500 pg/ml) of mouse/human recombinant MIF (rMIF) (R&D, Minneapolis, MN) and cultured another 5 days at 37°C. The cells

were fed with differentiation medium containing rMIF every other day during differentiation.

In addition, dissociated inner ear cultures were prepared from E13/14 mouse embryos as described in the previous reports (Bianchi et al., 2002, Bianchi et al., 2006) with minor modifications. Briefly, the inner ears were dissected from E 13-14 CD-1 mouse embryos (Hilltop Labs, Scottdale, PA). After removal of the surrounding mesenchyme and SAG, the inner ears were transferred to a dish of DME medium, followed by the separation of cochlear regions from vestibular ones. Pooled inner ear tissues (cochlear vs. vestibular) were rinsed three times with PBS, then incubated at 37°C in 0.1% trypsin in PBS for 10 minutes with DNase (80 U/ml) (Sigma, St. Louis, MO) added during the last 5 minutes of incubation. The inner ears were rinsed in PBS, then incubated in 0.1% trypsin inhibitor for 10 minutes at 37°C. The pooled cells were resuspended in the DME medium and further dissociated by trituration with fire polished pipets. Once the cells were sufficiently dissociated, they were plated onto poly-D-lysine coated 48-well plates (Fisher Scientific, Pittsburgh, PA) in defined culture medium at a density of 700,000 cell/cm^2. If used in immunohistochemical assays, the cells were fixed for 10-20 minutes in 10% neutral buffered formalin after the designated culture period.

Otocyst excision and otocyst CM collection: CM was also collected from chick otocysts at embryonic day 4-5 (E4-5) based on the method of Bianchi and Cohan (Bianchi and Cohan, 1993) with minor modifications. Briefly, eight

otocysts from E4-5 chick were collected and incubated in a 250µl of either CEF medium without serum, γ-interferon or DME (serum free) medium (Fisher Scientific, Pittsburgh, PA) at 37°C for 3 days. After centrifugation at 10,000 rpm at room temperature for 1 minute, the supernatant was transferred into a low protein binding microtest tube (DENVILLE Scientific INC, Metuchen, NJ) and stored at 4°C until use either for proteomic studies or in bioassays.

Neurite outgrowth and survival of SAG neurons *in vitro*: To determine the effects of conditioned media obtained either from 2B1DT cells or otocysts on SAG neurite outgrowth and survival, bioassays were performed as in Bianchi et al., 2005 with minor modifications (Bianchi et al., 2005). Briefly, whole SAGs were excised from chick embryos at E5 and placed on a poly-D-lysine (Fisher Scientific, Pittsburgh, PA) coated 96 well plate to evaluate SAG neurite outgrowth. The plate was then incubated at 37°C for 72 hours and the scoring mechanism in Bianchi et al., 2005 was applied to assess the extent of neurite outgrowth. To examine SAG neuron survival, individual SAG neurons were prepared by pooling dissociated cells from 25-30 ganglia. First, the isolated ganglia were washed with calcium-free PBS (3x) followed by centrifugation at 10,000 rpm for 1 minute and aspiration of supernatant. Then, the ganglia were incubated with PBS containing 0.025 % trypsin (Invitrogen, Carlsbad, CA) at 37°C for 10 minutes with inversion or swirling. After centrifugation and aspiration of trypsin-containing PBS, the cells were incubated with PBS containing 0.1 % trypsin inhibitor (Sigma, St. Louis, MO) at 37°C for 10 minutes. Ganglia were rinsed once with either CEF/DME medium followed by centrifugation and aspiration. Finally, the ganglia

were resuspended in defined medium and dissociated by trituration with a fire-polished glass pipet.

The individual cells were plated on poly-D-lysine coated 96 well plates at a density of 10,000 cells per well (determined by haemocytometer counts). Cells were incubated at 37°C until they became attached to the plate. After cell attachment, the medium was replaced with CM or control medium (identical medium which had not had otocyst or cell contact, but which had been incubated for the identical number of days) +/- γ-interferon and incubated at 37°C for 48-72 hours.

SAG assays were also performed with either mouse/human rMIF (R&D, Minneapolis, MN) in basal media or in bio-inactive IMO conditioned medium (prepared from IMO cell lines that do not produce ODF) at concentration range of 5 pg/ml – 500 ng/ml or 2 ng/ml -200 ng/ml respectively to determine if there was an effect on SAG neurite outgrowth and survival. SAG neurite outgrowth was observed and scored after 24, 48 and 72 hours based on a standard scale of 0-5 described by Bianchi et al. (2005) and the percentage of SAG neurons surviving was calculated after 48-72 hours following immunostaining. For this assay, all samples were done in triplicate, but randomly assigned to wells on the plate (e.g. no triplicate conditions were next to one another). Function-blocking anti-mouse MIF antibody (R&D, Minneapolis, MN) followed by protein G beads were added to either IMO-generated CM or otocyst-generated CM at 10-300 ng/ml.

Organotypic co-cultures of postnatal OC with SGNs: Organotypic co-culture assays of the OC with SGNs were performed to assess neurite outgrowth from WT SAGs or SG toward the cochleae isolated from either WT (Balb/C) mice or MIF KO mice. The inner ears were isolated from mice at postnatal day 3 (P3) as described by Chen et al. (Chen et al., 2009). The cochlea were then carefully isolated and immersed in cold PBS. After removal of the bone surrounding the cochlea, the lateral wall tissues, including the stria vascularis and spiral ligament, and the auditory nerve bundle were dissected away leaving the OC and SG, which were separated. The freshly excised OC explants either from WT or MIF KO mice were then placed with the freshly excised SGNs from WT mice into 35 mm culture dishes (Fisher Scientific, Pittsburgh, PA) coated with 15 µl of polymerized rat tail collagen solution (BD, Franklin Lakes, NJ) in 50 µl of DME medium. The small amount of medium was added to prevent the tissues from drying. The tissues were observed daily by light microscopy for the ensuing 7 days.

Preparation of sectioned cochlear epithelium: Mice were decapitated immediately after being euthanized with CO_2. Both temporal bones were then extracted and the cochleae were placed into 4% paraformaldehyde (PFA) (Sigma, St. Louis, MO) for fixation overnight at 4˚C on a Nutator (BD, Franklin Lakes, NJ) (Campos-Barros et al., 2000) with minor modifications. The next day, the cochleae were rinsed in PBS, followed by decalcification by placing them in 5% ethylenediaminetetraacetic acid (EDTA) at 4˚C on a Nutator. EDTA was changed daily until the cochleae were sufficiently decalcified (usually 5 days).

Following decalcification, the cochleae were placed in 30% sucrose in 1x PBS solution until they sank in the solution. After washing with fresh 30% sucrose solution, the cochleae were incubated in a 1:1 mixture of 30% sucrose solution and OCT until they sank. The cochleae were then transferred to embedding molds containing 100% OCT and were frozen on dry ice with isopentane. The frozen specimens were stored at -80˚C until cryosectioning. The tissues were sectioned at 8 µm and placed onto two sets of Superfrost slides (One set serves as the experimental and the other serves as control for MIF and MCP1 [stained for IgG (Zymed, San Francisco, CA)]. Sectioned tissues were stored at -20˚C until staining with MIF and MCP1.

Auditory brainstem response (**ABR) assessment:** Auditory brainstem responses (ABRs) were assessed for WT (Balb/C) (n=5) and MIF KO mice (n=4) at 4 weeks of age as described by Saul et al. (Saul et al., 2008). Briefly, animals were anesthetized with an intraperitoneal injection of ketamin (65 mg/kg), xylazine (3.5 mg/kg) and acepromazine (2 mg/kg). Body temperature was maintained with heating pads and heat lamps. Subdermal needle electrodes were placed at the skull vertex (active) and pinnae of the test (reference) and contralateral (ground) ears. ABRs were recorded in an electrically and acoustically shielded chamber (Acoustic Systems, Glendale Heights, IL) and Tucker-Davis Technologies (Alachua, FL) System II hardware and SigGen/Biosig software were used to present stimuli and record responses. Tones were generated by a Beyer driver

(Beyerdynamic, Mt. Prospect, IL) and were delivered by a speculum placed just inside the tragus. Stimuli were presented as 15 millisecond tone bursts, with 1 msec rise/fall times, and were repeated 10 times per second. Responses were collected at 12, 24 and 48 kHz for a range of stimulus levels, separated by 10 dB steps at higher intensities and 5 dB steps near threshold. Thresholds were interpolated between the lowest stimulus intensity producing a response, and the highest stimulus where no response was observed (5 dB increments).

SG neuronal density measurements: SG neuronal density measurements were performed by the Kresge Hearing Research Institute P30 core at the University of Michigan on WT (Balb/C, control), $MIF^{-/-}$, and $MCP1^{-/-}$ KO mice at 4, 8, and 12 weeks post-birth. Balb/C 4wk (n=5), Balb/C 8wk (n=3), Balb/C 12wk (n=6), $MIF^{-/-}$ 4wk (n=5), $MIF^{-/-}$ 8wk (n=5), $MIF^{-/-}$ 12wk (n=3), $MCP1^{-/-}$ 4wk (n=3), $MCP1^{-/-}$ 8wk (n=6), $MCP1^{-/-}$ 12wk (n=3). Briefly, mice were decapitated and their bullae were rapidly removed. The cochleae were opened at the base and apex and 4% PFA was gently introduced through scala tympani. Cochleae were then placed into fixative for 12-48 hours, followed by rinse in PBS. Cochleae were decalcified in 5% EDTA in PBS at room temperature for 24 hours, dehydrated through a graded series of alcohols and then processed for embedding into JB-4Plus, a glycol methacrylate plastic (Electron Microscopy Sciences, Hatfield, PA). Five micron plastic sections were cut in a paramodiolar plane and each section was picked up and put on a slide. Slides were rehydrated, dipped in Paragon [200ml 30% ethanol (Fisher Scientific, Pittsburgh, PA), 1.46 g toluidine blue (Fisher Scientific, Pittsburgh, PA), 0.54g basic fuchsin (Fisher Scientific,

Pittsburgh, PA)] for 1 minute, dehydrated in graded strengths of ethanol, dipped in Xylene (Fisher Scientific, Pittsburgh, PA) and coverslipped with Permount mounting medium. The twelve most mid-modiolar sections were selected and every other sections was used for quantitative assessment. Each selected slide was placed in the microscope and digital images were acquired into the Metamorph Image Analysis workstation (Molecular Devices, Sunnyvale, CA) under bright field optics. The most basal profile of the Rosenthal's canal was acquired at a low (2.5-10x) magnification. The outline of the profile of the Rosenthal canal was circles to determine the total area using the Metamorph Image Analysis software. Magnification was then increased (16-25X) for SG Cell counting. Every SG neuron within Rosenthals Canal was "clicked" and counted. SG Neuron density was then calculated by dividing the number of SG cells by the area measured. Each of the remaining 2 more apical profiles was then assessed as above and binned separately, proceeding from base to apex. The next section for the "assessment area" was then counted, with a total of six sections for each animal assessed.

RT-PCR assays for MIF and CD74: RT-PCR was carried out to detect the expression of MIF in the embryonic and adult inner ear using Jump Start Taq (Sigma, St. Louis, MO) following the manufacturer's instruction. Equal amounts of mRNA form each sample was used for this reaction. PCR conditions included an initial denaturation at 95°C for 1 min, 94°C for 30 sec, 55°C for 30 s and 72°C for 30 s and 35 cycles and final extension at 72°C for 5 min and 4°C holding temperature. PT-PCR was also performed to detect the expression of CD74 in

the embryonic SAG and adult SG neurons in the same manner, except using annealing temperature at 58°C. The PCR products were separated on 2.0% agarose gels and visualized using ethidium bromide under UV light. The primer sequences of MIF and CD74 are as follows:

MIF-Forward (F) = CCAGAACCGCAACTACAGTAAGC
MIF-Reverse (R) = TTGGCAGCGTTCATGTCGTAATAG

CD74-F = GCTTCCGAAATCTGCCAAACC
CD74-R = AAGAGCCACTGCTTCATCCAGC

For the loading control, water was added instead of reverse transcriptase.

2-D gel electrophoresis: 2-D gel electrophoretic analysis was performed to identify the factor(s) in bioactive 2B1DT cell conditioned medium when compared to a bio-inactive medium (either medium that had been incubated with IMO cell lines that do not produce ODF, or medium alone, without cell or otocyst exposure). Conditioned media were collected in the same manner as described above. Conditioned medium was also collected from otocysts of chick and mouse. For the chick, eight otocysts from embryonic chick were isolated at E day 4-5. These otocysts were placed in groups of 8 in 250 µl of serum-free medium under identical conditions to those under which the IMO CM was produced. The samples were then sent to the Proteome Center at the University of Michigan for 2-D gel analysis, spectroscopy and MALDI-TOF-TOF analysis,

proteomic analysis and protein identification through sequencing of selected spots cut from the 2-D gels (http://www.proteomeconsortium.org/).

Immunocytochemistry (ICC): ICC was performed with whole and dissociated SAG neurons, ES cells and dissociated inner ear cells (E13-14). In cultures of whole and dissociated SAG neurons, SAG neurites in the 96 well plates were fixed with 100 µl of 4 % PFA solution for 20 minutes followed by a careful washing of the plate three times with PBS. After aspirating PBS, 100 µl of PBS containing 3 % hydrogen peroxide was applied and incubated for 10 minutes. The plate was then incubated with 100 µl of PBT [PBS containing 1 % Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO) and 0.5 % Tween 20] for 1 hour followed by blocking with 100 µl of PBT containing 5 % Normal Goat Serum (NGS) (Invitrogen, Carlsbad, CA) for 30 minutes. Then, the plate was incubated with 100 µl of Monoclonal Antibody against Neuronal Class III β-Tubulin (Covance, Berkeley, CA) diluted with PBT containing 5 % NGS (1:500) at 4°C overnight. Twenty-four hours later, the plate was incubated with 100 µl of PBT containing 5 % NGS for 30 minutes after washing the plate with PBT (3x). Then, a secondary antibody, goat anti mouse, (BioRad, Hercules, CA) diluted with PBT (1:500) was applied and incubated for 3 hours. The plate was washed with PBT (3x) followed by PBS (1x). Plates were labeled with 20 µl of AEC substrate (Zymed, San Francisco, CA) for 5 minutes or until color developed. The reaction was then stopped by adding 20 µl of MilliQ water. Micrographs were taken using Nikon ACT-1 software on a Leitz (Leica) Diavert inverted microscope. The entire

experiment was performed under the hood at room temperature, except for the incubation with primary antibody at 4°C.

D3 mES cells (differentiated into neuron-like cells with MIF and undifferentiated) were immunostained in the same manner as SAG neurons, with a few modifications. Rabbit anti-neurofilament polyclonal antibody (1:400) (Chemicon, Temecula, CA) and goat anti-rabbit-HRP (1:500) (Zymed, San Francisco, CA) were used as a primary antibody and a secondary antibody respectively.

In cultures of the dissociated inner ear, ICC was performed in the same manner as described above and the cells were double labeled for myosin VI (a marker for sensory hair cells) (10 ug/ml, T. Hasson, University of California San Diego) and MIF (1:3000) (Santa Cruz biotechnology Inc., Santa Cruz, CA). For cytokeratin (a marker for supporting cells) (1:100) (Sigma, St. Louis, MO) and MIF as reported in the previous studies (Bianchi et al., 2002, Bianchi et al., 2006). Alexa Flour 488 goat anti-rabbit (1:2000) (Molecular Probes, Eugene, OR) and Alexa Flour 594 rabbit anti-goat (1:2000) (Molecular Probes, Eugene, OR) were used as the secondary antibodies for detection of myosin VI and MIF, while Alexa Flour 488 goat anti-rabbit (1:2000) (Molecular Probes, Eugene, OR) and Alexa Flour 555 goat anti-mouse (1:2000) (Molecular Probes, Eugene, OR) were used as the secondary antibodies for detection of MIF and cytokeratin. Confocal images were captured with a Zeiss LSM-5 Pascal microscope.

Sandwich ELISA for MIF: Sandwich ELISA assays were performed (Abcam, http://www.abcam.com/ps/pdf/protocols/Sandwich_ELISA.pdf) to determine MIF levels in conditioned media secreted from E5 chick otocysts and 2B1DT IMO cells. First, 100 µl of 2.0 µg/ml of an anti-human MIF IgG monoclonal antibody (R&D, Minneapolis, MN) was dissolved in diluent reagent (PBS containing 1 % BSA) and added to each well of a 96 well plate (Nalge Nunc International, Rochester, NY). The plate was left at room temperature overnight. Next morning, the plate was washed (3x) with 150 µl washing buffer (PBS containing 0.05 % Tween 20). Then, the plate was blocked with 200 µl of diluent reagent per well for 1 hour followed by washing with washing buffer in the same manner. Next, 100 µl of test sample per well, as well as 100 µl recombinant mouse MIF antigen per well at a concentration range of 0.125 – 1.75 ng/ml were incubated to determine a standard curve. This incubation was allowed to proceed for 2 hours. After removal of antigen followed by washing, 100 µl of 400 ng/ml Biotinylated Anti-human MIF antibody (R&D, Minneapolis, MN) was added to each well and incubated for 2 hours. Then, the plate was incubated with100 µl Streptavidin-horse radish peroxide (HRP) complex (1:1,000) per well for 2 hours after washing (3x). The plate was covered with aluminum foil to avoid exposure of the light-sensitive substrate. After incubation, the plate was washed again with washing buffer. Then, 100 µl of tetramethylbenzidine (TMB) (Promega, Madison, WI) was added to each well and incubated for 15 minutes. During the incubation, the plate was kept covered with aluminum foil. The reaction was stopped by adding 100 µl of Stop solution (1 N Hydrochloric acid) to each well. Finally, absorbance

was read at 450 nm using a Halogen BELLAPHOT plate reader (Fisher Scientific, Pittsburgh, PA). The entire assay was performed at room temperature and all samples were done in triplicate.

Immunohistochemistry: Embryonic sectioned inner ear tissues were labeled for MIF. Tissues were incubated at a 1:1 ratio of ice cold acetone and methanol for 1 minute followed by 5 minute air dry. After washing with PBST for 2 minutes twice, a barrier was circumscribed around the tissue slice on the slide with a pap pen (Fisher Scientific, Pittsburgh, PA) to minimize the reagents used. Then, the tissues were blocked against non-specific binding by the secondary antibody by incubation in 3% normal donkey serum for 30 minutes. The tissues were incubated in rabbit anti-MIF antibody (1:3000) (Zymed, South San Francisco, CA) for 1 hour. After rinsing with PBST three times, sections were incubated with goat anti-rabbit Alexa Flour 594 (1:200) (Molecular Probes, Eugene, OR) for 30 minutes. After washing three times with PBS, the sections were coversliped in Prolong Gold Antifade reagents (Invitrogen, Carlsbad, CA), warmed to room temperature and observed with a fluorescence microscope. Negative controls lacked primary antibody. All steps were done at room temperature in a humidified chamber.

Adult sectioned cochleae (WT mice) were stained in the same manner as the embryonic specimens, with a few modifications. Adult tissues were double-labeled for MIF and MCP1. After blocking with 3% normal donkey serum, the tissues were incubated in rabbit anti-MIF antibody (1:1000) (Santa Cruz

biotechnology Inc., Santa Cruz, CA) and goat anti-MCP1 antibody (1:100) (R&D, Minneapolis, MN) for 30 minutes followed by washing (3x/ x min/wash) in PBST. Then, the tissues were incubated in donkey anti-rabbit Alexa Flour 555 (1:200) (Molecular Probes, Eugene, OR) and donkey anti-goat Alexa Flour 488 (1:200) (Molecular Probes, Eugene, OR) for 30 minutes. Photographs were taken sequentially using a Leica phase contrast microscope (Leica, Hicksville, NY).

Statistical analysis: All data, except for the cytocochleogram analysis (OHC loss percentage), were analyzed by one-tail t-test. Multiple comparisons Kruskal-Wallis test with family alpha was used for the cytocochleogram analysis. A statistical probability of $P<0.05$ was considered significant in all data (Moore and McCabe, 2004).

Results

Conditioned medium (CM) from IMO cells and embryonic chick otocysts enhanced SAG neurite outgrowth and survival.

Earlier studies demonstrated that CM or "otocyst-derived factor" (ODF) from chick, mouse and rat promoted directional neurite outgrowth and survival of early stage statoacoustic ganglion (SAG) neurons (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993). SAG explants from E5 chick embryos were plated on poly-D-lysine-coated 96-well plates [as previously described; (Bianchi et al., 2005)). The explants were treated with serum free chick otocyst

CM (otocyst-generated ODF), serum free IMO 2B1 CM (IMO-generated ODF +/- γ -interferon), or serum free basal medium. After 48 hours in culture, SAG neurite outgrowth was scored using the scoring scale of 1 (no neurite outgrowth) to 5 (long, dense radial growth), developed by Bianchi et al. (Bianchi et al., 2005). Individual SAG explants treated with IMO-generated ODF or chick otocyst-generated ODF showed robust neurite outgrowth, with mean scores of 4.1 in otocyst-generated ODF and 3.9 in IMO-generated ODF after normalization (Figure 2.1-B, C and G). In contrast, the explants with basal medium or basal medium + γ -interferon showed little neurite outgrowth (Figure 2.1-A). Statistical analysis demonstrated that both ODFs produced outgrowth significantly greater than basal medium ($p < 0.05$).

Dissociated neuron assays were performed with E5 chick SAGs to evaluate the effect of ODFs on neuronal survival. Survival of SAG neurons was determined after 48-72 hours in culture after staining with anti-Tuj1 antibody (Figure 2.1-D, E, F and H). After 48 hrs, 13% and 18% neurons respectively were Tuj-1 positive in the presence of otocyst-generated ODF and IMO-generated ODF after normalization (Figure 2.1-H). Significant neuronal survival in ODFs was observed along with extended neuronal processes from individual cells compared to basal control medium, in which the cells were dead and no neurite outgrowth was observed ($p < 0.05$). Together, these bioassays confirmed that both ODFs promote SAG neurite outgrowth and survival, but that we were able to recapitulate the results reported in the early studies.

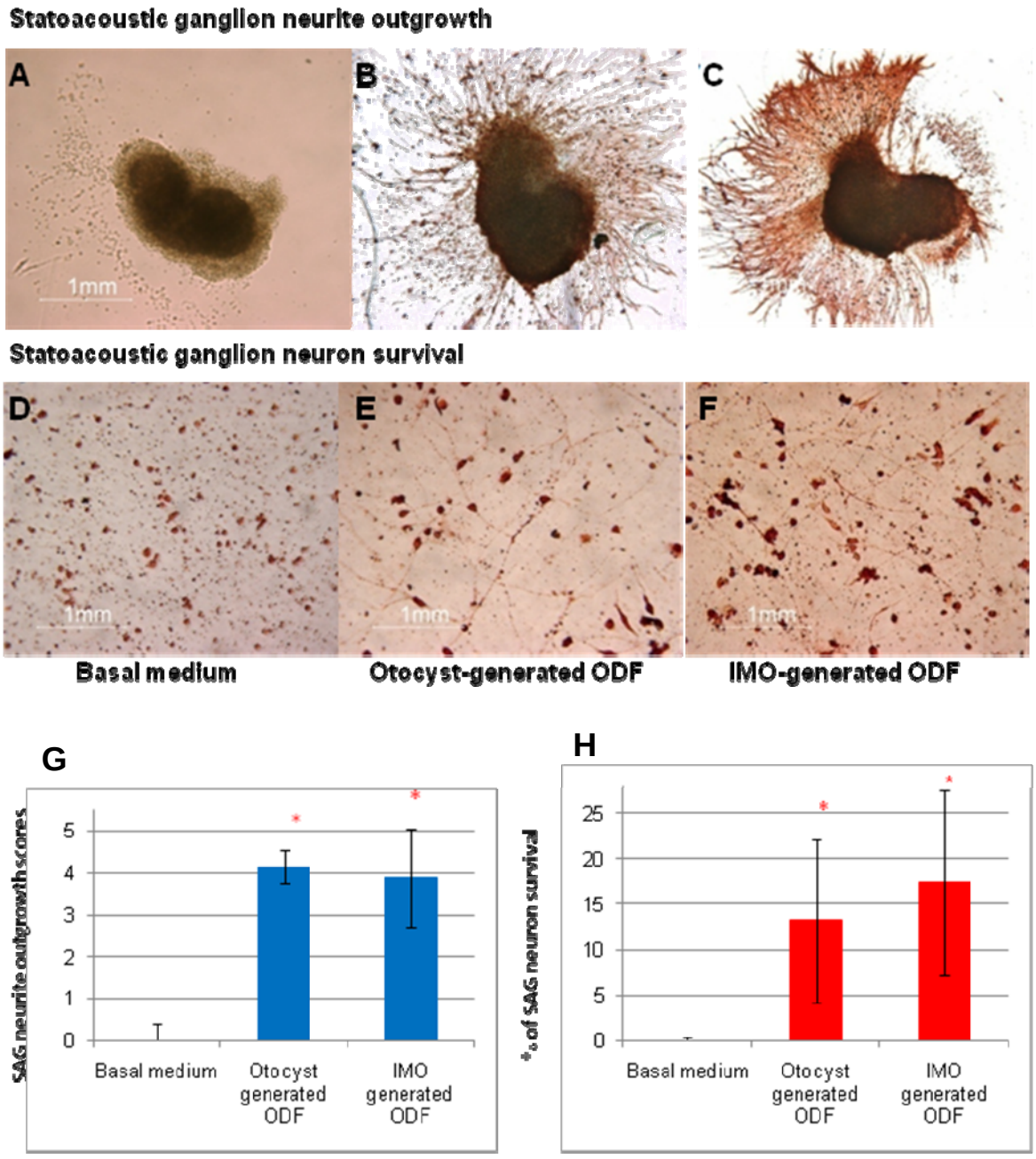


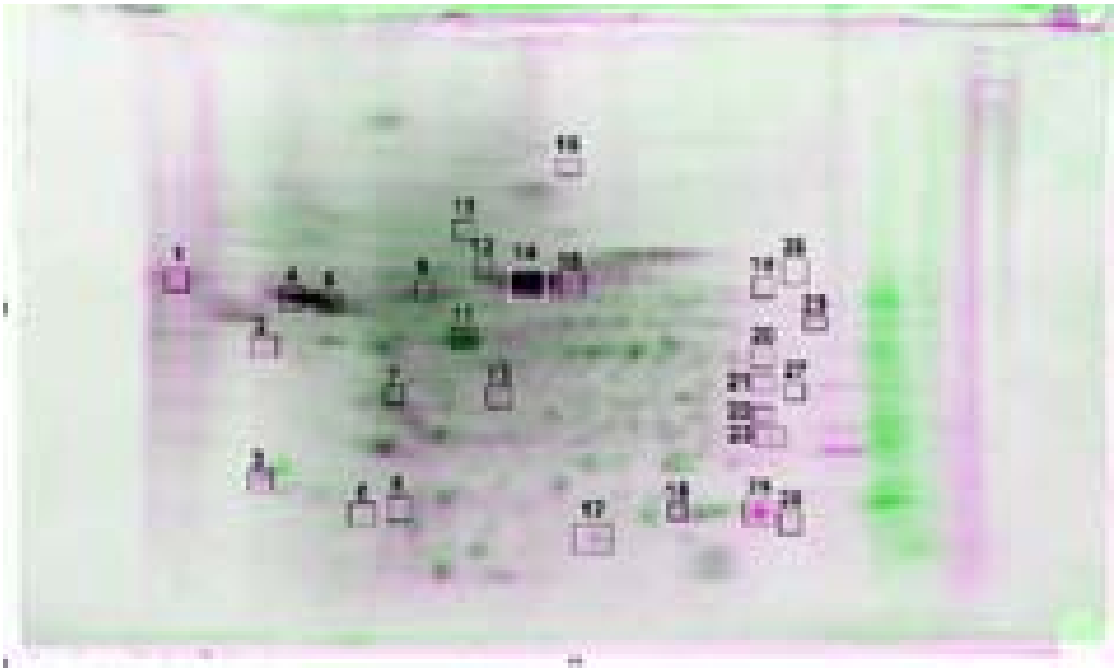
Figure 2.1: (Top row): Chick SAG Neurite Extension: Neurites extend from E5 chick SAG in the presence of conditioned medium from either otocyst-generated ODF (B) or IMO-generated ODF (C) compared to lack of neurite outgrowth in basal medium (A). (Middle row): Survival of Dissociated SAG neurons: Neurons from E5 chick survive and extend neuronal processes in Otocyst-generated ODF (E) or IMO-generated ODF (F). Dissociated chick SAG neurons die and do not

extend neurites in basal medium (D). (Bottom row): The quantification of extent of neurite outgrowth demonstrates that shows that both IMO- and otocyst-generated ODFs significantly enhance SAG neurite outgrowth (G). SAG neuronal survival in otocyst-generated or IMO-generated ODF is also significantly enhanced compared to control (H). ($p < 0.05$).

MIF is a major bioactive protein component of the ODF.

IMO-generated ODF identified in the previous assays as having bioactivity were then compared to inactive CM from IMO cells that do not produce ODF or to medium alone by 2D gel analysis and a subsequent proteomic screen in which components of the active fraction were identified on the gels, cut out, subjected to MALDI/TOF/TOF spectroscopy and sequenced by the University of Michigan Proteomics Core (http://www.med.umich.edu/mgpc/cores/ppc.htm#tdge). MIF was found in all the biologically active, but not inactive, samples (Figure 2.2) from both IMO-generated and otocyst-generated ODF. Proteomic analysis also confirmed the presence of MCP-1 (Figure 2.2), which we previously detected using a cytokine protein array (Bianchi et al., 2005). The arrays we used in the 2005 study did not include MIF. A yeast 2-Hybrid screen was also performed (data not shown) that also confirmed that MIF and MCP1 are potentially interacting proteins. This data will be reported elsewhere. The proteomic comparisons were also conducted with chick otocyst-generated ODF and the same results were found (data not shown). Following the identification of MIF and MCP-1 in ODFs, sandwich ELISA assays were performed to quantify both MIF and MCP1 in otocyst-generated ODF and IMO-generated ODF. The

concentrations of MIF [1.14 ng/ml (n = 39)] and MCP-1 [1.27 ng/ml (n = 6)] in otocyst-generated ODF were similar to those found in IMO-generated ODF, where the concentration of MIF was 2.43 ng/ml (n = 6), while that of MCP-1 was 1.22 ng/ml (n = 6).



Figures 2.2: Proteomic analysis of bioactive ODF from IMO CM demonstrates that both MIF and MCP1 are expressed in bioactive CM prepared from IMO-generated ODF. Two-D gel comparison of Active (represented in pink) vs. Inactive (represented in green) CM from IMO 2B1 (active) and IMO 3C6 (inactive) CM, determined in bioassays of E5 chick SAG neurite outgrowth and survival. The spots represented in the active but not inactive CM were cut out and identified by the Protein sequencing/Proteomics facility at U of M. Spot 25 is MCP1 and Spot 28 is MIF. These proteomic comparisons have been repeated with chick otocyst-generated ODF and similar results have been found.

Cytokines	Experimental approaches
Macrophage Migration Inhibitory Factor (MIF)	Proteomics Immunohistochemistry (IHC) In situ hybridization (ISH) PCR, Y2H
Monocyte Chemoattractant Protein1 (MCP1)	Cytokine array Proteomics IHC, ISH, Y2H

Table 2.1: Summary of the two major cytokines found in ODF and the experimental approaches used to identify and verify these cytokines.

IHC=Immunohistochemistry with an anti-MIF antibody and fluorescent secondary antibodies used to label the developing mouse inner ear between stages 11.5 and 17.5; PCR: real time quantitative PCR (see methods); Y2H a yeast two hybrid analysis using each cytokine in turn as bait (MCP1 or MIF) was also performed (data not shown).

Recombinant (r) MIF mimics the bioactivity of ODF in a dose-dependent manner.

To characterize MIF's role in neuronal extension and survival of SAG neurons in the developing inner ear, neuronal bioassays were conducted with various concentrations of either human or mouse recombinant (r) MIF. MIF concentrations in a range of 5 pg/ml to 500 ng/ml were chosen based on the results of ELISA assays, which showed MIF concentration at 1.0 to 2.5 pg/ml in ODFs. The concentration range chosen for testing was selected to be at least 10x higher and lower. Serum free basal medium and CM from E8-10 chick brain were used as negative and positive control respectively in the assays. The effect

of rMIF on SAG neurite outgrowth and survival was dose-dependent (Figure 2.3). After 48 hours in culture, 5 pg/ml and 5 ng/ml of rMIF significantly enhanced SAG neurite outgrowth, while 50 pg/ml MIF promoted SAG neuron survival significantly compared to basal control ($p < 0.05$). In contrast, higher concentrations (500 ng/ml) of rMIF either had no effect or inhibited both SAG neurite outgrowth and survival after 48 hours in culture (see discussion for proposed explanation).

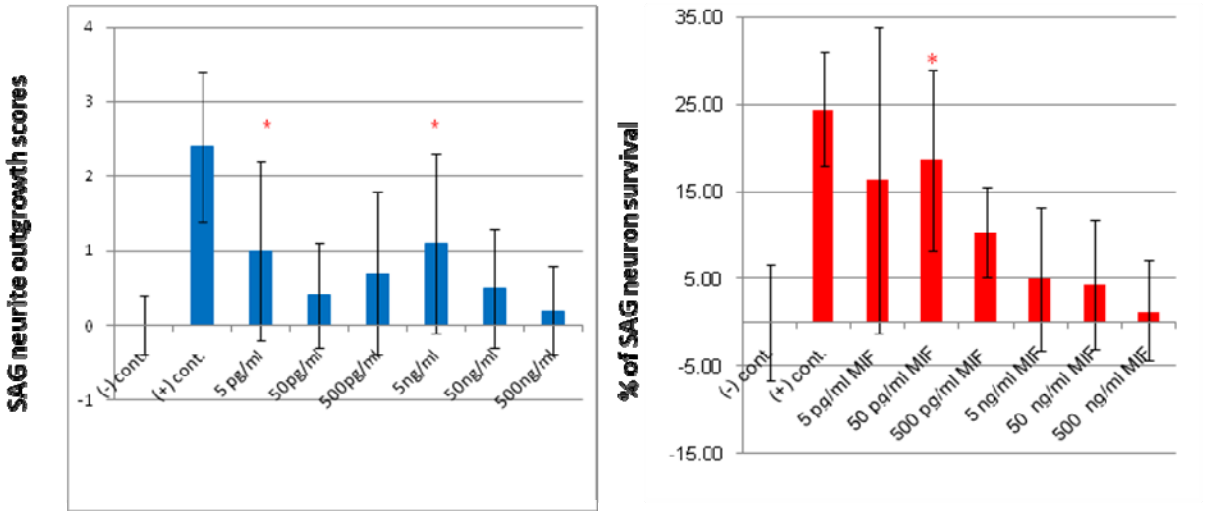


Figure 2.3: rMIF from either human (R&D, Mineneapolis, MN) or mouse (R&D, Mineneapolis, MN) enhances both SAG neurite ougrowth and survival in a dose-dependent manner. After 48 hours in culture, 5 pg/ml and 5 ng/ml of rMIF significantly enhanced SAG neurite outgrowth compared to control ($p < 0.05$) (left plot). 50 pg/ml of rMIF promoted SAG neuron survival significantly compared to basal control ($p < 0.05$) (right plot). In contrast, higher concentrations (500 ng/ml) of rMIF have no effect or inhibited both SAG neurite outgrowth and survival after 48 hours in culture. Basal medium was used as the negative control and CM from E8-10 chick brain was used as the positive control. ($p < 0.05$), n= 20

Recombinant MIF enhanced postnatal SG neurite outgrowth.

Postnatal mouse SGNs were also examined for any effect of rMIF on neurite outgrowth/regrowth. SGNs were isolated from the WT (Balb/C) mice at postnatal day 3 (P3) and cultured in serum free basal medium containing 5 ng/ml or 50 ng/ml rMIF. As in assays of the embryonic SAG, serum free basal medium was used as the negative control and CM from E8-10 chick brains was used as the positive control in the assays. After 48 hours in culture, SGNs in 50 ng/ml rMIF demonstrated significant neurite outgrowth compared to the negative control ($p < 0.05$) (Figure 2.4). However, SG in 5 ng/ml rMIF also showed some degree of outgrowth, however, it was not statistically significant. The results indicate that rMIF can promote neurite outgrowth not only from the embryonic SAG stage but also from postnatal SG.

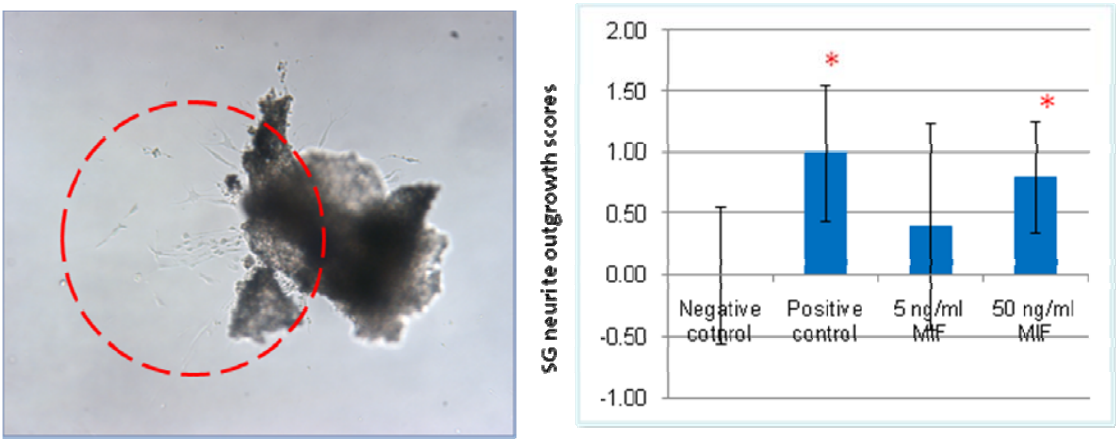


Figure 2.4: Mouse/human rMIF promoted WT (Balb/C) postnatal SG neurite outgrowth. After 48 hours in culture, 50 ng/ml rMIF significantly enhanced SG neurite outgrowth ($p < 0.05$) compared to the negative control (basal medium). CM from E8-10 chick brain was used as the positive control. N=9.

MIF at as low a concentration as 1 pg/ml promotes the neuronal differentiation of mouse embryonic stem cells (mESC).

To further explore the role of MIF in neuronal development, we examined the effect of MIF on neuronal development in the mouse D3 ES cell line (Doetschman et al., 1985). Various concentrations (100 to 50 ng/ml illustrated in Figure 2.5) of rMIF were introduced to ES cells. However, as low as 1 pg/ml of rMIF caused a subset of the ES cells, but not all, to become neuron-like after 48 hours in culture. The cells treated with rMIF developed long neurites that were neurofilament positive (Figure 2.5, D-I). In contrast, the cells in the absence of rMIF kept proliferating (Figure 2.5-A).

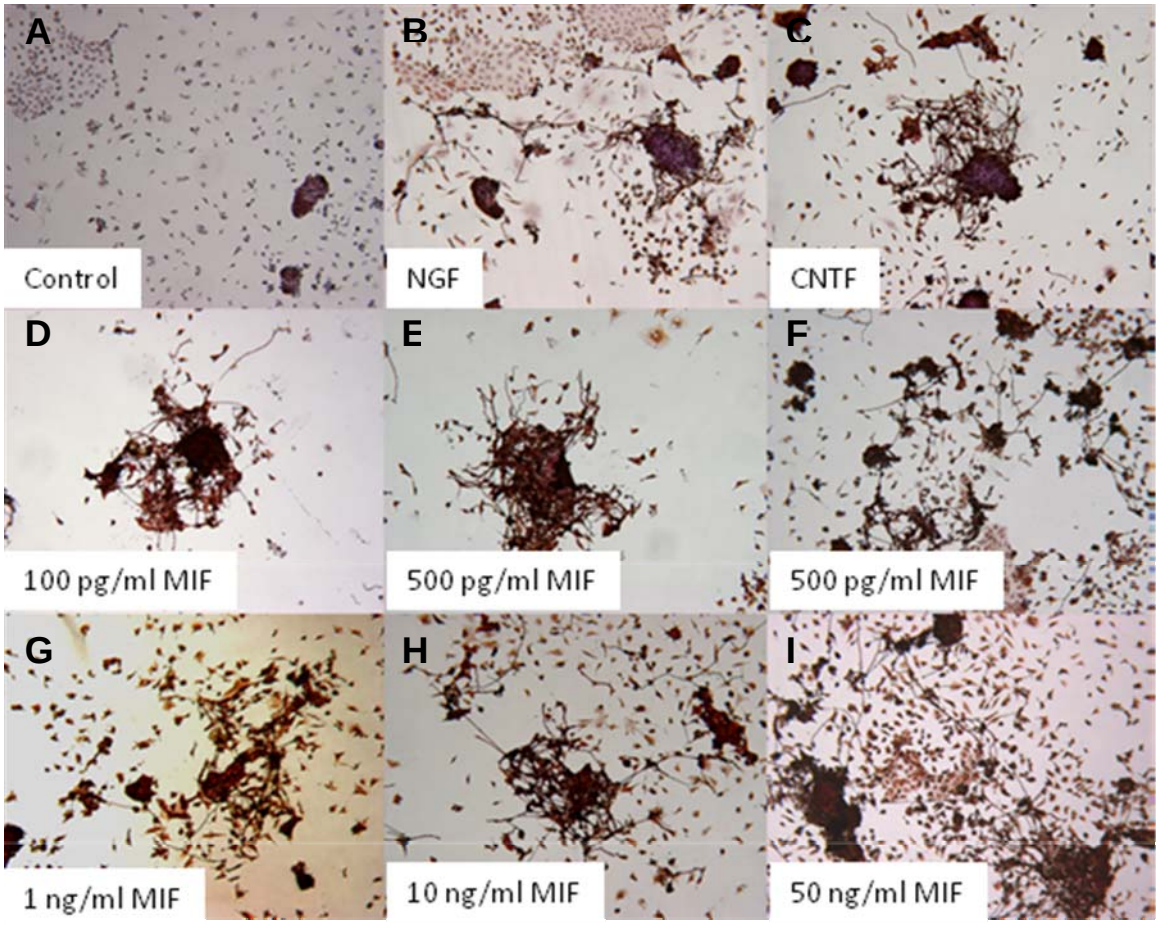


Figure 2.5: Mouse D3 ES cells treated with MIF at the concentrations specified took on a neuron-like morphology within 48 hrs, elaborated long neurites and could be labeled with an antibody to neurofilament and a secondary HRP antibody. Phase contrast (Leica) photograph (4x). Note that not all stem cells have become neuron neuron-like.

Function-blocking antibodies to MIF inhibit ODF-induced SAG neurite outgrowth and prevent neuron survival.

To determine whether MIF was an essential bioactive component of SAG neurite outgrowth and survival, functional-blocking assays were performed with chick SAG neurons. rMIF was pretreated with anti-MIF antibody followed by

protein G beads to remove MIF activity. SAG explants treated with 10 ng/ml of rMIF produced neurite outgrowth with an average score of 4.1 (n=8), while the explants treated with MIF that had been pre-treated with the function-blocking anti-MIF antibody produced little if any neurite outgrowth (n=7) as shown in Figure 2.6 (left). In the assays of dissociated SAG neurons, measuring neuronal survival, IMO-generated ODF and 10 ng/ml rMIF demonstrated 67% (n=31) and 77% (n=17) neuronal survival respectively (Figure 2.6, right). The antibody reduced dissociated SAG neuronal survival to background levels when used to treat rMIF (n=6), whereas some residual neuronal survival was seen after treatment of IMO-generated ODF with this function-blocking antibody (n=6) (see discussion). Taken together, the results demonstrate that MIF is both necessary and sufficient to promote outgrowth and survival of SAG neurons.

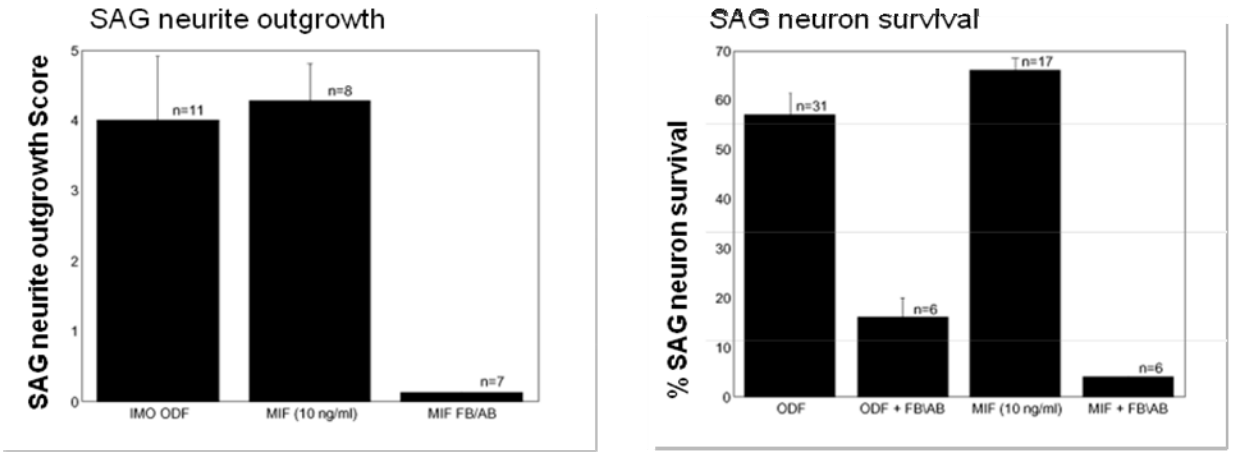


Figure 2.6: Function-blocking antibody-treated MIF, used in assays of SAG neurite outgrowth and survival. 10 ng/ml rMIF mimicked the bioactivity of IMO-generated ODF. Pre-treatment of MIF with a function-blocking anti-MIF antibody (R&D, Mineneapolis, MN) and protein G beads, substantially inhibited MIF's neurite outgrowth-promoting activity. Scoring of neurite outgrowth is according to Bianchi et al., 2005 (Left). SAG neuronal survival was promoted by both IMO-generated ODF and 10 ng/ml rMIF. The same function-blocking antibody, which was used for the blockade of MIF's ability to inhibit SAG neurite outgrowth was used to treat either rMIF or IMO-generated ODF. The antibody reduces neuronal survival to background levels if used to treat rMIF, but some residual neuronal survival is seen after treatment of IMO-generated ODF with the function-blocking antibody and protein G beads (Right). We hypothesize that this reflects the presence of additional bioactive cytokines such as MCP1 (see discussion).

MIF is expressed in sensory epithelial regions of the developing and mature inner ear.

Embryonic and adult mouse inner ears were examined for expression of MIF mRNA by RT-PCR and MIF protein by western blotting and immunohistochemistry. Expression of MIF mRNA in the otic vesicle from E9.5 mice has been reported (Kobayashi et al., 1999). We have recently detected

MIF protein in IMO cells, isolated from E9.5 Immortomouse otocysts (discussed in Appendix). Thus, our laboratory examined its expression at the mRNA level in the mouse E14 ear, during embryonic otic development and in adult inner ear tissues. RT-PCR experiments demonstrated that MIF mRNA was expressed in both embryonic and adult inner ear tissues, including the cochlea (Figure 2.7) and ampullae (data not shown).

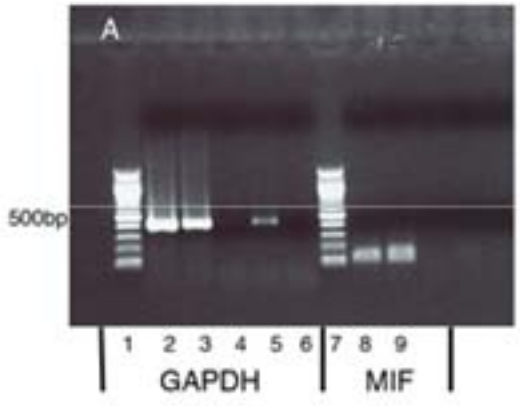


Figure 2.7: MIF mRNA expression in cochlear tissues and SGNs. Cochleae were removed from adult WT (Balb/c) mice. SG were separated from the remaining sensory epithelium. RNA was isolated from the tissues and RT-PCR performed. GAPDH expression, used as a control for loading, was seen in SGN (lane 2), sensory epithelium (lane 3), no GAPDH in the water control (lane 4), -RT in SGN and sensory epithelium (lanes 5 and 6, respectively). MIF expression is present in both the SG (lane 8) and sensory epithelium (Lane 9). Lanes 1 and 7 are the 100bp DNA ladder.

The expression of MIF protein was examined in the E15.5 mouse inner ear. Its expression was detected in the developing inner ear, including the cochlea, utricle, saccule, lateral canal, latera ampulla and crux (Figure 2.8-B and C).

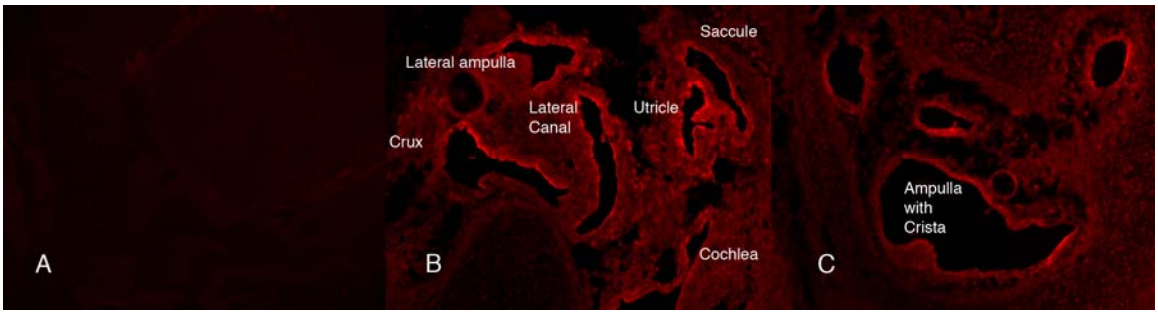


Figure 2.8: MIF protein expression in the embryonic (E) day 15.5 mouse inner ear. (A). Control: Secondary antibody alone. No background labeling is seen. (B and C). MIF expression is detected in the developing inner ear, including the cochlea, utricle, saccule, lateral canal, lateral ampulla and the crux. (772 ms exposure; Image at 20x magnification).

To determine the expression of MIF in the mature mouse inner ear, whole mount cochleae from 4 week (wk) old WT (Balb/c) mice (n=4) were immunostained. As shown in Figure 2.9, MIF is expressed in supporting cells (SC) that cup the inner and outer HC (IHC and OHC, respectively). Its expression was also detected in the stria vascularis (StV), spiral limbus (SL), and spiral ligament (SpL). The cytokine MCP1 was expressed in the IHC and OHC of the Organ of Corti, as well as in the StV (Figure 2.9). Overlapping staining (yellow) was expected as the SC cup the HC to support them.

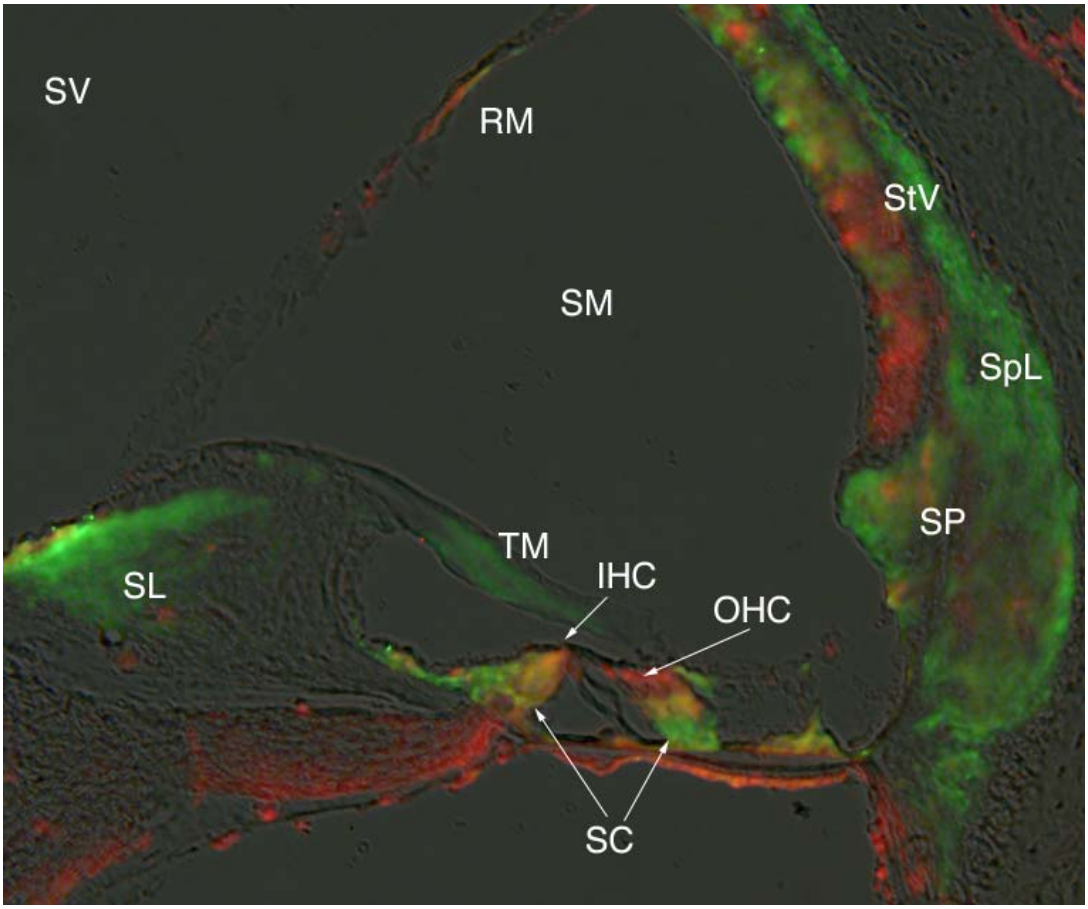


Figure 2.9: Expression of MIF and MCP1 proteins in the cochlea of 4 week Balb/C mice. Cryosections of Balb/c cochleae were labeled for MIF (green) and MCP1 (red). MIF is expressed in SL, SpL, StV, SP and SC. MCP1 expression is found in IHC and OHC as well as in StV and basement membranes. The region of yellow overlap indicates the portion of the SC that directly contact and cup the IHC and OHC. Reisners membrane (RM), scala media (SM), scala vestibuli (SV), tectoral membrane (TM). Image at 20x.

Expression of MIF in Cultured Inner Ear "Domes" from 13/14 day Mouse Embryos

To examine the differential expression of MIF in inner ear cells in more detail, cultures of inner ear cells, which are dissociated and subsequently form inner ear "domes" were analyzed as previously described (Bianchi et al., 2002, Bianchi et al., 2006). As in our previous studies, domes formed with a central mass of HC resting upon a SC layer. Cytokeratin labeled the outer borders of SC (Figure 2.10-A, red), whereas myosin 7A labeled HC in the centrally located region above the plane of the SC (Figure 2.10-B, green). In contrast, MIF was co-localized to the SC layer of the dome, with cytokeratin localized to the cellular borders (Figure 2.10-B, red) and MIF appearing in the cytoplasm (Figure 2.10-B, green). The central regions of the domes (above the focal plane), that contain HC were not labeled with MIF (Figure 2.10-B).

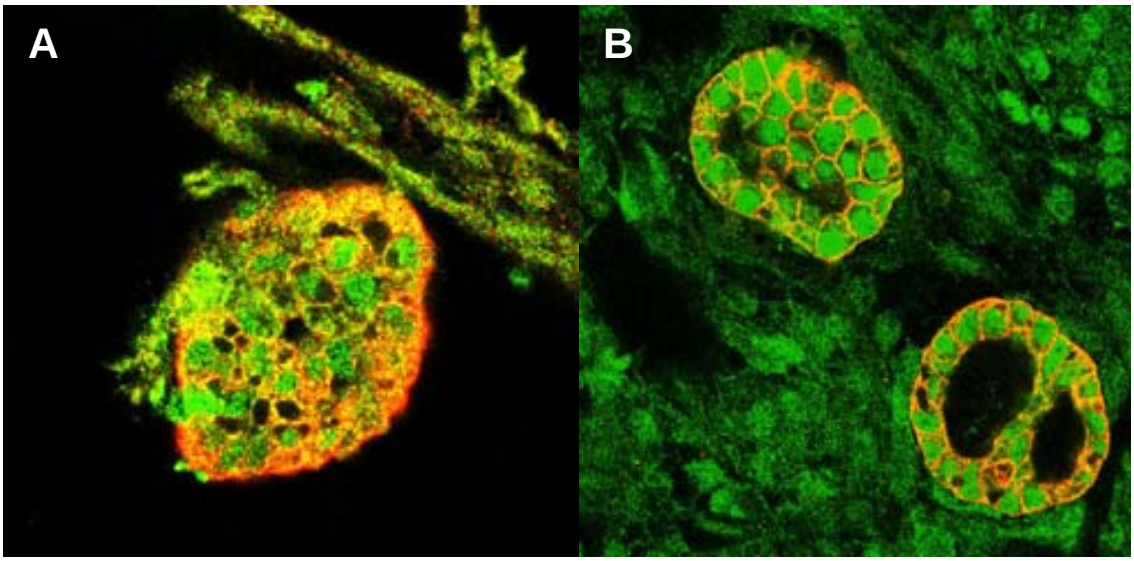


Figure 2.10: Ear domes (made as in Bianchi et al., 2002, 2006) were produced and cultured from E13-14 mouse embryos. (A). Double-labeled fluorescent immunocytochemistry shows myosin 7A positive hair cells primarily in the central region of the domes (green), whereas MIF (red) is detected in the surrounding (and underlying) SC layer (20x). (B). Double labeled fluorescent immunocytochemistry with anti-cytokeratin and anti-MIF (20x). SC are labeled with cytokeratin (red) in the underlying layer of the domes, while MIF expression is detected in the cytoplasmic region of these same cells. In domes, the upper layer of HC is not immunoreactive in these preparations. Note: MIF signal is encircled by cytokeratin signal. Red: anti-Cytokeratin; Green: anti-MIF.

CD74, a MIF receptor, is expressed in developing SAG and postnatal SG neurons at the mRNA level.

RT-PCR was conducted to examine the expression of mRNA for CD74, a MIF receptor, on E14 mouse SAG neurons, adult mouse SG neurons and the mouse sensory epithelium (Figure 2.11). CD74 mRNA was expressed both in the embryonic SAG (Figure 2.11-A) and the adult SG neurons as well as the adult sensory epithelium in the cochlea (Figure 2.11-B).

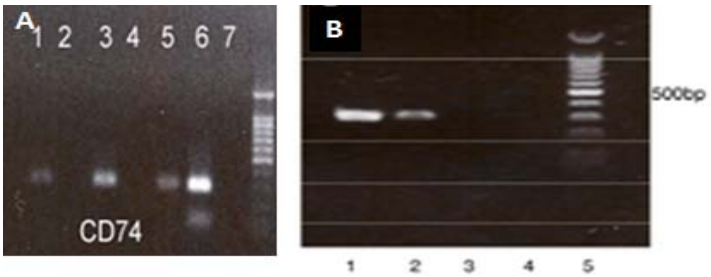


Figure 2.11: Expression of CD74 in embryonic and adult inner ear at the mRNA level. Expression of CD74 mRNA is present in the E14.5 WT (Balb/C) mouse SAG (lane1) (A). Expression of CD74 is present in the adult SGNs (lane 1) and the sensory epithelium (lane 2), but not in the water control (lane 3). Lane 4=blank. Lane 5= 100bp DNA ladder (NEB) (B).

Characterization of the MIF Knock-out (MIF KO) Mouse

Hearing: Auditory Brain Stem Response (ABRs) Recordings from MIF KO and WT Mice

Auditory brain stem response (ABR) testing was performed to determine whether loss of MIF affects hearing in the MIF KO mice. Hearing loss began as early as 4 weeks in MIF KO mice (Table 2.2). The most dramatic hearing loss was noted at 48kHz. MIF KO mice had statistically elevated thresholds at this frequency compared to WT mice. A statistically significant difference was seen at 24kHz as well. Furthermore, the MIF thresholds at 48 kHz remained elevated, but constant as the KO mice aged through 12 weeks. Balb/c mice, which served as the control mice in this experiment, are known to have age related hearing loss as time progresses and this was seen at all frequencies in the control animals.

Frequency	Genotype	Mean dB SPL + SD
12 kHz	WT (n=5)	28 ± 3
	MIF KO (n=4)	36 ± 2
24 kHz	WT (n=5)	24 ± 2
	MIF KO (n=4)	43 ± 2
48 kHz	WT (n=5)	29 ± 14
	MIF KO (n=4)	58 ± 2

Table 2.2: ABR Thresholds in WT and MIF KO mice at 4 weeks. The most dramatic hearing loss is seen at 48kHz, the high-frequency detecting basal region of the cochlea. MIF KO mice have statistically elevated thresholds compared to WT mice in the 48 kHz region.

Morphological changes in MIF KO mouse inner ears

To determine if hearing loss was a result of altered innervation patterns, missing neurons or obvious phenotypic changes in the SG, spiral ganglion neuron counts were performed on Balb/c (control) and on MIF and MCP1 KO mice at 4, 8 and 12 weeks of age. The density of neurons was determined by cochlear turn, and is shown in Figure 2.12. At 4 wks, MIF KO mice had lower cell densities than Balb/c mice in the P1 (12 kHz) and P3 (48kHz) turns. In addition, immunostaining assays were performed: In MIF KO mice, in the basal turn of the cochlea (organ of Corti) in the 48 kHz range, cochleograms showed that both OHC and IHC were missing, although small numbers of supporting cells remained in MIF KO mice (Figure 2.13).

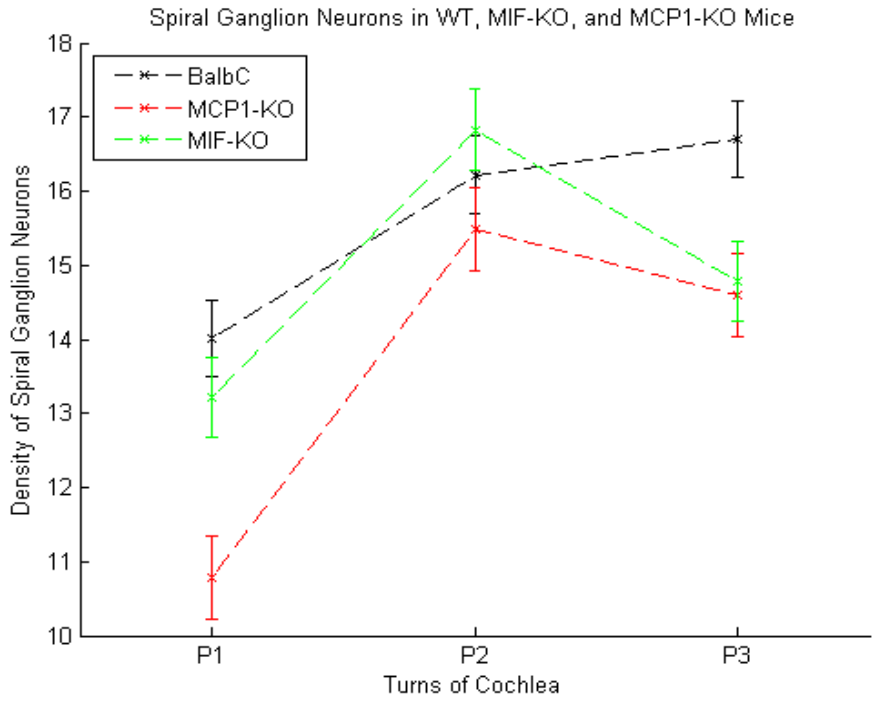


Figure 2.12: Density of spiral ganglion neurons in WT, MIF and MCP1 KO mice at 4 weeks of age. MIF KO mice demonstrate statistically lower average cell densities than Balb/c mice in the P1 (12 kHz) and P3 (48kHz) turns.

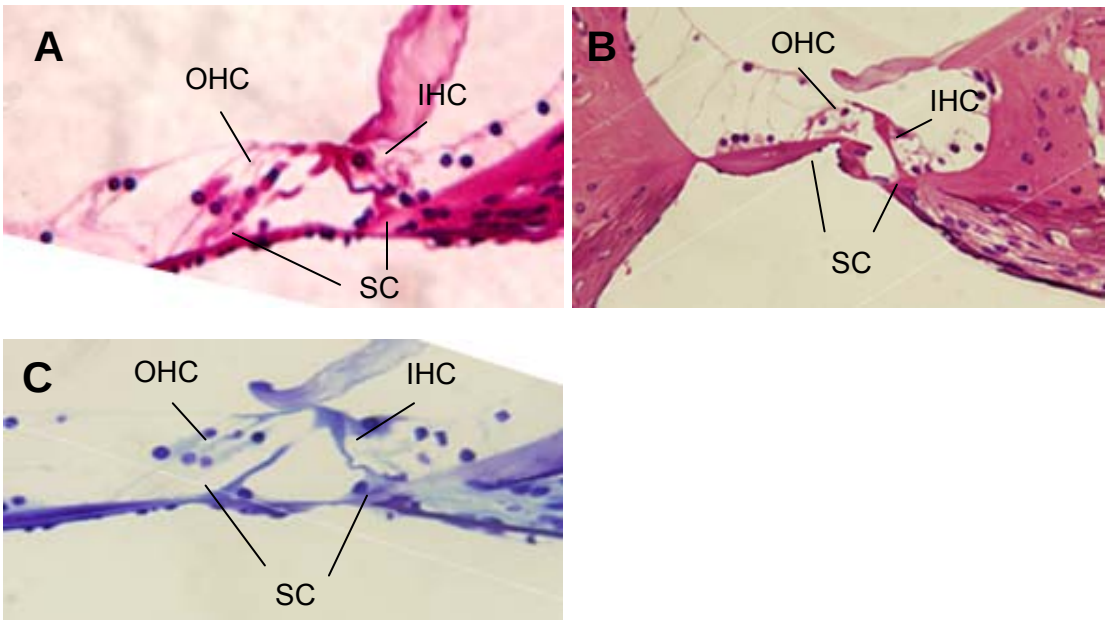


Figure 2.13: **A.** MIF KO mouse basal turn of the OC in the 48 KHz range (same region as in WT mice as shown in Figure 2.9) shows missing OHC and IHC, although SC are still present. **B**. Similar findings are seen in the MCP1 KO animals. In cochleograms, 7 out of 9 animals at 4 weeks of age show this loss of HC at 48 kHz. **C.** Control OC at 8 weeks.

Expression of neurofilament and myosin 7a in whole mount vestibular tissue and cochlea in WT and MIF KO mice

Whole mount vestibular and cochlear tissues from 4wk old WT (Balb/c) mice were analyzed by epifluorescence for normal innervation patterns and the presence of HC, using antibodies to neurofilament and myosin 7a, respectively. Figure 2.14 shows analysis of neurofilament and myosin 7a expression in the cochlear epithelium of WT and MIF KO mice in basal high frequency region. The results demonstrated that both IHC and OHC were phenotypically normal in both

the apex (data not shown) and hook (basal) regions of the OC in WT mice. However, in the comparable sections from a MIF KO mouse, there was an absence of OHC in some regions of the OC (Figure 2.15). These missing OHC were seen in the entire regions of the cochlea in MIF KO mice, with most loss in the apical low frequency region (P1) (mean % loss, 7.10 ± 1.19%), suggesting that normal expression of MIF is required for normal OHC patterning.

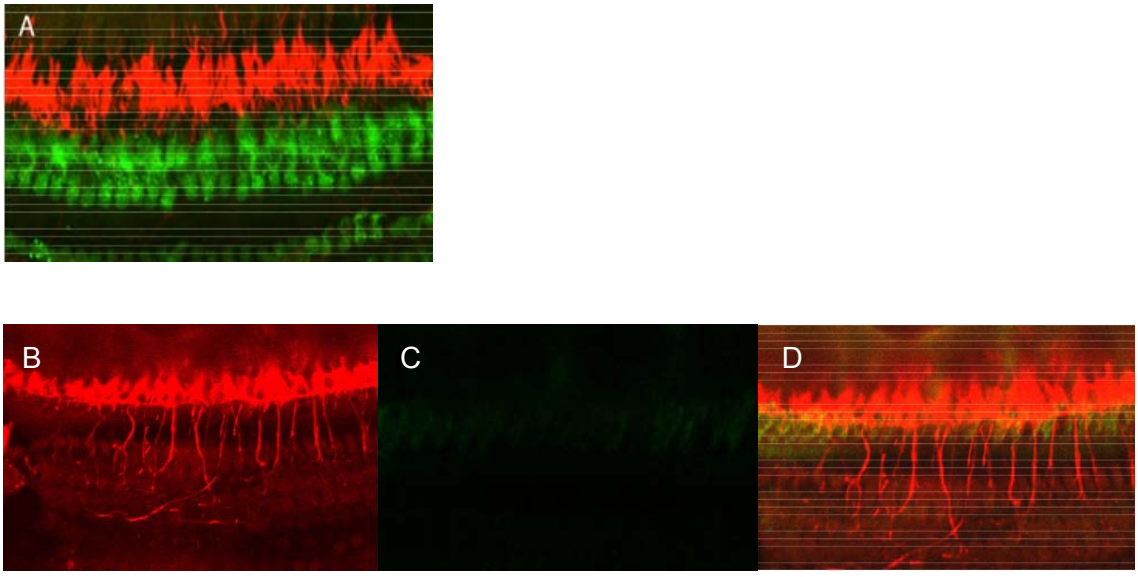


Figure 2.14: Epifluorescence of OC for neurofilament (a neuronal marker, red) and myosin 7a (a HC marker, green) in WT (Balb/C) (A) and MIF KO (B-D) mice. (A): Both IHC and OHC in the cochlea are phenotypically normal in both the apex and hook regions of the OC in WT mice. (B-D): There are lower neuron density (B and D) and missing HC (C and D) in the MIF KO OC.

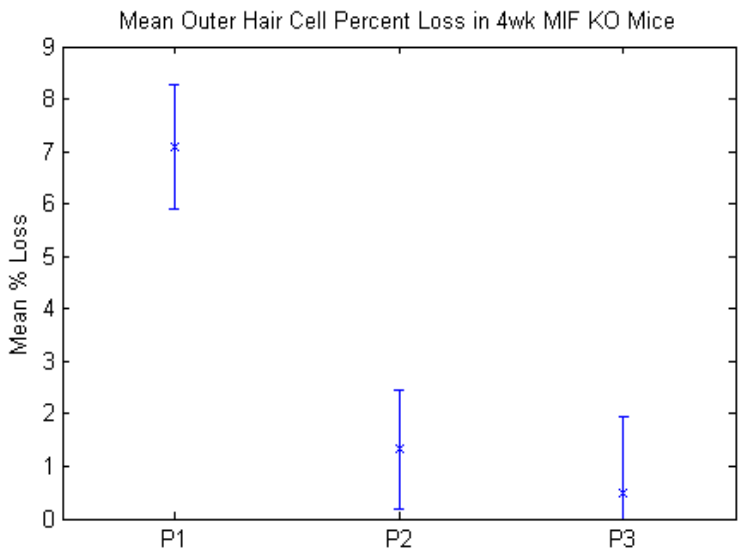
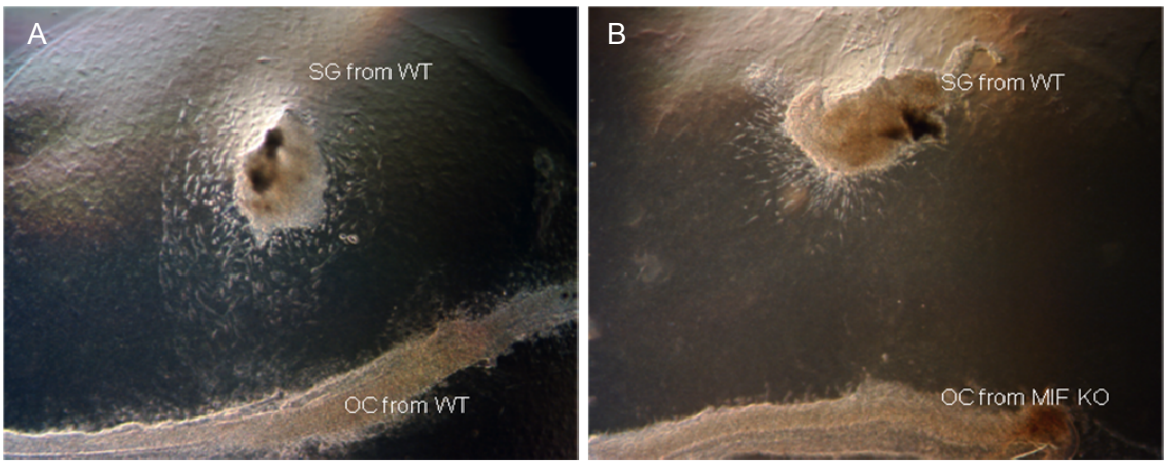


Figure 2.15: Mean OHC loss percentage in MIF KO mice compared to WT mice at 4 weeks of age. Mean % loss of OHC is 7.10 ± 1.19 in P1, 1.33 ± 1.13 in P2 and 0.49 ± 1.46 in P3. ($p<0.05$), n = 4.

Neurites from spiral ganglion explants extend directionally toward the WT OC, but not toward the MIF KO OC.

To further determine if MIF affects inner ear neuronal development or maturation at post-natal stages, co-culture assays of the cochlea with SGNs were conducted. Freshly excised OC from WT and MIF KO mice at P3 were cultured with freshly excised SG from P3 WT mice. After 5 days of culturing in basal medium, directional SG neurite outgrowth was observed toward the WT OC, while only random neurite extension that did not reach the OC was seen in MIF KO OC as shown in Figure 2.16. The results present further evidence that MIF functions as an essential component in an initial neurite outgrowth not only

embryonic SAG, but also postnatal SG. Immunostaining of the SG explants from WT P4 and the neurite outgrowth for Tuj1 confirmed that both were neurons (Figure 2.16-C).



OC and SG from WT at postnatal day 3 (P3) (after 5 days)

OC from MIFKO and SG from WT at P3 (After 5 days)

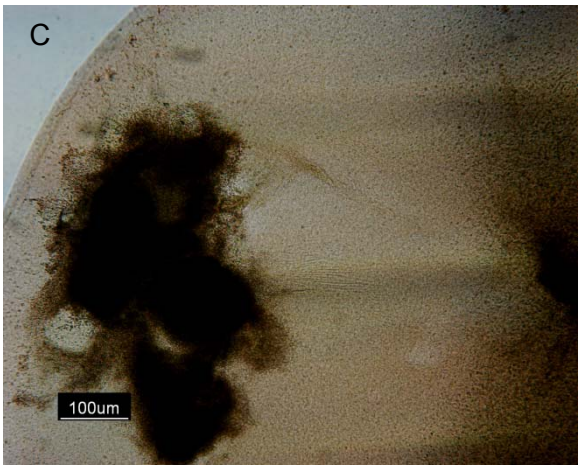


Figure 2.16: Co-culture assays of OC with SG. Freshly excised OC from P3 WT (A) and P3 MIF KO mice (B) are cultured with freshly excised SG from P3 WT mice. Directional SG neurite outgrowth is seen toward the WT mouse cochlea (A). Only random neurite extension that does not reach the OC is observed in cultures of WTSG and cochleae from MIF KO mice (B). The SG explants from WT P4 mouse are stained for Tuj1 (C). The explants show SG neurite outgrowth toward WT OC.

Discussion

The role of classic “neurotrophic factors”, in the development of the inner ear has been studied extensively (Malgrange et al., 1996, Staecker et al., 1996, Marzella et al., 1997, Ylikoski et al., 1998, Tessarollo et al., 2004). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) (Malgrange et al., 1996, Staecker et al., 1996, Tessarollo et al., 2004) and glial cell line-derived neurotrophic factor (GDNF) (Ylikoski et al., 1998) in the development and maintenance of the SAG has been a focal point of the research in many laboratories interested in both ganglionic neuronal development and regeneration. However, there is mounting evidence from a great many laboratories, including ours, that these factors have little to do with the very early stages of SAG development. Our research has identified the major bioactive components of ODF, which provides the earliest trophic cues to developing SAG neurons. These components are immune system cytokines, rather than “classical” neurotrophins. These cytokines are crucial for directional neurite outgrowth and neuron survival in the early inner ear (see also Bianchi et al., 2005). MIF and MCP1 are produced in otic crest cells of the developing otocyst (data not shown). As the otic epithelium matures, MIF begins to be produced by SC and MCP1 by the sensory HC of the inner ear (Figure 2.9).

The receptors for both of these cytokines [CD74 for MIF and CCR2 for MCP1 (Bianchi et al., 2005)] are expressed on the neuroblast on neurons of the SAG and, most significantly on adult SGNs.

We have additional evidence that MIF can also direct the development of mouse embryonic stem cells into neurons, a finding that could be used in repair processes when these neurons are lost or damaged. Furthermore, MIF is involved in sensory HC development, probably through its influence on SC development. We and others (Fekete et al., 1998), hypothesize that SC and HC come from a common precursor during development. What is even more important is that the findings of this study—that "inflammatory" cytokines act as neurotrophins in the developing nervous system—are not only relevant to an understanding of SAG development in the inner ear, but are of general relevance to the development of all neural and sensory systems (Barald and Bianchi, in preparation).

MIF is the most extensively investigated of the ODF cytokines in nervous system development and regeneration. MIF is expressed in the developing nervous system, eye and inner ear of *Xenopus* (Suzuki et al., 2004), mouse (Kobayashi et al., 1999) and zebrafish (Ito et al., 2008). *MIF* mRNA is expressed in the mouse developing inner ear at embryonic day 9.5, just prior to neural innervation (Kobayashi et al., 1999), and we have found that it is expressed in SC in the adult mouse (Figure 2.9).

Exposing isolated SAGs to exogenous recombinant MIF (from either a mouse or a human source) promotes neurite outgrowth and survival comparable to that observed with active ODF fractions. Addition of function-blocking MIF antibodies blocks ODF-induced SAG neurite outgrowth and survival. Taken

together, these results suggest that cytokines/ chemokines play an important role in otic innervation. However, concentrations of MIF that have positive effects on the survival of dissociated neurons of the SAG are different from those that affect neurite outgrowth from whole ganglia (Figure 2.3). In the dissociated cell assays of survival, the amount of MIF in the culture equally reaches each cell. Therefore, lower concentrations of MIF have adverse effects on cell survival. In the cultures of the whole ganglia, however, cells are clustered together and other cell types, including glial cells are present in the undissociated ganglia. Not only does the full effect of the MIF in the medium not reach every cell, but because other cell types are present, several explanations for both the effects and the variability can be offered. First, the effects on the neurons in the whole explant cultures could be mediated through effects of cytokines on the glial cells. The effects on the neurons could be both primary (direct effects of MIF) and secondary effects (through the effects of MIF on the glial cells and glial cell production of factors. Second, Schwann cells themselves produce MIF (Huang et al., 2002a, Huang et al., 2002b, Nishio et al., 2002), of unknown concentration, so that the final concentration of MIF in the cultures could be quite different from that which is exogenously added. Although the effects of MIF on neurite outgrowth in “whole” ganglia, appear to be biphasic, (Figure 2.3a), increasing concentrations of MIF have increasingly inhibitory effects on dissociated neuronal survival (Figure 2.3b). We also found this to be true of MCP1; increasing concentrations of rMCP1 had similarly inhibitory effects on SAG neuronal survival (Bianchi et al., 2005). We must not lose sight of the fact that MIF is not the only cytokine in ODF; nor is it

the only cytokine made by Schwann cells/glial cells. MCP1 is also found in ODF and is made by Schwann cells/Glial cells (Vincent et al., 2005).

Such cytokines are potentially interactive, even synergistic at specific concentrations (Wu and Bradshaw, 1996, Kavanaugh, 2002). We have not yet explored this relationship between, for example, MIF (made by SC) and MCP1 (made by HC) in any detail. If it proves to be the case, as we and others speculate that SC and HC are derived from a common precursor cell, the expression patterns of cytokines in the postulated precursor cells and the differentiating "daughters" could become a focus of future work. In addition, the MIF KO mouse appears to form a relatively "normal" inner ear (Figure 2.14). A careful developmental study of the developing inner ear of these animals must be done, particularly in tracing the expression patterns of other cytokines known to be present in ODF (in addition to MCP1, RANTES and TNFα are present in ODF; Bianchi et al, 2005). These cytokines are both (Chow et al., 2001)binders to other cytokine receptors (Chow et al., 2001, Budagian et al., 2005) and can have synergistic effects (Kadokami et al., 2001, Telekes et al., 2005, Hirano et al., 2007); the absence of MIF could well be compensated for by the presence of these additional cytokines. Cytokines are well known to have "knife-edge" differences between concentrations that have positive and negative effects. This is true of their effects on neuronal regeneration (Liefner et al., 1998) and in the immune system (Kleemann et al., 2000). The variability we saw in the neurite outgrowth assays (Figure 2.3) could be due to cytokine accessibility issues in which the "actual" concentration of the cytokine seen by individual neurons in the

explanted ganglion (and how many of these neurons are affected/exposed) differs appreciably from ganglion to ganglion. Correlating ganglionic dimensions with outgrowth could possibly clarify some of these issues, since presumably the larger the diameter of the explanted ganglion, the greater the surface area and the greater the accessibility of neurons on the outside to the cytokine in the medium. Cytokines are also notoriously "sticky" and adhere to plastic, substrates (natural and artificial).

If MIF and MCP1 specifically regulate SC and HC innervation respectively, one would expect to find MIF expression in SC and MCP1 in HC, as we do. In addition, their receptors should be present on the immature SAG neurons and the possibly the mature SG. Immunohistochemistry demonstrates that MIF is expressed in SC and MCP1 in the inner and outer HC of 4wk old WT mice. RT-PCR shows the presence of a MIF receptor, CD74, and the MCP1 receptor CCR2 in the developing SAG (data not shown), suggesting a capability of HC and SC to secrete, and the SAG to detect and migrate toward, the source of these chemokines, the otic crest in the otocyst and the HC/SC in the maturing inner ear. Adult SGNs also express these receptors, opening a promising avenue for auditory system regeneration and possibly cochlear prostehsis/implant potentiation.

It is highly likely that other chemokines and their receptors are involved with cochlear innervation as well. The receptors for cytokines identified in ODF, including RANTES (receptors are CCR1 and additional receptors for MIF

(CXCR4/CXCR2) (Bernhagen et al., 2007) and TNF-α (TNFR1/TNFR2) (Zou et al., 2005), are all expressed in developing and adult otic tissue. Determining the interactions among these chemokines and their receptors will be crucial to determining the exact mechanisms by which these factors direct cochlear innervation.

Not only are these chemokines expressed in the ear and the ganglion, but the absence of these factors results in profound hearing loss in KO mice. ABR testing done on 4wk, 8wk, and 12 wk old MIF, MCP1, CCR2 (MCP1 receptor) KO and WT mice indicated a profound hearing loss at the high frequency region of the cochlea at 48kHz as early as 4wks in all the KO mice tested (both cytokine [MIF and MCP1] and cytokine R [CCR1, CCR5, CCR2] KO mice). After ABR testing, analysis of the innervation and cochlear structure of MIF and MCP1 KO mice demonstrated abnormal auditory nerve structure as well as some missing IHC and OHC. Rosenthal's canal was frequently disorganized when compared to Balb/c control tissues. The absence of functional MIF and/or MCP1 proteins during development produces structural and physiological abnormalities, supporting the hypothesis that these chemokines play a crucial role in recruiting SG neurons to allow for proper innervation of HC, at least in the high frequency basal turn of the cochlea. This research provides the first direct link between chemokine secretion and innervation of the inner ear. Discovering that cytokines play important roles in development of the inner ear is fully in accord with a major new focus in cytokine biology and neuroimmunology (Siemion et al., 2005, Wrona, 2006). Cytokines and chemokines (cytokines with known attractant

properties) were initially identified and characterized for their critical roles in the immune system, particularly in mediating T cell function (Gonzalo et al., 1996). Cytokines/chemokines are relatively small molecular weight proteins initially identified for their ability to attract and activate immune cells by interacting with specific cytokine receptors (Coughlan et al., 2000). Recent studies have demonstrated that cytokines/chemokines are also present in **developing and adult neural systems**. These proteins are now being implicated in a variety of roles including neuronal migration, outgrowth, and survival (Meng et al., 1999, Bajetto et al., 2001, Chalasani et al., 2003, Belmadani et al., 2005). In some cases, chemokines were found to regulate axonal outgrowth by inhibiting the repellent effects of other molecules such as semaphorins and slit-2 (Chalasani et al., 2003). Not only are cytokines/chemokines absolutely required for neuronal development (Suzuki et al., 2004), they are also critical in neuronal repair processes after axotomy in both the peripheral and central nervous systems (Bajetto et al., 2001, Koda et al., 2004). Our finding that immune system "inflammatory" cytokines behave as neurotrophins in the early developing nervous system indicates that, evolutionarily and developmentally, such cytokines are multifaceted and pleiotrophic.

In the future, exogenous MIF or MCP1 expression in the adult cochlea could be used to reinnervate damaged cochleae and restore hearing. Neuron loss is a significant contributing factor in hearing loss and is notable in ageing human populations. Cochlear implants, presently the only treatment for many forms of deafness, depend on the preservation of the auditory system's SGN.

The larger the numbers of functional SGNs remaining at the time of cochlear implant the greater the probability of success in restoring function (Altschuler et al., 2008). MIF could therefore play a significant role in SGN maintenance or regeneration in therapeutic efforts to maintain or restore hearing function.

It is possible that other chemokines in secreted ODF are required for innervation in the regions of the cochlea required for low and mid-frequency hearing. One can envision a tonotopic map that correlates individual chemokine and/or neurotrophin expression with defined frequency intervals. If such a patterning mechanism exists, it would provide a very detailed mechanism for therapeutic regeneration of HC in specific regions of the cochlea.

References

Altschuler RA, O'Shea KS, Miller JM (Stem cell transplantation for auditory nerve replacement. Hear Res 242:110-116.2008).

Ard MD, Morest DK, Hauger SH (Trophic interactions between the cochleovestibular ganglion of the chick embryo and its synaptic targets in culture. Neuroscience 16:151-170.1985).

Armstrong BD, Hu Z, Abad C, Yamamoto M, Rodriguez WI, Cheng J, Tam J, Gomariz RP, Patterson PH, Waschek JA (Lymphocyte regulation of neuropeptide gene expression after neuronal injury. J Neurosci Res 74:240-247.2003).

Bajetto A, Bonavia R, Barbero S, Florio T, Schettini G (Chemokines and their receptors in the central nervous system. Front Neuroendocrinol 22:147-184.2001).

Barald KF, Lindberg KH, Hardiman K, Kavka AI, Lewis JE, Victor JC, Gardner CA, Poniatowski A (Immortalized cell lines from embryonic avian and murine otocysts: tools for molecular studies of the developing inner ear. Int J Dev Neurosci 15:523-540.1997).

Belmadani A, Tran PB, Ren D, Assimacopoulos S, Grove EA, Miller RJ (The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J Neurosci 25:3995-4003.2005).

Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C (MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med 13:587-596.2007).

Bianchi E, Denti S, Granata A, Bossi G, Geginat J, Villa A, Rogge L, Pardi R (Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. Nature 404:617-621.2000).

Bianchi LM, Cohan CS (Developmental regulation of a neurite-promoting factor influencing statoacoustic neurons. Brain Res Dev Brain Res 64:167-174.1991).

Bianchi LM, Cohan CS (Effects of the neurotrophins and CNTF on developing statoacoustic neurons: comparison with an otocyst-derived factor. Dev Biol 159:353-365.1993).

Bianchi LM, Daruwalla Z, Roth TM, Attia NP, Lukacs NW, Richards AL, White IO, Allen SJ, Barald KF (Immortalized mouse inner ear cell lines demonstrate a role for chemokines in promoting the growth of developing statoacoustic ganglion neurons. J Assoc Res Otolaryngol 6:355-367.2005).

Bianchi LM, Dolnick R, Medd A, Cohan CS (Developmental changes in growth factors released by the embryonic inner ear. Exp Neurol 150:98-106.1998).

Bianchi LM, Huri D, White IO (Embryonic inner ear cells use migratory mechanisms to establish cell patterns in vitro. J Neurosci Res 83:191-198.2006).

Bianchi LM, Person AL, Penney EB (Embryonic inner ear cells reaggregate into specific patterns in vitro. J Assoc Res Otolaryngol 3:418-429.2002).

Budagian V, Bulanova E, Orinska Z, Thon L, Mamat U, Bellosta P, Basilico C, Adam D, Paus R, Bulfone-Paus S (A promiscuous liaison between IL-15 receptor and Axl receptor tyrosine kinase in cell death control. EMBO J 24:4260-4270.2005).

Campos-Barros A, Amma LL, Faris JS, Shailam R, Kelley MW, Forrest D (Type 2 iodothyronine deiodinase expression in the cochlea before the onset of hearing. Proc Natl Acad Sci U S A 97:1287-1292.2000).

Chalasani SH, Baribaud F, Coughlan CM, Sunshine MJ, Lee VM, Doms RW, Littman DR, Raper JA (The chemokine stromal cell-derived factor-1 promotes the survival of embryonic retinal ganglion cells. J Neurosci 23:4601-4612.2003).

Chen FQ, Schacht J, Sha SH (Aminoglycoside-induced histone deacetylation and hair cell death in the mouse cochlea. J Neurochem 108:1226-1236.2009).

Chow D, He X, Snow AL, Rose-John S, Garcia KC (Structure of an extracellular gp130 cytokine receptor signaling complex. Science 291:2150-2155.2001).

Coughlan CM, McManus CM, Sharron M, Gao Z, Murphy D, Jaffer S, Choe W, Chen W, Hesselgesser J, Gaylord H, Kalyuzhny A, Lee VM, Wolf B, Doms RW, Kolson DL (Expression of multiple functional chemokine receptors

and monocyte chemoattractant protein-1 in human neurons. Neuroscience 97:591-600.2000).

Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R (The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol 87:27-45.1985).

Fekete DM, Muthukumar S, Karagogeos D (Hair cells and supporting cells share a common progenitor in the avian inner ear. J Neurosci 18:7811-7821.1998).

Germiller JA, Smiley EC, Ellis AD, Hoff JS, Deshmukh I, Allen SJ, Barald KF (Molecular characterization of conditionally immortalized cell lines derived from mouse early embryonic inner ear. Dev Dyn 231:815-827.2004).

Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez AC, Siegelman MH, Cybulsky M, Gutierrez-Ramos JC (Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. J Clin Invest 98:2332-2345.1996).

Hirano Y, Shichijo M, Deguchi M, Nagira M, Suzuki N, Nishitani Y, Hattori M, Arimura A (Synergistic effect of PGD2 via prostanoid DP receptor on TNF-alpha-induced production of MCP-1 and IL-8 in human monocytic THP-1 cells. Eur J Pharmacol 560:81-88.2007).

Huang T, Qin J, Xiong S, Yu L, Huo X, Liao H, Li J, Liu D ([Expression of macrophage migration inhibitory factor mRNA in Schwann cells]. Zhonghua Wai Ke Za Zhi 40:699-701.2002a).

Huang T, Qin JQ, Huo XK, Yu L, Xiong SH, Liu DY, Zhong SZ (Changes in content of macrophage migration inhibitory factor secreted by Schwann cells after peripheral nerve injury. Di Yi Jun Yi Da Xue Xue Bao 22:493-495.2002b).

Ito K, Yoshiura Y, Ototake M, Nakanishi T (Macrophage migration inhibitory factor (MIF) is essential for development of zebrafish, Danio rerio. Dev Comp Immunol 32:664-672.2008).

Kadokami T, McTiernan CF, Kubota T, Frye CS, Bounoutas GS, Robbins PD, Watkins SC, Feldman AM (Effects of soluble TNF receptor treatment on lipopolysaccharide-induced myocardial cytokine expression. Am J Physiol Heart Circ Physiol 280:H2281-2291.2001).

Kavanaugh A (Combination cytokine therapy: the next generation of rheumatoid arthritis therapy? Arthritis Rheum 47:87-92.2002).

Kleemann R, Hausser A, Geiger G, Mischke R, Burger-Kentischer A, Flieger O, Johannes FJ, Roger T, Calandra T, Kapurniotu A, Grell M, Finkelmeier D, Brunner H, Bernhagen J (Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. Nature 408:211-216.2000).

Kobayashi S, Satomura K, Levsky JM, Sreenath T, Wistow GJ, Semba I, Shum L, Slavkin HC, Kulkarni AB (Expression pattern of macrophage migration inhibitory factor during embryogenesis. Mech Dev 84:153-156.1999).

Koda M, Nishio Y, Hashimoto M, Kamada T, Koshizuka S, Yoshinaga K, Onodera S, Nishihira J, Moriya H, Yamazaki M (Up-regulation of macrophage migration-inhibitory factor expression after compression-induced spinal cord injury in rats. Acta Neuropathol 108:31-36.2004).

Lefebvre PP, Leprince P, Weber T, Rigo JM, Delree P, Moonen G (Neuronotrophic effect of developing otic vesicle on cochleo-vestibular neurons: evidence for nerve growth factor involvement. Brain Res 507:254-260.1990).

Liefner M, Maruschak B, Bruck W (Concentration-dependent effects of pentoxifylline on migration and myelin phagocytosis by macrophages. J Neuroimmunol 89:97-103.1998).

Malgrange B, Lefebvre P, Van de Water TR, Staecker H, Moonen G (Effects of neurotrophins on early auditory neurones in cell culture. Neuroreport 7:913-917.1996).

Marzella PL, Clark GM, Shepherd RK, Bartlett PF, Kilpatrick TJ (LIF potentiates the NT-3-mediated survival of spiral ganglia neurones in vitro. Neuroreport 8:1641-1644.1997).

Meng SZ, Oka A, Takashima S (Developmental expression of monocyte chemoattractant protein-1 in the human cerebellum and brainstem. Brain Dev 21:30-35.1999).

Nishio Y, Nishihira J, Ishibashi T, Kato H, Minami A (Role of macrophage migration inhibitory factor (MIF) in peripheral nerve regeneration: anti-MIF antibody induces delay of nerve regeneration and the apoptosis of Schwann cells. Mol Med 8:509-520.2002).

Roth TM, Ramamurthy P, Ebisu F, Lisak RP, Bealmear BM, Barald KF (A mouse embryonic stem cell model of Schwann cell differentiation for studies of the role of neurofibromatosis type 1 in Schwann cell development and tumor formation. Glia 55:1123-1133.2007).

Saul SM, Brzezinski JAt, Altschuler RA, Shore SE, Rudolph DD, Kabara LL, Halsey KE, Hufnagel RB, Zhou J, Dolan DF, Glaser T (Math5 expression and function in the central auditory system. Mol Cell Neurosci 37:153-169.2008).

Siemion IZ, Kluczyk A, Cebrat M (The peptide molecular links between the central nervous and the immune systems. Amino Acids 29:161-176.2005).

Staecker H, Galinovic-Schwartz V, Liu W, Lefebvre P, Kopke R, Malgrange B, Moonen G, Van De Water TR (The role of the neurotrophins in maturation and maintenance of postnatal auditory innervation. Am J Otol 17:486-492.1996).

Suzuki M, Takamura Y, Maeno M, Tochinai S, Iyaguchi D, Tanaka I, Nishihira J, Ishibashi T (Xenopus laevis macrophage migration inhibitory factor is essential for axis formation and neural development. J Biol Chem 279:21406-21414.2004).

Telekes A, Kiss-Toth E, Nagy T, Qwarnstrom EE, Kusz E, Polgar T, Resetar A, Dower SK, Duda E (Synergistic effect of Avemar on proinflammatory cytokine production and Ras-mediated cell activation. Ann N Y Acad Sci 1051:515-528.2005).

Tessarollo L, Coppola V, Fritzsch B (NT-3 replacement with brain-derived neurotrophic factor redirects vestibular nerve fibers to the cochlea. J Neurosci 24:2575-2584.2004).

Thompson DL, Gerlach-Bank LM, Barald KF, Koenig RJ (Retinoic acid repression of bone morphogenetic protein 4 in inner ear development. Mol Cell Biol 23:2277-2286.2003).

Vincent AJ, Taylor JM, Choi-Lundberg DL, West AK, Chuah MI (Genetic expression profile of olfactory ensheathing cells is distinct from that of Schwann cells and astrocytes. Glia 51:132-147.2005).

Wrona D (Neural-immune interactions: an integrative view of the bidirectional relationship between the brain and immune systems. J Neuroimmunol 172:38-58.2006).

Wu YY, Bradshaw RA (Synergistic induction of neurite outgrowth by nerve growth factor or epidermal growth factor and interleukin-6 in PC12 cells. J Biol Chem 271:13033-13039.1996).

Ylikoski J, Pirvola U, Virkkala J, Suvanto P, Liang XQ, Magal E, Altschuler R, Miller JM, Saarma M (Guinea pig auditory neurons are protected by glial cell line-derived growth factor from degeneration after noise trauma. Hear Res 124:17-26.1998).

Zou J, Pyykko I, Sutinen P, Toppila E (Vibration induced hearing loss in guinea pig cochlea: expression of TNF-alpha and VEGF. Hear Res 202:13-20.2005).

Moore D and McCabe G (Introduction to the practice of statistics. 5th edition, 2004).

This chapter is being submitted for publication, with the same title as the chapter, but with the following authors: Lisa M. Gerlach-Bank*[1,6], Fumi Ebisu*[1,6], Dov Lerman-Sinkoff[1,3], Elizabeth C. Smiley[1], Yu-chi Shen[1], Poornapriya Ramamurthy[1,3], Deborah L. Thompson[1], Therese M. Roth[1], Christine R. Beck[1], Matthew Flynn[1], Ryan S. Teller[1], Luming Feng[1], G. Nicholas Llewellyn[1], Katie E. Holmes[1], Brandon Holmes[2], Cyrrene Sharples[2], Jaeda Coutinho[2], Stephanie A. Linn[1], Andrew P. Chervenak[1], David F. Dolan[4], Jennifer Benson[4], Ariane Kanicki[4], Richard Altschuler[4], Alicia Koch[7], Ethan M. Jewett[1, 8], John A. Germiller[5], Lynne M. Bianchi[1,2] and Kate F. Barald[1,3, 6]

- Joint first authors

1. Department of Cell and Developmental Biology, University of Michigan Ann Arbor MI 48109-2200
2. Department of Biology, Oberlin College, Oberlin OH
3. Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, MI
4. Kresge Hearing Research Institute, Department of Otolaryngology, Head and Neck Surgery, University of Michigan Medical School, Ann Arbor, MI,
5. Children's Hospital of Philadelphia, Department of Pediatric Otolaryngology and University of Pennsylvania
6. Program in Neuroscience University of Michian, Ann Arbor, MI
7. Department of Rheumatology, University of Michigan, Ann Arbor, MI
8. Bioinformatics Program, Center for Computational Biology and Medicine, University of Michigan Medical School, University of Michigan, Ann Arbor, MI

Chapter III

Conclusions and future work

Sensorineural hearing loss (SNHL) is the most common form of deafness in humans and derives, in most cases, either from primary degeneration of the spiral ganglion neurons (SGNs) or from secondary degeneration of these neurons, due to the loss of cochlear sensory hair cell (Martinez-Monedero et al., 2006, Friedman and Avraham, 2009). Cochlear implants (CI) are currently the only treatment for SNHL (Roehm and Hansen, 2005, Altschuler et al., 2008, Shibata et al., 2010). CI provide an increasingly successful therapy to restore hearing, particularly speech recognition, for those suffering from profound deafness (Altschuler et al., 2008, Shibata et al., 2010). Nevertheless, the efficacy of these cochlear prostheses is dependent upon the functionality of residual neurons and the ability of the CI to stimulate those neurons, so that the presence of some conserved nerve functionality is essential for CI functionality (Altschuler et al., 2008, Serin et al., 2009, Shibata et al., 2010). Preserving SGN or finding a cellular replacement for lost or damaged SGN therefore is a priority if CI function is to be enhanced. Ultimately, inducing restoration of hearing function through regeneration of hair cells (HC), neurons or both would be a more satisfactory way to resolve this problem. Nevertheless, despite a great deal of work in many laboratories (Shibata et al., 2010), finding a stem cell source in the

mammalian inner ear to replace lost cell population has been unsuccessful. Such a population/source could be used to induce regeneration, either through transdifferentiation of supporting cells (SC) or other cell types into HC or by replacing lost or damaged cell populations with appropriately differentiated stem cells (Hernandez et al., 2007, Hori et al., 2007, Diensthuber et al., 2009, Oshima et al., 2009).

Innervation target-derived or cellular pathway-derived growth and survival factors are essential for the normal development and survival of neurons (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993, Bianchi et al., 1998, Bianchi et al., 2005). In the developing inner ear, the otocyst is the precursor of the inner ear sensory organs as well as the innervations of the sensory organs (Barald and Kelley, 2004). Early studies in both the rat (embryonic day (E)11-14) and the chick (E4-6) demonstrated that during the early stages of auditory development, cells in the antero-ventral region of the otocyst secrete soluble and diffusible factor(s), which have been collectively termed Otocyst Derived Factor(s) (ODF), which influence both directional neurite outgrowth and survival (the definition of a “neurotrophin”) of neurons, which are the precursor to both the auditory ganglion and the vestibular ganglion in the embryonic statoacoustic ganglion (SAG) (Bianchi and Cohan, 1991, 1993). The identity/identities of the biologically active components of ODF had not been fully characterized until our recent proteomic studies identified the bioactive components of ODF as immune system cytokines, including macrophage

migration inhibitory factor (MIF) (Gerlach-Bank, Ebisu et al, in preparation), Monocyte chemoattractant protein 1 (MCP-1, also called JE or CCL2) (Bianchi et al., 2005), gamma interferon, TNF-alpha and RANTES. However, of all of these cytokine components found in ODF, only MIF acts as a sufficient and necessary molecule that promotes both SAG neurite outgrowth and the survival of dissociated SAG neurons (Gerlach-Bank, Ebisu et al, in preparation).

MIF is a ubiquitously expressed "pleiotropic" cytokine since it plays multiple roles including in the immune system and the pathogenesis and progression of inflammatory diseases (Bernhagen et al., 1993, Calandra and Roger, 2003, El-Turk et al., 2008). Studies demonstrated that MIF is implicated in carcinogenesis and demonstrate a link between chronic inflammation and various types of cancers (Lue et al., 2007). More recently, several studies found vital additional roles for MIF in the early development (neurulation) as well as repair mechanisms of peripheral nerves and skin after injury by enhancing cell proliferation and migration (Nishio et al., 1999, Abe et al., 2000, Nishio et al., 2002, Suzuki et al., 2004, Shimizu, 2005, Dewor et al., 2007, Velnar et al., 2009).

Based on all of the intriguing roles that MIF plays in neurulation, neuronal development, pathfinding and cell migration as well as the discovery that MIF is a major component of the otocyst-generated ODF, we hypothesized that MIF plays a key instructional role in inner ear neuronal and sensory cell development. The objective of this dissertation was to determine if there was a role for MIF in inner

ear neuronal and sensory cell development using chicks and mice as model systems both *in vitro* and *in vivo*.

As mentioned previously, the otocyst secretes ODF that supports the neurite outgrowth and survival of SAG neurons. Because so little ODF can be obtained from the embryonic otocyst, our laboratory developed immortalized otocyst cell lines from the E9.5 Immortomouse otocyst (IMO) cells (Barald et al., 1997). These early studies found that IMO cells secrete a functionally bioactive ODF equivalent (Thompson et al., 2003, Germiller et al., 2004, Bianchi et al., 2005). Neuronal culture bioassays with SAG explants in the presence of IMO-generated ODF, conditioned medium (CM) from IMO cells cultured in basal medium (serum-free without γ-interferon), and otocyst-generated ODF, CM produced by incubating E 4 or E5 chick otocysts in the same basal medium, demonstrated that both ODFs promoted the extension of neurites from ganglionic explants and the survival of dissociated SAG neurons significantly compared to control (basal medium), confirming that IMO-generated ODF is “bioequivalent” to otocyst-generated ODF. Moreover, these results demonstrate that IMO-generated ODF can be used for further experiments to characterize ODF bioactivity. The identified active IMO-generated ODF and inactive CM from IMO cell lines without ODF activity were subjected to proteomic analysis. MIF was found in the bioactive ODF samples from both mouse and chick otocysts (data not shown), as well as MCP1 (Bianchi et al., 2005). Following the identification of MIF and MCP1, we quantified the concentrations of MIF and MCP1 both in IMO- and otocyst-generated ODFs. We found that the concentrations MIF and

MCP1 were very similar in both ODFs, in a range of 1.0 to 2.5 ng/ml. This is similar to the concentration of MIF produced by the rat spinal cord neurons (Chalimoniuk et al., 2006).

In the neuronal bioassays with mouse or human recombinant MIF (rMIF), we found that rMIF mimics the bioactivity of ODF in a dose-dependent manner. Concentrations of rMIF at 5 pg/ml and 5 ng/ml significantly enhanced chick SAG neurite outgrowth, while 50 pg/ml of rMIF also significantly promoted SAG neuronal survival. In contrast, higher concentrations (e.g.500 ng/ml) of rMIF had no effect or inhibited both SAG neurite outgrowth and survival. Lue et al. demonstrated the dose-dependent rMIF bioactivity in ERK signaling activation, where rMIF activated ERK phosphorylation in fibroblast at lower concentrations in the range of 50-100 ng/ml, while higher concentrations exceeding 1000 ng/ml were inhibitory in these cells (Lue et al., 2006), supporting our findings that too high a concentration is inhibitory and injurious. This finding presents a caveat to any proposed MIF-inducible regenerative attempts to restore functional connections in the adult mammalian inner ear using MIF, since rather small increases in MIF could prove deleterious and induce an inflammatory response that could do irreparable damage to the neuronal cells in the spiral ganglion (SG) that continue to carry CD74 receptors on their cell surfaces into adulthood (Gerlach-Bank, Ebisu et al., in preparation). We observed significant SG neurite outgrowth in the presence of 50 ng/ml of rMIF compared to control, indicating that rMIF at this range can still promote neurite outgrowth not only in the embryonic SAG stage but also in the postnatal SG stage.

In addition, we examined MIF's bioactive effect on cultures of mouse embryonic stem (mES) cells. We found that concentrations as low as 1 pg/ml of rMIF induced a subset of the cells to become neurofilament positive cells with a neuron-like morphology, further demonstrating a role for this cytokine in neuronal development. Studies to examine how closely these MIF-induced ES-derived neurons resemble early or mature inner ear neurons both chemically and physiologically are underway (Ramamurthy et al, in preparation). To further explore the role of MIF in neuronal development, we performed functional-blocking assays using anti-MIF antibodies on SAG neurons. Blockade of MIF activity reduced MIF-induced SAG neurite outgrowth to background levels. The antibody also reduced MIF-induced SAG neuronal survival to basal levels, whereas some residual neuronal survival was seen after treatment of IMO-generated ODF with this function-blocking antibody, indicating that additional factor(s), which we speculate to be MCP1, in the ODF also play a role in inner ear neuronal development. Together, we demonstrated that MIF appears to be both necessary and sufficient to promote outgrowth and survival of inner ear neurons, from early to late stages.

We detected the expression of MIF in the mouse inner ear at both the mRNA and protein levels in sensory epithelial regions of both the developing and mature inner ear. A group detected MIF mRNA expression in the otic vesicle/otocyst at E9.5 (Kobayashi et al., 1999). We detected MIF protein expression in IMO cells, which were isolated from E9.5 Immortomouse otocysts. At later embryonic stages, we detected MIF mRNA and protein in inner ear

tissues. We also found MIF mRNA in the adult inner ear tissues, including the cochlea. Immunostaining assays with adult inner ear tissues demonstrated the expression of MIF and MCP1 proteins in the SC and HC respectively. It has been suggested that hair cells possess a migratory mechanism to help achieve their final position in the organ of Corti (Bianchi et al., 2002, Bianchi et al., 2006). An *in vitro* assay in which dissociated embryonic inner ear otocyst cells reaggregate in culture to form structures called “ear domes” (Bianchi et al., 2006) demonstrated that MIF activity segregated with the cytokeratin-labeled supporting cells and that MCP1 labeling segregated with the Myosin 7a-expressing HC . Our previous study demonstrated the co-expression of MCP1 with myosin 7A, a HC marker, in dissociated embryonic inner ear cells, as well as in “domes”. The directional chemotactic activity of MCP1 towards isolated human blood monocytes and the ability of IMO-generated ODF to mimic this activity were also demonstrated in the earlier study (Bianchi et al., 2005). In the studies described in this dissertation, we used immunohistochemistry to localize cytokeratin, a SC marker, to the outer border of SC, while myosin 7A labeled HC in the centrally located region above the plane of the SC. MIF was co-localized to the cytoplasm in the SC of the dome, with cytokeratin forming the cellular borders. In contrast, the central regions of the domes, that contain HC, were not labeled with MIF antibodies. These results provide further evidence for cell specific expression patterns of MIF in SC and MCP1 in HC, as well as an ability of dissociated HC and SC to recapitulate an otocyst like pattern through

reaggregation and sorting in a tissue culture dish (Bianchi et al, 2006; Bianchi et al, 2005; Gerlach-Bank, Ebisu et al, in preparation).

In characterization of MIF knock-out (KO) mice, we found that MIF KO mice developed hearing impairment at 4 weeks of age, with the most profound hearing loss at 48 kHz. The lower average density of SG neurons was also observed in the P1 (12 kHz) and P3 (48 kHz) turns of the cochlea in MIF KO mice compared to wild-type (WT) mice at 4 weeks of age. Immunostaining assays showed that both inner hair cells (IHC) and outer hair cells (OHC), as well as SC were missing some, but not all, in MIF KO mice at 4 weeks of age. Cochleograms and an anatomical analysis demonstrated that MIF KO mice exhibited disorganized auditory nerve structure, with some OHC and IHC missing, suggesting the requirement of MIF expression for normal development and patterning of HC and SC, as well as normal neuronal development. Furthermore, co-culture assays of post-natal organ of Corti explants, which expressed MIF, with SGN explants compared with the same WT explants and organ of Corti from MIF KO animals demonstrated an ongoing role of MIF in the directional neurite outgrowth of mature inner ear neurons toward the cochlea. Taken together, these results suggest that normal expression of MIF is essential for normal physiological and morphological inner ear development and innervation.

Future studies will focus on the effects of MCP1 and its possible synergy with MIF in MIF-induced SAG/SG neurite outgrowth and survival. Our previous study demonstrated that MCP1 alone had little effect on SAG neurite outgrowth

(Bianchi et al., 2005). However, blockade of MCP1 bioactivity in IMO-generated ODF reduced ODF-induced SAG neurite outgrowth and the addition of recombinant MCP1 (rMCP1, 20 ng/ml) significantly remedied the inhibition of the outgrowth; however, too much rMCP1 (200 ng/ml) had reduced effects on neurite outgrowth (Bianchi et al., 2005). In the current study, we found that MIF alone enhanced SAG neurite outgrowth, however, the maximum lengths of neurite outgrowth induced by rMIF was less than that induced by ODFs. Furthermore, blockade of MIF activity in ODF failed to completely block ODF-induced SAG neurite outgrowth. These results suggest that another component of ODF, likely MCP1, is capable of interacting with MIF to promote/synergize in SAG outgrowth. We will examine this possibility by performing neuronal outgrowth and survival assays in the presence of rMIF and rMCP1. We will then perform function blocking assay with IMO- and otocyst-generated ODF pretreated with anti-MIF and anti-MCP1 antibodies or RNAi constructs to mouse MIF and MCP1. We have recently found that siRNA against mouse MIF (0.25 μg/ul) significantly reduced MIF expression in IMO cells.

Based on our observation on that MIF KO mice developed hearing impairment at 4 weeks of age, with the most profound hearing loss in the 48 kHz region along with lower SG neuronal density at this region, additional future studies include determining whether there is any potential involvement of MIF in the establishment of the tonotopic map, a sound detection gradient, by immune-labeling of the cochlea for MIF. We detected MIF expression both in the developing and mature inner ear in WT mice. We observed normal HC

development in WT mice, while abnormal HC development in MIF KO mice, suggesting its involvement in HC development/maturation probably through effects on SC. We will perform loss-of-function assays using RNAi knock-down techniques to determine MIFs role in both neuronal and SC/HC development/maturation.

In addition, we will examine the downstream signal pathway of MIF. We observed a dose-dependent bioactivity of rMIF. The lower concentrations of rMIF promoted both SAG neurite outgrowth and survival, while higher concentration was inhibitory. A group found that lower concentrations of MIF activated ERK phosphorylation, while higher concentration inhibited it (Lue et al., 2006). The expression of phosphorylated ERK will be examined after the treatment of inner ear neurons with various concentrations of rMIF. In addition to ERK, Kleemann et al. discovered the direct interaction of MIF with Jab1 when the extracellular MIF concentrations are high (Kleemann et al., 2000). As mentioned previously, Jab1 functions in the activation of AP-1, a transcription factor that activates pro-inflammatory genes, and in promoting the degradation of $p27^{kip1}$, a protein that controls the cell cycle. Many studies (Chen and Segil, 1999, Chen et al., 2003, McKenzie et al., 2004) have implicated $p27^{kip1}$ in inner ear development. We will investigate whether MIF plays a role in $p27^{kip1}$ regulation via Jab1. It is known that the physical interaction of MIF with Jab1 modulates the role of Jab1. Immunostaining assays will be performed to examine whether MIF and Jab1 are co-localized in the presence of high concentrations of rMIF. As mentioned above, an effect of MCP1 was also dose-dependent. This highly concentration

dependent phenomenon in MCP1 needs to be investigated in much more detail as well.

Based on the limited observations discussed above, the possible developmental scenario of the ear in the MIF KO mouse that results in the majority SC being missing in the adult high frequency detecting cochlear regions suggests that they are abnormally developed in the MIF KO mouse. SC and HC are hypothesized to come from the same precursors (Fekete et al., 1998, Rivolta and Holley, 2002), therefore, few or no HC are developed in this region either. The neurotrophins and/or cytokines responsible for neuronal development and for maintenance of neuronal connections are secreted by HC and SC, as described previously. Since some SC are still present in the MIF KO mouse, different neurotrophins(s) must also be secreted from SC in the MIF KO mouse to maintain at least some neuronal survival. However, since there is also HC loss, the auditory nerves, which are known to carry receptors for other neurotrophins and cytokines (Gerlach-Bank, Ebisu et al, in preparation) might not be able to project toward HC to innervate them or the connections, if made, might not be maintained. The consequence is that the MIF KO mouse develops hearing impairment in a specific cochlear region (high frequency). It is certainly possible to speculate that different cytokine/neurotrophin combinations are responsible for establishing and maintaining the tonotopic map. It is possible that different combinations are responsible for the different frequency-detecting regions of the cochlea and that these dependencies are both redundant and

overlapping to some extent. Only careful examination and experimentation will discern whether this is the case.

In this dissertation, we also examined a role of CD74, the most characterized receptor for MIF, in inner ear neuronal development. We detected its expression both in the embryonic SAG neurons and the adult SG neurons in mice at the mRNA level. Blockade of its activity with RNAi significantly, but not completely, reduced chick SAG neurite outgrowth and survival, suggesting that CD74 is required at least for chick embryonic SAG neurite outgrowth and survival.

Further studies will be performed to determine its effect on SAG/SG neurite outgrowth and survival in mice. A recent study identified additional receptors for MIF, CXCR2 and CXCR4 (Bernhagen et al., 2007). The failure of complete blockade of neurite outgrowth and survival with CD74 siRNA suggests the possible involvement of additional receptors and the CXCR receptors are potentially good candidates for this interaction. We will clarify this possibility by assessing their expression both in the embryonic SAG and postnatal/adult SG neurons both in the mouse and the chick. Similar studies are being done in the zebrafish by others in the lab (Shen et al., in preparation). If we detect their expression in SAG/SG, we will then examine their role in inner ear neuronal development and maintenance by functionally blocking their activity using RNAi techniques. Additionally, we will investigate whether damaged SG neurons still retain or are capable of re-expressing the three proposed MIF receptors by

performing immunoassays after the treatment of SG neurons with ototoxic drug(s).

The significance of this project is that these approaches could provide new therapeutic avenues to treat hearing loss. If damaged SG neurons retain or are capable of re-expressing cytokine receptors responsible for SGN survival and outgrowth during development, MIF could be used to maintain SGN survival or potentiate integration of remaining sensory HC with a CI.

Nevertheless, there are a few things that one must include consideration when introducing rMIF and other cytokines.

First, carefully controlled concentrations of cytokines, particularly MIF and MCP1 that do not produce an inflammatory response must be introduced as we observed that the low concentrations of the cytokines have effects on the auditory system, while the high concentrations of those have deleterious effects. Tumor necrosis factor-α (TNF-α), another proinflammatory cytokine, which is known to be released by SC (Satoh et al., 2003), is apparently involved in a positive feedback mechanism with other inflammation-associated cytokines to promote hair cell damage caused by inflammatory cytokines, including some of the IGF factors. However, we believe that those effects are undoubtedly seen at higher concentrations of TNF-alpha and other "inflammatory" cytokines, than the very low concentrations of such cytokines that produce the positive neurotrophic and neurite-outgrowth stimulating effects seen at lower cytokine concentrations. The additional studies by different group support our ideas. One group investigated the expression levels of TNF- α as well as γ -interferon in patients

with mixed connective tissue disease (MCTD) with SNHL and compared these levels with MCTD patients without SNHL (Hajas et al., 2009). The authors found that serum levels of TNF- α and γ -interferon increased in MCTD patients with SNHL compared to those without SNHL (Hajas et al., 2009). Another group demonstrated that blockade of TNF-α with a TNF-α antibody reduced the extent of cochlear injury and hearing loss associated with Streptococcus pneumoniae meningitis in Mongolian gerbils (Aminpour et al., 2005).

Second, these studies also highlight the remarkable similarities and molecular interactions between the developing immune and nervous systems and also underscore the fact that molecules like MIF, which play vital roles in development can also play a role in both disease (e.g. multiple sclerosis) and carcinogenesis (Shimizu et al., 1999, Fingerle-Rowson et al., 2003, Nishihira et al., 2003, Honda et al., 2009). Since many therapies for auto-immune diseases, multiple sclerosis and other inflammatory disorders like arthritis depend on the potential use of anti-MIF function blocking antibodies, these strategies, too must be carefully considered, especially if such treatments or strategies adversely affect adult neuronal populations that respond to MIF or if MIF in low concentrations are needed to maintain a functioning auditory and possibly vestibular system. Anti-cancer drugs like cisplatin and anti-bacterial aminoglycoside antibiotics have severe consequences for hearing; it is possible that anti-MIF therapeutics could have similar deleterious effects.

The additional issue of cytokines and their receptors is their redundancy and promiscuity. Many individual cytokines are themselves pleiotropic and many

of them have overlapping functions (Leonard, 1994, Ozaki and Leonard, 2002). Cytokine pleiotropy and redundancy can be explained by the ability of certain cytokines to signal via more than one type of receptor complex and by an individual receptor component being shared by more than one cytokine (Ozaki and Leonard, 2002). As mentioned above, in the case of MIF, it has a high affinity for CD74 and its binding to CD74 activates the ERK-MAP kinase signaling pathway for cell proliferation. The additional receptors for MIF are CXCR2/4, which MIF acts as a noncognate ligand of these receptors in inflammatory and artherogenic cell recruitment (Bernhagen et al., 2007). The authors observed that blockade of MIF, but not of the canonical ligands of CXCR2 or CXCR4, in mice with advanced atherosclerosis led to plaque regression and reduced monocyte and T-cell content in plaques (Bernhagen et al., 2007). We are presently investigating the role of the CXCR receptors in mif signaling cascades in the zebrafish (Shen et al., in preparation). An additional example of cytokine receptors' promiscuous binding includes IL-10R2 (Hinck, 2010). IL-10R2 is a shared cell surface receptor required for the activation of class II cytokines, such as IL-10, IL-22, IL-26, IL-28 and IL-29, which play critical roles in host defense (Yoon et al., 2010). Yoon et al. (2010) identified IL-10R2 residues and the ligand-specific- ternary complexes with two cytokines, IL-22 and cmvIL-10. There is a key structural determinants shared with class I cytokines, yet promiscuity in binding is seen with the class II cytokines, IL-22 and cmvIL-10. Binding of IL-22 to ligand specific receptor, IL-10R1, and the common receptor, IL-10R2, results in immune suppression and anti-inflammatory sequellae. By

contrast, binding of cmvIL-10 to the ligand specific receptor, IL-22, and the common receptor, IL-10R2, results in acute-phase response and innate immunity.

Finally, how could one introduce rMIF into a diseased or damaged inner ear? Such introduction would be dependent on the condition of SGNs. If many of SGNs survive or are in good condition, one could introduce exogenous MIF into either a gel coating a CI or by "coating" a CI with MIF-producing cells (such as Schwann cells, which produce MIF) to potentiate neuronal contact with the CI. If most of SGNs are lost, it is possible that stem cells induced to become neuron-like through exposure to the "right" concentrations of MIF could be transplanted into the inner ear to potentiate a CI. In such a scenario, the stem cells would be induced pluripotent stem cells, derived from the patient and to be efficacious, these neuron-like cells must a) not become tumors and b) must have the same physiological and molecular properties as the neurons that they might replace. These experiments, determining how inner ear neuron-like the MIF-induced ES cells are, are underway in our laboratory.

References

Abe R, Shimizu T, Ohkawara A, Nishihira J (Enhancement of macrophage migration inhibitory factor (MIF) expression in injured epidermis and cultured fibroblasts. Biochim Biophys Acta 1500:1-9.2000).

Altschuler RA, O'Shea KS, Miller JM (Stem cell transplantation for auditory nerve replacement. Hear Res 242:110-116.2008).

Aminpour S, Tinling SP, Brodie HA (Role of tumor necrosis factor-alpha in sensorineural hearing loss after bacterial meningitis. Otol Neurotol 26:602-609.2005).

Ard MD, Morest DK, Hauger SH (Trophic interactions between the cochleovestibular ganglion of the chick embryo and its synaptic targets in culture. Neuroscience 16:151-170.1985).

Barald KF, Lindberg KH, Hardiman K, Kavka AI, Lewis JE, Victor JC, Gardner CA, Poniatowski A (Immortalized cell lines from embryonic avian and murine otocysts: tools for molecular studies of the developing inner ear. Int J Dev Neurosci 15:523-540.1997).

Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R (MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365:756-759.1993).

Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C (MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med 13:587-596.2007).

Bianchi LM, Cohan CS (Developmental regulation of a neurite-promoting factor influencing statoacoustic neurons. Brain Res Dev Brain Res 64:167-174.1991).

Bianchi LM, Cohan CS (Effects of the neurotrophins and CNTF on developing statoacoustic neurons: comparison with an otocyst-derived factor. Dev Biol 159:353-365.1993).

Bianchi LM, Daruwalla Z, Roth TM, Attia NP, Lukacs NW, Richards AL, White IO, Allen SJ, Barald KF (Immortalized mouse inner ear cell lines demonstrate a role for chemokines in promoting the growth of developing statoacoustic ganglion neurons. J Assoc Res Otolaryngol 6:355-367.2005).

Bianchi LM, Dolnick R, Medd A, Cohan CS (Developmental changes in growth factors released by the embryonic inner ear. Exp Neurol 150:98-106.1998).

Bianchi LM, Huri D, White IO (Embryonic inner ear cells use migratory mechanisms to establish cell patterns in vitro. J Neurosci Res 83:191-198.2006).

Bianchi LM, Person AL, Penney EB (Embryonic inner ear cells reaggregate into specific patterns in vitro. J Assoc Res Otolaryngol 3:418-429.2002).

Calandra T, Roger T (Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 3:791-800.2003).

Chalimoniuk M, King-Pospisil K, Metz CN, Toborek M (Macrophage migration inhibitory factor induces cell death and decreases neuronal nitric oxide expression in spinal cord neurons. Neuroscience 139:1117-1128.2006).
Chen P, Segil N (p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. Development 126:1581-1590.1999).
Chen P, Zindy F, Abdala C, Liu F, Li X, Roussel MF, Segil N (Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. Nat Cell Biol 5:422-426.2003).
Dewor M, Steffens G, Krohn R, Weber C, Baron J, Bernhagen J (Macrophage migration inhibitory factor (MIF) promotes fibroblast migration in scratch-wounded monolayers in vitro. FEBS Lett 581:4734-4742.2007).
Diensthuber M, Oshima K, Heller S (Stem/progenitor cells derived from the cochlear sensory epithelium give rise to spheres with distinct morphologies and features. J Assoc Res Otolaryngol 10:173-190.2009).
El-Turk F, Cascella M, Ouertatani-Sakouhi H, Narayanan RL, Leng L, Bucala R, Zweckstetter M, Rothlisberger U, Lashuel HA (The conformational flexibility of the carboxy terminal residues 105-114 is a key modulator of the catalytic activity and stability of macrophage migration inhibitory factor. Biochemistry 47:10740-10756.2008).
Fekete DM, Muthukumar S, Karagogeos D (Hair cells and supporting cells share a common progenitor in the avian inner ear. J Neurosci 18:7811-7821.1998).
Fingerle-Rowson G, Petrenko O, Metz CN, Forsthuber TG, Mitchell R, Huss R, Moll U, Muller W, Bucala R (The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. Proc Natl Acad Sci U S A 100:9354-9359.2003).
Friedman LM, Avraham KB (MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mamm Genome 20:581-603.2009).
Germiller JA, Smiley EC, Ellis AD, Hoff JS, Deshmukh I, Allen SJ, Barald KF (Molecular characterization of conditionally immortalized cell lines derived from mouse early embryonic inner ear. Dev Dyn 231:815-827.2004).
Hajas A, Szodoray P, Barath S, Sipka S, Rezes S, Zeher M, Sziklai I, Szegedi G, Bodolay E (Sensorineural hearing loss in patients with mixed connective tissue disease: immunological markers and cytokine levels. J Rheumatol 36:1930-1936.2009).
Hernandez PP, Olivari FA, Sarrazin AF, Sandoval PC, Allende ML (Regeneration in zebrafish lateral line neuromasts: expression of the neural progenitor cell marker sox2 and proliferation-dependent and-independent mechanisms of hair cell renewal. Dev Neurobiol 67:637-654.2007).
Hinck AP (Class II cytokine common receptors: something old, something new. Structure 18:551-552.2010).
Honda A, Abe R, Yoshihisa Y, Makino T, Matsunaga K, Nishihira J, Shimizu H, Shimizu T (Deficient deletion of apoptotic cells by macrophage migration

inhibitory factor (MIF) overexpression accelerates photocarcinogenesis. Carcinogenesis 30:1597-1605.2009).
Hori R, Nakagawa T, Sakamoto T, Matsuoka Y, Takebayashi S, Ito J (Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea. Neuroreport 18:1911-1914.2007).
Kleemann R, Hausser A, Geiger G, Mischke R, Burger-Kentischer A, Flieger O, Johannes FJ, Roger T, Calandra T, Kapurniotu A, Grell M, Finkelmeier D, Brunner H, Bernhagen J (Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. Nature 408:211-216.2000).
Kobayashi S, Satomura K, Levsky JM, Sreenath T, Wistow GJ, Semba I, Shum L, Slavkin HC, Kulkarni AB (Expression pattern of macrophage migration inhibitory factor during embryogenesis. Mech Dev 84:153-156.1999).
Lefebvre PP, Leprince P, Weber T, Rigo JM, Delree P, Moonen G (Neuronotrophic effect of developing otic vesicle on cochleo-vestibular neurons: evidence for nerve growth factor involvement. Brain Res 507:254-260.1990).
Leonard WJ (The defective gene in X-linked severe combined immunodeficiency encodes a shared interleukin receptor subunit: implications for cytokine pleiotropy and redundancy. Curr Opin Immunol 6:631-635.1994).
Lue H, Kapurniotu A, Fingerle-Rowson G, Roger T, Leng L, Thiele M, Calandra T, Bucala R, Bernhagen J (Rapid and transient activation of the ERK MAPK signalling pathway by macrophage migration inhibitory factor (MIF) and dependence on JAB1/CSN5 and Src kinase activity. Cell Signal 18:688-703.2006).
Lue H, Thiele M, Franz J, Dahl E, Speckgens S, Leng L, Fingerle-Rowson G, Bucala R, Luscher B, Bernhagen J (Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. Oncogene 26:5046-5059.2007).
Martinez-Monedero R, Corrales CE, Cuajungco MP, Heller S, Edge AS (Reinnervation of hair cells by auditory neurons after selective removal of spiral ganglion neurons. J Neurobiol 66:319-331.2006).
McKenzie E, Krupin A, Kelley MW (Cellular growth and rearrangement during the development of the mammalian organ of Corti. Dev Dyn 229:802-812.2004).
Nishihira J, Ishibashi T, Fukushima T, Sun B, Sato Y, Todo S (Macrophage migration inhibitory factor (MIF): Its potential role in tumor growth and tumor-associated angiogenesis. Ann N Y Acad Sci 995:171-182.2003).
Nishio Y, Minami A, Kato H, Kaneda K, Nishihira J (Identification of macrophage migration inhibitory factor (MIF) in rat peripheral nerves: its possible involvement in nerve regeneration. Biochim Biophys Acta 1453:74-82.1999).
Nishio Y, Nishihira J, Ishibashi T, Kato H, Minami A (Role of macrophage migration inhibitory factor (MIF) in peripheral nerve regeneration: anti-MIF

antibody induces delay of nerve regeneration and the apoptosis of Schwann cells. Mol Med 8:509-520.2002).
Oshima K, Senn P, Heller S (Isolation of sphere-forming stem cells from the mouse inner ear. Methods Mol Biol 493:141-162.2009).
Ozaki K, Leonard WJ (Cytokine and cytokine receptor pleiotropy and redundancy. J Biol Chem 277:29355-29358.2002).
Rivolta MN, Holley MC (Asymmetric segregation of mitochondria and mortalin correlates with the multi-lineage potential of inner ear sensory cell progenitors in vitro. Brain Res Dev Brain Res 133:49-56.2002).
Roehm PC, Hansen MR (Strategies to preserve or regenerate spiral ganglion neurons. Curr Opin Otolaryngol Head Neck Surg 13:294-300.2005).
Serin GM, Derinsu U, Sari M, Gergin O, Ciprut A, Akdas F, Batman C (Cochlear implantation in patients with bilateral cochlear trauma. Am J Otolaryngol.2009).
Shibata SB, Cortez SR, Beyer LA, Wiler JA, Di Polo A, Pfingst BE, Raphael Y (Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae. Exp Neurol 223:464-472.2010).
Shimizu T (Role of macrophage migration inhibitory factor (MIF) in the skin. J Dermatol Sci 37:65-73.2005).
Shimizu T, Abe R, Nakamura H, Ohkawara A, Suzuki M, Nishihira J (High expression of macrophage migration inhibitory factor in human melanoma cells and its role in tumor cell growth and angiogenesis. Biochem Biophys Res Commun 264:751-758.1999).
Suzuki M, Takamura Y, Maeno M, Tochinai S, Iyaguchi D, Tanaka I, Nishihira J, Ishibashi T (Xenopus laevis macrophage migration inhibitory factor is essential for axis formation and neural development. J Biol Chem 279:21406-21414.2004).
Thompson DL, Gerlach-Bank LM, Barald KF, Koenig RJ (Retinoic acid repression of bone morphogenetic protein 4 in inner ear development. Mol Cell Biol 23:2277-2286.2003).
Velnar T, Bailey T, Smrkolj V (The wound healing process: an overview of the cellular and molecular mechanisms. J Int Med Res 37:1528-1542.2009).
Yoon SI, Jones BC, Logsdon NJ, Harris BD, Deshpande A, Radaeva S, Halloran BA, Gao B, Walter MR (Structure and mechanism of receptor sharing by the IL-10R2 common chain. Structure 18:638-648.2010).

Appendix

Suppression of the expression of CD74, a receptor for macrophage migration inhibitory factor (MIF), inhibits inner ear statoacoustic ganglion (SAG) neurite outgrowth and SAG neuronal survival in vitro

Abstract

CD74 is one of the known receptors for Macrophage Migration Inhibitory Factor (MIF), an important immune system cytokine. MIF has now been identified as the major "neurotrophic" molecule on which the earliest stages of inner ear development depends. We have shown that MIF is produced by the embryonic otocyst and secreted as part of the otocyst derived factor (ODF) and that it is both necessary and sufficient to promote directional neurite outgrowth from nascent statoacoustic ganglion neurons (SAG) and to serve as a survival factor for these neurons (Gerlach-Bank, Ebisu et al., in preparation and Chapter 2). We also demonstrated that MIF is produced by both the embryonic otocyst during SAG neuritogenesis and innervation and by the Supporting Cells (SC) of the mature inner ear. Both embryonic SAG neurons and mature Spiral Ganglion neurons (SGNs), the mature form of these cells, express CD74 receptors and respond to MIF. Directional neurite outgrowth from both SAG and spiral ganglion

(SG) explants and survival of both these dissociated neuronal populations is highly MIF concentration-dependent. Low concentrations have positive effects: higher concentrations are inhibitory. Here we demonstrate that blocking expression of the CD74 receptor with siRNA inhibits both neurite outgrowth and neuronal survival of SAG. These results demonstrate that both MIF and its CD74 receptor play critical roles in the establishment of connections between the SAG and the inner ear.

Introduction

Release of neurotrophic factors by target tissues is essential for the neuronal populations that innervate these tissues and organs to initiate directional neurite outgrowth toward the targets (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993). In the early developing inner ear, cells in the otic crest region of the otocyst secrete a factor called Otocyst Derived Factor (ODF) (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993). ODF has been implicated as a promoter of both directional neurite outgrowth and survival of early stage statoacoustic ganglion (SAG) neurons, which at earlier stages, had migrated away from the otic crest region and then send out processes to innervate the nascent inner ear. In a previous study, we reported that Macrophage Migration Inhibitory Factor (MIF), an immune system cytokine, is a major component of ODF and also acts as a major directional neurite outgrowth factor and survival factor for developing SAG neurons. Additionally,

we have demonstrated that MIF functions as an essential component of normal inner ear neuronal development and innervation. We also found MIF expression both in cells of the developing otocyst and in supporting cells (SC) of the adult cochlea. One known receptor for MIF found in mammals is CD74 (Leng et al., 2003). In the zebrafish, the CD74 homolog is called Major Histocompatibility Complex (MHC) class II invariant chain, of which there are two variants, iclp 1 and 2 in the zebrafish. Binding of MIF to CD74 is known to be necessary for activation of MIF-mediated ERK phosphorylation via a Ras-Raf-MEK dependent pathway, for cell proliferation in cell populations that express CD74, and for both immune system and neuronal cells' differentiation and gene induction. CD74 is also expressed both on embryonic SAG and on adult spiral ganglion (SG) neurons. In the current study, we asked whether a role could be demonstrated for CD74 in inner ear neuronal development. We hypothesized that CD74 is necessary for initiation of MIF-induced SAG neurite outgrowth. We have now demonstrated that knockdown of CD74 with specific RNAi inhibited ODF-induced SAG neurite outgrowth and survival significantly, indicating a critical role for CD74 in inner ear neuronal development. In addition, we detected MIF expression in an Immortomouse otocyst (IMO) cell line obtained from a transgenic mouse at embryonic day 9.5, which is a critical period for inner ear morphogenesis and neurogenesis and MIF siRNA also successfully knocked down its expression.

Sensorineural hearing loss (SNHL) is the most common form of deafness in humans and derives, in most cases, either from primary degeneration of spiral ganglion neurons (SGNs) or from secondary degeneration of these neurons, which is seen after cochlear sensory hair cell (HC) loss (Martinez-Monedero et al., 2006, Friedman and Avraham, 2009). Although new HC production is quite common in cold-blooded vertebrates and birds (Corwin and Cotanche, 1988, Ryals and Rubel, 1988, Balak et al., 1990, Lombarte et al., 1993, Raphael et al., 2007, Brignull et al., 2009), the mammalian cochlea has no ability to spontaneously regenerate sensory auditory HC after birth (Matsui and Cotanche, 2004, Raphael et al., 2007, Stone and Cotanche, 2007, Beisel et al., 2008, Brignull et al., 2009). Cochlear implants (CI) are surgically implanted devices that bypass a nonfunctional cochlea and improve hearing function by directly electrically stimulating any remaining auditory nerves that project toward the cochlea (Altschuler et al., 2008, Shibata et al., 2010). Since no HC regeneration occurs in mammals, CI are currently the only treatment for SNHL (Roehm and Hansen, 2005, Altschuler et al., 2008, Shibata et al., 2010). Nevertheless, the successful function of a CI depends on activation of auditory nerves, so the presence of these nerves and conserved functionality are essential for CI function (Altschuler et al., 2008, Serin et al., 2009, Shibata et al., 2010).

In the developing inner ear, the otocyst is a precursor of all inner ear organs, including sensory HC and neurons of the auditory and vestibular systems, and the structures that house them. Soon after the otocyst forms, individual

neuroblasts delaminate from the anteroventral region of the otocyst called the otic crest (Hemond and Morest, 1991) and coalesce to form the developing SAG neurons, a precursor of the SG and the vestibular ganglion (VG), which provide the sensory innervation for the auditory system and vestibular structures respectively (Barald and Kelley, 2004). Early studies found that directional neurite outgrowth of SAG neurons toward the otocyst was mediated by ODF, which is secreted by cells in the otocyst (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993). We previously found that among the bioactive components of ODF, MIF is a major component (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2). Immunohistochemical and functional assays have demonstrated that MIF is expressed in both the developing and the postnatal/adult inner ear and functions as an essential factor for morphogenesis and neurogenesis in the auditory system (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2).

CD74 is one of the functional receptors reported for MIF and has been shown to be necessary for activation of the extracellular signal regulated kinase1/2 MAP kinase (ERK1/2-MAPK) cascade as well as for cell proliferation (Leng et al., 2003). We have found that CD74 is expressed on both developing SAG and adult SG neurons in mice and chicks (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2) and its homologues iclp1 and 2 are expressed on SAG neurons in the zebrafish (Shen et al., submitted).

Based on our data and that of others (Leng et al., 2003), we hypothesized that CD74 is required for neuronal development (and possibly maintenance) in the inner ear. We used RNAi techniques to knock-down CD74 expression in cultured avian embryonic SAG explants and measured neurite outgrowth and survival in the presence of either CD74 siRNA or the missense control. We report here that CD74 siRNA exposure for 48 hrs resulted in significant suppression of CD74 expression and concomitantly reduced SAG neurite outgrowth and survival significantly. Additionally, we found that MIF is produced by some clones of IMO cell lines (Barald et al., 1997), which we obtained from H2kbtsA58 transgenic mice (Barald et al., 1997, Holley et al., 1997) at embryonic day (E) 9.5, which is an appropriate time for it to play a role in inner ear morphogenesis and neurogenesis. This result indicates that MIF is expressed at least as early as E9.5 in mice and provides further evidence that MIF plays an essential role of in inner ear development. We also demonstrated that mouse MIF siRNA constructs successfully suppressed MIF expression in IMO cells, one source of ODF, which can be used in future experiments to further characterize the role of MIF in inner ear development and maintenance.

Materials and Methods

Design of siRNAs: RNA interference techniques were used to knock down the expression of mouse MIF protein and avian CD74 protein by introducing a homologous double stranded (ds) RNA. The nucleotide sequences of dsRNA

and complimentary dsRNA for mouse MIF mRNA and avian CD74 mRNAs were as follows:

Mouse MIF siRNA duplex: 5'-CCGCAACUACAGUAAGCUGdTdT-3' and 5'-CAGCUUACUGUAGUUGCGGdTdT- 3'.
A control RNA duplex: 5'-GCGCGCUUUGUAGGAUUCGdTdT-3' and 5'-CGAAUCCUACAAAGCGCGCdTdT-3'.

Avian CD74 siRNA duplex: 5'-GCAACAAGACUGAGGAUCAAATdTd-3' and 5'-UUUGAUCCUCAGUCUUGUUGCTdTd-3'.

A control RNA duplex: 5'- GCAAGAAGACAGAGGUUCAAATdTd-3' and 5'-UUUGAACCUCUGUCUUGUUGCTdTd-3'.

All constructs were confirmed by enzymatic digestion (Hind III) and DNA sequence analysis (DNA sequencing core, University of Michigan).

Cell Culture of cells for siRNA transfection: IMO 2B1DT cells that express MIF and the chick brain cells that express CD74 receptor (Bryan et al., 2008) were used to titrate the siRNA concentrations necessary to achieve maximal inhibition of MIF and CD74 protein, as determined by western blotting.

IMO 2B1DT cells: The IMO cell line, IMO 2B1DT was cultured as previously described (Barald et al., 1997, Thompson et al., 2003, Germiller et al., 2004) with minor modifications to accommodate the transfection process. Transfection of mouse MIF siRNA and its missense control into these cells was followed by

western blotting assays to examine the effects on MIF levels. 1-3 × 10^5 cells/well of 2B1DT cells were grown in chick embryo fibroblast (CEF) medium containing 15 % fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1 unit of γ-interferon (Chemicon International, Inc., Temecula, CA) on 6-well culture plates (Fisher Scientific, Pittsburgh, PA) overnight at 37°C prior to transfection.

Chick brain cells: Primary cultures of chick brain cells were also cultured in Dulbecco's modified Eagle's (DME) medium (Invitrogen, Carlsbad, CA) containing 15% FBS and 1 unit of γ-interferon for the transfection of avian CD74 siRNA and its missense control followed by western blotting assays to examine the effects on CD74 levels. The brain cells were excised from E5-8 chick embryos. The cells were then treated with 1 ml trypsin solution [100 µl trypsin (Invitrogen, Carlsbad, CA) and 900 µl of Phosphate Buffered Saline (PBS) (Invitrogen, Carlsbad, CA)] for 10 minutes at 37°C, followed by repetitive pipetting to dissociate the cells. After washing the dissociated cells with PBS, the cells were resuspended in the DME medium. About 1-3 × 10^5 cells/well were placed on the 6-well plates coated with poly-d-lysine (BD, Franklin Lakes, NJ). The cells were incubated for 24 hours at 37°C prior to transfection.

Transfection of siRNA: IMO 2B1DT cells were transfected either with mouse MIF siRNA or its missense control and the chick brain cells were transfected either with avian CD74 siRNA or its missense control using a transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. Twenty four hours post-transfection, western blotting assays were performed with IMO

2B1DT cells and chick brain cells to examine any effect of these siRNAs on MIF and CD74 levels respectively.

Western blotting assay: Western blotting assays were performed as described by Takakura et al. (Takakura et al., 2008) with minor modifications. Protein samples were prepared by lysing IMO 2B1DT cells or chick brain cells in lysis buffer containing complete protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN). After addition of sample buffer followed by boiling, gel electrophoresis was performed using 12% precast SDS-polyacrylamide gels (BioRad, Hercules, CA). Proteins were transferred onto a nitrocellulose membrane (Millipore, Billerica, MA) at 100 voltages for 1 hour. After blocking with 2% non-fat Milk (Nestle, Solon, OH), membranes were probed with the following antibodies. For the detection of mouse MIF expression, anti-MIF antibody (1:3,000) (Zymed, South San Francisco, CA) was followed by anti-rabbit IgG peroxidase conjugate secondary antibody (1:40,000) (Zymed, South San Francisco, CA). For the detection of avian CD74 expression, anti-avian CD74 antibody (1:200) (Santa Cruz, La Jolla, CA) was followed by goat anti-mouse HRP-conjugated secondary antibody (1:10,000) (BioRad, Hercules, CA). Membranes were also probed with anti-GAPDH antibody (1:10,000) (Millipore, Billerica, MA) followed by goat anti-mouse HRP-conjugated secondary antibody (1:10,000) (BioRad, Hercules, CA) to assess protein loading. Membranes were then developed by enhanced chemiluminescence (ECL) (Amersham Life Science Ltd, Bucks., UK) followed by exposure to photographic film (Kodak, Fisher Scientific, Pittsburgh, PA) to observe the expression of the proteins. Each

experiment was repeated three times. Quantification of bands was done with Scion Image software (Frederick, MD) and normalized to GAPDH.

Otocyst excision and otocyst conditioned medium (also called otocyst-generated ODF) collection: To produce otocyst-generated ODF, conditioned medium (CM) was collected from chick otocysts at E4 based on the method of Bianchi et al. (Bianchi and Cohan, 1993, Bianchi et al., 1998) with minor modifications. Briefly, eight otocysts from E4 chick were collected and incubated in 250µl of serum and γ-interferon free DME medium (basal medium) at 37°C for 3 days. After centrifugation at 10,000 rpm at room temperature for 3 minutes, the supernatant was transferred into a low protein binding microtest tube (DENVILLE Scientific INC, Metuchen, NJ) and stored at 4°C until used.

Neuronal cell culture-based bioassays for neurite outgrowth and survival in the presence of avian CD74 siRNA or its missense control: SAG bioassays were performed as in Bianchi et al. (Bianchi et al., 2005) with minor modifications to determine whether there was an effect of CD74 siRNA on ODF-induced SAG neurite outgrowth and survival. For SAG neurite outgrowth, whole SAGs were excised from chick embryos at E5 and placed on a poly-D-lysine coated 96 well plate. For the assays, SAG neurite outgrowth in basal medium was used as the negative control and that in the otocyst-generated ODF was used as the positive control. After 2-3 hours, SAGs that had been cultured in the otocyst-generated ODF were transfected either with avian CD74 siRNA (2.0 µg/µl) or its missense control (2.0 µg/µl). Forty-eight hours post-transfection, SAG neurite outgrowth in the presence of ODF or medium alone was observed and scored based on our

previously published standard scale of 0-5 (Bianchi et al., 2005). To examine SAG neuron survival, individual SAG neurons were prepared by pooling dissociated cells from 25-30 ganglia. First, the isolated ganglia were washed with calcium-free PBS (3x) followed by centrifugation at 10,000 rpm for 1 minute and aspiration of supernatant. Then, the ganglia were incubated with PBS containing 0.025 % trypsin (Invitrogen, Carlsbad, CA) at 37°C for 10 minutes. After centrifugation and aspiration of trypsin-containing PBS, the cells were incubated with PBS containing 0.1 % trypsin inhibitor (Sigma, St. Louis, MO) at 37°C for 10 minutes. Ganglia were rinsed once with basal medium followed by centrifugation and aspiration. Finally, the ganglia were resuspended in basal medium and dissociated by trituration with a fire-polished glass pipet. The individual cells were plated on poly-D-lysine coated 96 well plates at a density of 10,000 cells per well (determined by haemocytometer counts). Cells were incubated at 37°C until they became attached to the plate. After cell attachment, the medium was replaced with otocyst-generated ODF or basal medium. A subset of the wells containing cells cultured in otocyst-generated ODF were transfected either with avian CD74 siRNA (2.0 µg/µl) or its missense control (2.0 µg/µl) and incubated at 37°C for 48-72 hours. All samples were assessed in triplicate in three separate experiments.

Immunocytochemistry (ICC): ICC was performed with dissociated SAG neurons. Dissociated SAG neurons in the 96 well plates were fixed with 100 µl of 4 % paraformaldehyde (PFA) solution for 20 minutes followed by careful washing of the plate three times with PBS. After aspirating PBS, 100 µl of PBS containing

3 % hydrogen peroxide was applied and incubated for 10 minutes. The plate was then incubated with 100 µl of PBT [PBS containing 1 % Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO) and 0.5 % Tween 20] for 1 hour followed by blocking with 100 µl PBT containing 5 % Normal Goat Serum (NGS) (Invitrogen, Carlsbad, CA) for 30 minutes. Then, the plate was incubated with 100 µl of Monoclonal Antibody against Neuronal Class III β-Tubulin (Covance, Berkeley, CA) diluted with PBT containing 5 % NGS (1:500) at 4°C overnight. Twenty-four hours later, the plate was incubated with 100 µl of PBT containing 5 % NGS for 30 minutes after washing the plate with PBT (3x). Then, a secondary antibody, goat anti mouse, (BioRad, Hercules, CA) diluted with PBT (1:500) was applied and incubated for 3 hours. The plate was washed with PBT (3x) followed by PBS (1x). Plates were labeled with 20 µl of AEC substrate (Zymed, San Francisco, CA) for 5 minutes or until color developed. The reaction was then stopped by adding 20 µl of MilliQ water. Micrographs were taken using Nikon ACT-1 software on a Leitz (Leica) Diavert inverted microscope. The entire experiment was performed under the hood at room temperature, except for the incubation with primary antibody at 4°C.

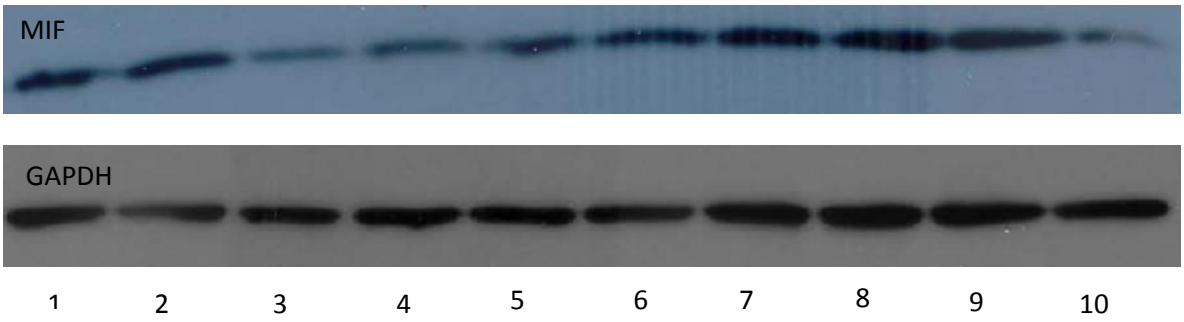
Statistical analysis: The data were analyzed by one-tail t-test. A statistical probability of $P<0.05$ was considered significant.

Results

siRNA to mouse MIF reduced the expression of MIF in IMO 2B1DT cells

IMO 2B1DT cells are a subcloned cell line isolated from E9.5 murine otocysts of the H2kbtsA58 transgenic mouse, which we have used to study certain aspects of sensory cell development in the inner ear (Barald et al., 1997, Thompson et al., 2003, Germiller et al., 2004). They also serve as a source of a bioequivalent ODF, which contains MIF (Bianchi et al., 2005; Gerlach-Bank, Ebisu et al., in preparation, Chapter 2). Earlier studies in many labs have demonstrated that cells in the otocyst secrete a factor called ODF, which enhances both directional neurite outgrowth and survival of early stage SAG neurons (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993). We had previously demonstrated that CM obtained from the IMO 2B1DT cells (IMO-generated ODF) has a bioequivalent ODF effect to that of ODF obtained from E4 chick otocysts (otocyst-generated ODF) on both SAG neurite outgrowth and survival (Bianchi et al., 2005; Gerlach-Bank, Ebisu et al., in preparation, Chapter 2). Both IMO- and otocyst-generated ODFs contain MIF (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2). In the current study, we examined the expression of MIF in 2B1DT cells with western blotting assays, followed by measuring the effect of the mouse MIF siRNA constructs on 2B1DT cells' production of MIF by introducing varying concentrations (0.25 to 1.5 μg/μl) of the siRNA constructs 24 hours after plating the cells. We found that the optimal concentration of mouse MIF siRNA necessary to suppress MIF expression

significantly (by about 30%) when compared to the missense control was 0.25 μg/μl (Figure A.1), indicating that siRNA techniques will be helpful for further characterization of the role of MIF in inner ear development and maintenance.



1. No transfection
2. GFP, 1.0 µg/µl
3. siRNA to mouse MIF, 0.25 µg/µl
4. siRNA to mouse MIF, 0.5 µg/µl
5. siRNA to mouse MIF, 1.0 µg/µl
6. siRNA to mouse MIF, 1.5 µg/µl
7. Missense, 0.25 µg/µl
8. Missense, 0.5 µg/µl
9. Missense, 1.0 µg/µl
10. Missense, 1.5 µg/µl

Figure A.1: Western blots were performed after cultured IMO cells, which exude MIF into conditioned serum-free medium, were treated with various concentrations of siRNA to mouse MIF or with its missense control for 24 hours in culture.

siRNA/missense, 0.25 µg/µl	0.72
siRNA/missense, 0.5 µg/µl	1.02
siRNA/missense, 1.0 µg/µl	0.89
siRNA/missense, 1.5 µg/µl	3.06

Table A.1: The ratio of siRNA/missense at various concentrations. 0.25 µg/µl of siRNA produced the most effective reduction in the expression of MIF compared to its missense control.

Exposure to CD74 siRNA for 48 hours significantly reduced ODF-induced SAG neurite outgrowth and survival

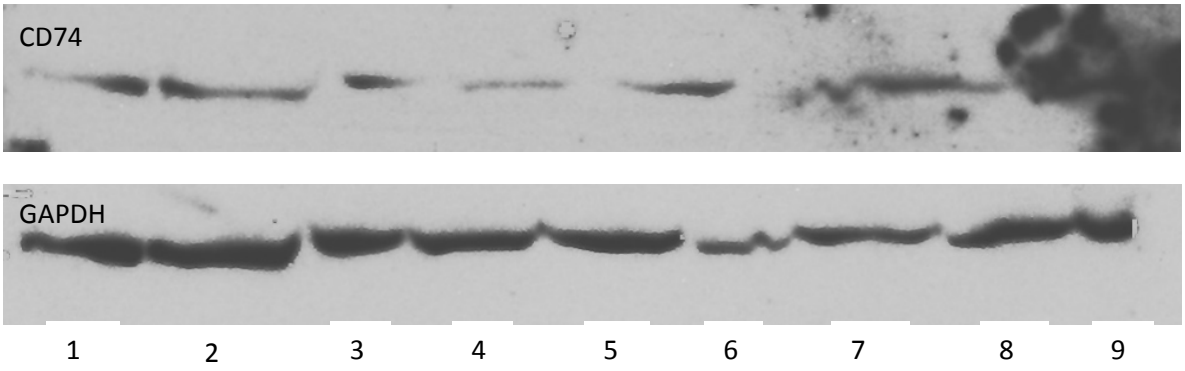
CD74 has been implicated as a surface receptor for MIF (Leng et al., 2003). Binding of MIF to CD74 is necessary for the activation of the MIF-mediated ERK1/2-MAPK signaling pathway, cell proliferation and gene induction in mouse embryonic fibroblasts. We constructed avian CD74 siRNA and its missense control and examined their effects on CD74 expression levels using the chick brain, since it expresses CD74 (Bryan et al., 2008) and we are able to obtain enough cells to perform Western blotting assays. The western blotting assays demonstrated that avian CD74 siRNA constructs at concentrations from 0.5 μg/μl to 2.0 μg/μl almost equally reduced CD74 expression when compared to its missense control as shown in Figure A.2 (16-27% reduction). We then performed neuronal bioassays with chick SAG explants to determine an optimal concentration of the siRNA on SAG neurite outgrowth using the same concentration range (from 0.5 μg/μl to 2.0 μg/μl). We decided to use the concentration at 2.0 μg/μl for the following experiments.

To determine whether CD74 is necessary for chick SAG neurite outgrowth in response to ODF, we performed neuronal cell culture assays either with avian CD74 siRNA or missense control in otocyst-generated ODF. We excised SAGs from E5 chick embryos and cultured them in otocyst-generated ODF for a few hours or until the explants attached to the plate at 37°C. Basal medium and otocyst-generated ODF without added siRNA were used as the negative control and the positive control respectively. After a few hours in otocyst-generated ODF,

we introduced either 2.0 µg/µl avian CD74 siRNA or 2.0 µg/µl of its missense control into the cultured SAG explants in ODF. The SAGs were cultured for an additional 48 hours at 37°C. We then observed and scored SAG neurite outgrowth using scoring criteria reported earlier (Bianchi et al., 2005). We observed that the SAG explants exposed to 2.0 µg/µl avian CD74 siRNA had significantly reduced SAG neurite outgrowth, with an average neurite outgrowth score of 1.0 ± 1.32 (Figure A.3-A), compared to those explants exposed to the same concentration of the missense control, which had average neurite outgrowth scores of 3.11± 1.27 (about 70% reduction, Figure A.3-B), after 48 hours exposure to the siRNA. SAG explants in otocyst-generated ODF (positive control) produced neurite outgrowth, with average scores of 1.67 ± 1.22, while the explants in basal medium (negative control) produced little or no neurite outgrowth (average scores = 0). Statistical analysis of this data showed that SAG explants treated with siRNA to CD74 significantly reduced the outgrowth compared to the positive control (about 40% reduction).

The SAG neuronal survival assays were performed in the same manner as the SAG neurite outgrowth assays with a few modifications. Dissociated SAG neurons were used for the survival assays and after 48 hours exposure to siRNA or its missense, both vital cells and total cells were counted to obtain the percentage of cell survival. We observed that the SAG neurons exposed to 2.0 µg/µl avian CD74 siRNA for 48 hours had significantly lower survival (5.1 ± 21.81% neuronal survival on average, Figure A.3-C) compared to those treated with 2.0 µg/µl missense control (24.6 ± 20.35% cell survival on average, about

80% reduction) (Figure A.3-D). Statistical analysis also demonstrated that cells treated with the siRNA (2.0 µg/µl) significantly reduced neuronal cell survival compared to the positive control (average cell survival of 32.4 ± 13.66%, about 85% reduction). These results suggest that CD74 is involved in both chick SAG neurite outgrowth and dissociated neuron survival.



1. No transfection
2. siRNA to avian CD74, 0.5 ug/ul
3. siRNA to avian CD74, 1.0 ug/ul
4. siRNA to avian CD74, 1.5 ug/ul
5. siRNA to avian CD74, 2.0 ug/ul
6. Missense, 0.5 ug/ul
7. Missense, 1.0 ug/ul
8. Missense, 1.5 ug/ul
9. Missense, 2.0 ug/ul

Figure A.2: Western blots were performed after cultured dissociated E5-8 chick brain cells that express CD74, a receptor for MIF, to examine the effect of avian CD74 siRNA and its missense on expression. The brain cells were treated with various concentrations of siRNA to avian CD74 or with its missense control for 24 hours in culture.

0.5 ug/ul siRNA/control	0.27
1.0 ug/ul siRNA/control	0.19
1.5 ug/ul siRNA/control	0.16
2.0 ug/ul siRNA/control	0.23

Table A.2: The ratio of siRNA/missense at various concentrations. All concentrations of siRNA to avian CD74 almost equally reduced the expression of Avian CD74 expression compared to the missense control.

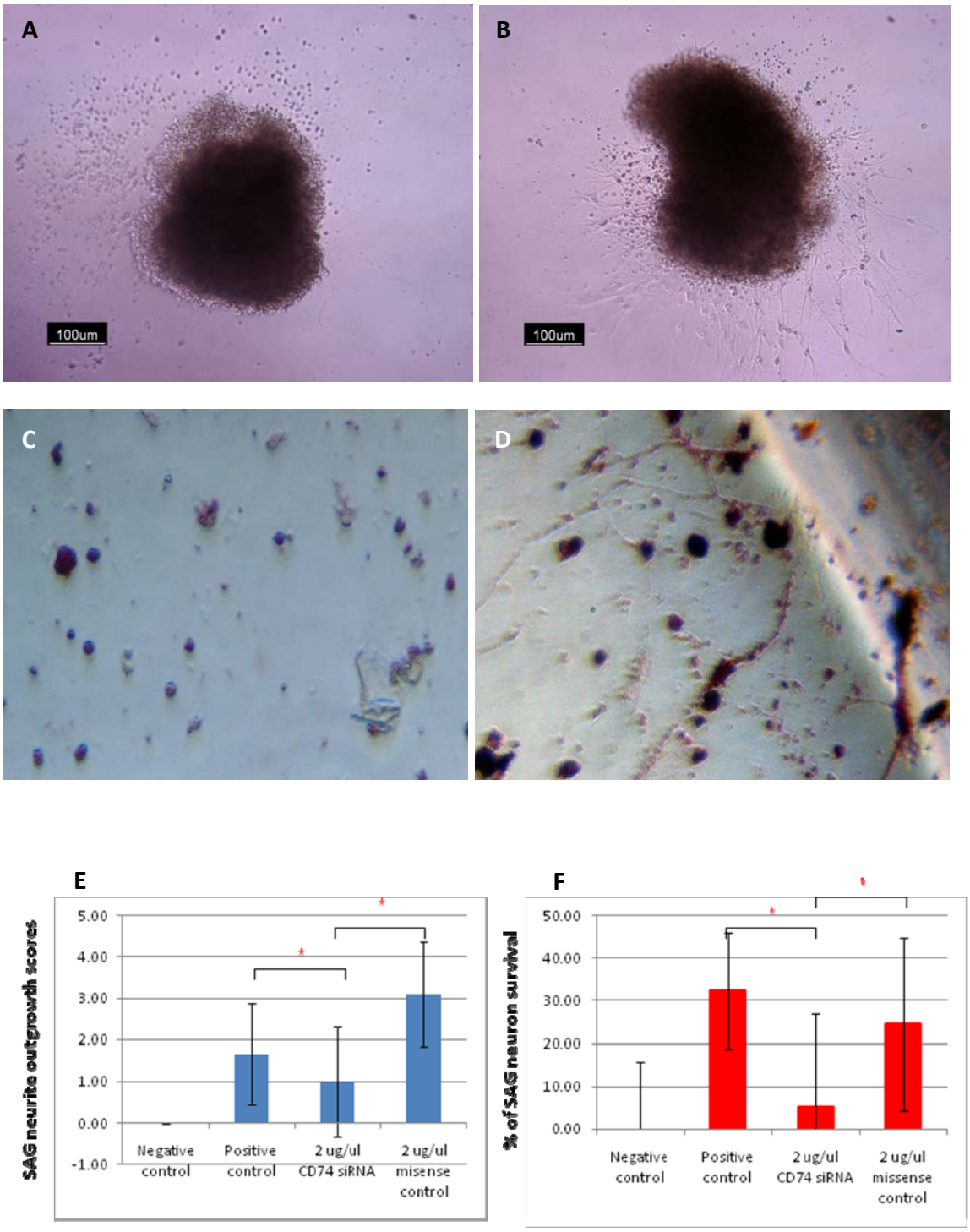


Figure A.3: Avian CD74 siRNA reduced SAG neurite outgrowth and survival: Neuronal bioassays using E5 chick whole SAGs and dissociated SAGs from the same stage embryos were exposed to conditioned medium generated from chick otocysts (called otocyst-generated ODF) at E4 to determine the effect of blocking CD74 with siRNA. SAG explants treated with otocyst-generated ODF

in the presence of 2.0 µg/µl avian CD74 siRNA show little SAG neurite outgrowth (A). In contrast, SAG explants treated with otocyst-generated ODF in the presence of 2.0 µg/µl of the missense control show robust SAG neurite outgrowth (B). Dissociated SAG neurons treated with otocyst-generated ODF in the presence of 2.0 µg/µl avian CD74 siRNA demonstrate lower cell survival (C). In contrast, dissociated SAG neurons treated with otocyst-generated ODF in the presence of 2.0 µg/µl missense control show higher neuronal survival (D). Statistical analysis demonstrates that 2 µg/µl of siRNA significantly reduced both SAG neurite outgrowth (E) and survival (F) compared to its missense control ($P < 0.05$). Serum and γ-interferon free basal medium is used as negative control and otocyst-generated ODF is used as positive control. n=9 (SAG neurite outgrowth). n=12 (dissociated SAG neuronal survival).

Discussion

MIF is a ubiquitously expressed pleiotropic cytokine (Nishihira, 2000, Bifulco et al., 2008, Rendon et al., 2009), which plays multiple roles in many different cell types, including a prominent role in T cell development and maturation in the immune system (Bernhagen et al., 1993, Bacher et al., 1996, Bernhagen et al., 1996, Bozza et al., 1999, Roger et al., 2001, Santos et al., 2008, Tohyama et al., 2008). MIF has also been shown to play a vital role in neuronal development (Suzuki et al., 2004) and in peripheral nerve regeneration (Nishio et al., 2002). A receptor for MIF, discovered by Leng et al. (2003) was found to be a type II transmembrane protein also called MHC class II invariant chain, CD74 (Leng et al., 2003). These authors determined that MIF binds to CD74 with high affinity, and the expression of CD74 is necessary for MIF-mediated activation of the ERK1/2-MAPK cascade, for cell proliferation and differentiation in mouse embryonic fibroblasts. We have characterized two CD74-like receptors, iclp1 and iclp2, which are expressed on nascent SAG in the

zebrafish (Shen et al, in preparation). Inhibition of iclp1 and 2 with antisense oligonucleotide morpholinos leads to the reduction in size of the SAG in the zebrafish (Shen et al, in preparation).

Target-derived growth and survival factors that act as neurotrophins are essential for the development, neurogenesis and pathway selection of the neuronal populations that innervate these target tissues. Earlier studies have demonstrated that the cells of the otocyst, which is a precursor of all inner ear sensory organs, including the sensory HC and their underlying supporting cells (SC) and of the neurons that innervate the HC, secretes a factor called ODF during early stage inner ear development (Ard et al., 1985, Lefebvre et al., 1990, Bianchi and Cohan, 1991, 1993). The ODF secreted by the otocyst mediates directional neurite outgrowth and survival of SAG neurons; the SAG contains precursors of both SG neurons and VG neurons (Barald and Kelley, 2004).

We have recently demonstrated that the bioactive components of ODF include MIF, and MIF functions as an essential molecular neurotrophin in normal inner ear development (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2). We have detected the expression of MIF both in the developing otocyst and in adult SC of the cochlea and that of CD74 both in developing SAG and postnatal/adult SG neurons in mammals (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2) and its homologues iclp1 and 2 in the developing zebrafish SAG (Shen et al, in preparation). Moreover, MIF knock-out (KO) mice showed significant hearing impairment in the high frequency region (48 KHz) of

the cochlea with concomitant loss of SGNs in this region of the cochlea (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2). Based on these observations and data, we also anticipated an essential role for the MIF receptor, CD74 in inner ear neuronal development.

Thus, in the current study, we asked whether CD74 could be demonstrated to play a role in inner ear SAG neurite outgrowth and survival. In cultured SAG bioassays, we demonstrated that CD74 siRNA significantly suppressed ODF-mediated SAG neurite outgrowth by almost 70% compared to the CD74 missense siRNA control (average scores of 1.0 compared to 3.11, respectively) as shown in Figures A.3-A and B. We also demonstrated that CD74 siRNA significantly reduced ODF-induced SAG neuronal survival by almost 80% compared to the CD74 missense siRNA control (average percentage of cell survival = 5.1% compared to 24.6%) as shown in Figures A.3-C and D.

Nevertheless, suppression of CD74 expression alone failed to completely inhibit ODF-induced SAG neurite outgrowth and survival, suggesting that there may be additional receptor(s) for MIF that play a role in mediating neurite outgrowth and survival and/or the different factor(s) in ODF may have the same effect as MIF in promoting SAG neurite outgrowth and survival.

A recent study has identified two additional functional receptors for MIF, CXC chemokine receptors named CXCR2 and CXCR4 (Bernhagen et al., 2007). According to these authors, MIF acts as a noncognate ligand for these receptors and competes with their cognate ligands, such as CXCL 1 and 8 for CXCR2

(Bernhagen et al., 2007, Schober et al., 2008, Beswick and Reyes, 2009) and CXCL12 for CXCR4 (Schober et al., 2008). CXCR2 is commonly expressed on macrophages and functions in recruiting leukocytes to sites of infection (Bernhagen et al., 2007, Beswick and Reyes, 2009). Besides its expression in the immune system, CXCR2 expression has been found in the nervous system (Horuk et al., 1997, Giovannelli et al., 1998, Luan et al., 2001, Valles et al., 2006). Developmental studies have shown that CXCR2 is expressed throughout the mouse brain during early development (Luan et al., 2001) and $CXCR2^{-/-}$ mice exhibited reduced numbers of cells in the oligodendrocyte lineage, myelination, and white matter in the vertebrate CNS (Padovani-Claudio et al., 2006). CXCR4 is a G-protein coupled receptor for stromal cell-derived factor-1 (SDF-1/CXCL12) (Ganju et al., 1998, Vera et al., 2008), which is functionally expressed on the cell surface of various types of cancer cells, and also plays a role in cell proliferation and migration of these cells (Burger and Kipps, 2006). In the developing mouse CNS, CXCR4 expression is detected as early as E8.5 and its expression is also found in adulthood (McGrath et al., 1999, Tissir et al., 2004, Lieberam et al., 2005). Abnormal development of the cerebellum (Zou et al., 1998), the hippocampal dentate gyrus (Lu et al., 2002) and uncontrolled initial motor axon trajectories (Lieberam et al., 2005) have also been reported in CXCR4 KO mice. These results suggest the importance of CXCR2 and CXCR4 in the development and/or maintenance of the CNS, and prompt us to speculate that they also may be involved in inner ear neuronal development. We will examine the possible involvement of these receptors by assessing their expression both in the

embryonic SAG and postnatal/adult SG neurons both in the mouse and the chick. If we detect their expression in SAG/SG, we will then examine their role in inner ear neuronal development and maintenance by functionally blocking their expression using similar siRNA strategies to those described in this report. In the present study, we examined only the role of CD74 and that only during embryonic stages. As previously reported and mentioned above, the expression of CD74 was detected in the postnatal/adult SGNs as well (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2). Thus, its role in postnatal/adult SGNs needs to be determined in similar functional assays. We have identified a quartet of bioactive cytokine components in ODF by performing proteomic assays and cytokine array studies (Gerlach-Bank, Ebisu et al., in preparation; Ebisu et al, in preparation), including MIF (Gerlach-Bank, Ebisu et al., in preparation, Chapter 2) and MCP-1 (Bianchi et al., 2005). The remaining identified factors and their receptors also need to be examined for their possible role(s) in neurite extension and neuron survival in the inner ear.

In the present study, we also showed that MIF protein is expressed as early as E9.5 in the developing inner ear in mice, since the IMO cell line IMO2B1DT was derived from this stage of the developing Immortomouse. Additionally, we demonstrated that mouse MIF siRNA successfully reduced its expression in IMO cells. This helps to validate IMO cells as a valid source of ODF-like activity.

References

Altschuler RA, O'Shea KS, Miller JM (Stem cell transplantation for auditory nerve replacement. Hear Res 242:110-116.2008).

Ard MD, Morest DK, Hauger SH (Trophic interactions between the cochleovestibular ganglion of the chick embryo and its synaptic targets in culture. Neuroscience 16:151-170.1985).

Bacher M, Metz CN, Calandra T, Mayer K, Chesney J, Lohoff M, Gemsa D, Donnelly T, Bucala R (An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. Proc Natl Acad Sci U S A 93:7849-7854.1996).

Balak KJ, Corwin JT, Jones JE (Regenerated hair cells can originate from supporting cell progeny: evidence from phototoxicity and laser ablation experiments in the lateral line system. J Neurosci 10:2502-2512.1990).

Barald KF, Kelley MW (From placode to polarization: new tunes in inner ear development. Development 131:4119-4130.2004).

Barald KF, Lindberg KH, Hardiman K, Kavka AI, Lewis JE, Victor JC, Gardner CA, Poniatowski A (Immortalized cell lines from embryonic avian and murine otocysts: tools for molecular studies of the developing inner ear. Int J Dev Neurosci 15:523-540.1997).

Beisel K, Hansen L, Soukup G, Fritzsch B (Regenerating cochlear hair cells: quo vadis stem cell. Cell Tissue Res 333:373-379.2008).

Bernhagen J, Bacher M, Calandra T, Metz CN, Doty SB, Donnelly T, Bucala R (An essential role for macrophage migration inhibitory factor in the tuberculin delayed-type hypersensitivity reaction. J Exp Med 183:277-282.1996).

Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R (MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365:756-759.1993).

Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C (MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med 13:587-596.2007).

Beswick EJ, Reyes VE (CD74 in antigen presentation, inflammation, and cancers of the gastrointestinal tract. World J Gastroenterol 15:2855-2861.2009).

Bianchi LM, Cohan CS (Developmental regulation of a neurite-promoting factor influencing statoacoustic neurons. Brain Res Dev Brain Res 64:167-174.1991).

Bianchi LM, Cohan CS (Effects of the neurotrophins and CNTF on developing statoacoustic neurons: comparison with an otocyst-derived factor. Dev Biol 159:353-365.1993).

Bianchi LM, Daruwalla Z, Roth TM, Attia NP, Lukacs NW, Richards AL, White IO, Allen SJ, Barald KF (Immortalized mouse inner ear cell lines demonstrate

a role for chemokines in promoting the growth of developing statoacoustic ganglion neurons. J Assoc Res Otolaryngol 6:355-367.2005).
Bianchi LM, Dolnick R, Medd A, Cohan CS (Developmental changes in growth factors released by the embryonic inner ear. Exp Neurol 150:98-106.1998).
Bifulco C, McDaniel K, Leng L, Bucala R (Tumor growth-promoting properties of macrophage migration inhibitory factor. Curr Pharm Des 14:3790-3801.2008).
Bozza M, Satoskar AR, Lin G, Lu B, Humbles AA, Gerard C, David JR (Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J Exp Med 189:341-346.1999).
Brignull HR, Raible DW, Stone JS (Feathers and fins: non-mammalian models for hair cell regeneration. Brain Res 1277:12-23.2009).
Bryan KJ, Zhu X, Harris PL, Perry G, Castellani RJ, Smith MA, Casadesus G (Expression of CD74 is increased in neurofibrillary tangles in Alzheimer's disease. Mol Neurodegener 3:13.2008).
Burger JA, Kipps TJ (CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107:1761-1767.2006).
Corwin JT, Cotanche DA (Regeneration of sensory hair cells after acoustic trauma. Science 240:1772-1774.1988).
Friedman LM, Avraham KB (MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mamm Genome 20:581-603.2009).
Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, Newman W, Groopman JE (The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. J Biol Chem 273:23169-23175.1998).
Germiller JA, Smiley EC, Ellis AD, Hoff JS, Deshmukh I, Allen SJ, Barald KF (Molecular characterization of conditionally immortalized cell lines derived from mouse early embryonic inner ear. Dev Dyn 231:815-827.2004).
Giovannelli A, Limatola C, Ragozzino D, Mileo AM, Ruggieri A, Ciotti MT, Mercanti D, Santoni A, Eusebi F (CXC chemokines interleukin-8 (IL-8) and growth-related gene product alpha (GROalpha) modulate Purkinje neuron activity in mouse cerebellum. J Neuroimmunol 92:122-132.1998).
Hemond SG, Morest DK (Ganglion formation from the otic placode and the otic crest in the chick embryo: mitosis, migration, and the basal lamina. Anat Embryol (Berl) 184:1-13.1991).
Holley MC, Nishida Y, Grix N (Conditional immortalization of hair cells from the inner ear. Int J Dev Neurosci 15:541-552.1997).
Horuk R, Martin AW, Wang Z, Schweitzer L, Gerassimides A, Guo H, Lu Z, Hesselgesser J, Perez HD, Kim J, Parker J, Hadley TJ, Peiper SC (Expression of chemokine receptors by subsets of neurons in the central nervous system. J Immunol 158:2882-2890.1997).
Lefebvre PP, Leprince P, Weber T, Rigo JM, Delree P, Moonen G (Neuronotrophic effect of developing otic vesicle on cochleo-vestibular

neurons: evidence for nerve growth factor involvement. Brain Res 507:254-260.1990).
Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery T, Chen Y, Mitchell RA, Bucala R (MIF signal transduction initiated by binding to CD74. J Exp Med 197:1467-1476.2003).
Lieberam I, Agalliu D, Nagasawa T, Ericson J, Jessell TM (A Cxcl12-CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. Neuron 47:667-679.2005).
Lombarte A, Yan HY, Popper AN, Chang JS, Platt C (Damage and regeneration of hair cell ciliary bundles in a fish ear following treatment with gentamicin. Hear Res 64:166-174.1993).
Lu M, Grove EA, Miller RJ (Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. Proc Natl Acad Sci U S A 99:7090-7095.2002).
Luan J, Furuta Y, Du J, Richmond A (Developmental expression of two CXC chemokines, MIP-2 and KC, and their receptors. Cytokine 14:253-263.2001).
Martinez-Monedero R, Corrales CE, Cuajungco MP, Heller S, Edge AS (Reinnervation of hair cells by auditory neurons after selective removal of spiral ganglion neurons. J Neurobiol 66:319-331.2006).
Matsui JI, Cotanche DA (Sensory hair cell death and regeneration: two halves of the same equation. Curr Opin Otolaryngol Head Neck Surg 12:418-425.2004).
McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J (Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol 213:442-456.1999).
Nishihira J (Macrophage migration inhibitory factor (MIF): its essential role in the immune system and cell growth. J Interferon Cytokine Res 20:751-762.2000).
Nishio Y, Nishihira J, Ishibashi T, Kato H, Minami A (Role of macrophage migration inhibitory factor (MIF) in peripheral nerve regeneration: anti-MIF antibody induces delay of nerve regeneration and the apoptosis of Schwann cells. Mol Med 8:509-520.2002).
Padovani-Claudio DA, Liu L, Ransohoff RM, Miller RH (Alterations in the oligodendrocyte lineage, myelin, and white matter in adult mice lacking the chemokine receptor CXCR2. Glia 54:471-483.2006).
Raphael Y, Kim YH, Osumi Y, Izumikawa M (Non-sensory cells in the deafened organ of Corti: approaches for repair. Int J Dev Biol 51:649-654.2007).
Rendon BE, Willer SS, Zundel W, Mitchell RA (Mechanisms of macrophage migration inhibitory factor (MIF)-dependent tumor microenvironmental adaptation. Exp Mol Pathol 86:180-185.2009).
Roehm PC, Hansen MR (Strategies to preserve or regenerate spiral ganglion neurons. Curr Opin Otolaryngol Head Neck Surg 13:294-300.2005).

Roger T, David J, Glauser MP, Calandra T (MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature 414:920-924.2001).
Ryals BM, Rubel EW (Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240:1774-1776.1988).
Santos LL, Dacumos A, Yamana J, Sharma L, Morand EF (Reduced arthritis in MIF deficient mice is associated with reduced T cell activation: down-regulation of ERK MAP kinase phosphorylation. Clin Exp Immunol 152:372-380.2008).
Schober A, Bernhagen J, Weber C (Chemokine-like functions of MIF in atherosclerosis. J Mol Med 86:761-770.2008).
Serin GM, Derinsu U, Sari M, Gergin O, Ciprut A, Akdas F, Batman C (Cochlear implantation in patients with bilateral cochlear trauma. Am J Otolaryngol.2009).
Shibata SB, Cortez SR, Beyer LA, Wiler JA, Di Polo A, Pfingst BE, Raphael Y (Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae. Exp Neurol 223:464-472.2010).
Stone JS, Cotanche DA (Hair cell regeneration in the avian auditory epithelium. Int J Dev Biol 51:633-647.2007).
Suzuki M, Takamura Y, Maeno M, Tochinai S, Iyaguchi D, Tanaka I, Nishihira J, Ishibashi T (Xenopus laevis macrophage migration inhibitory factor is essential for axis formation and neural development. J Biol Chem 279:21406-21414.2004).
Takakura S, Mitsutake N, Nakashima M, Namba H, Saenko VA, Rogounovitch TI, Nakazawa Y, Hayashi T, Ohtsuru A, Yamashita S (Oncogenic role of miR-17-92 cluster in anaplastic thyroid cancer cells. Cancer Sci 99:1147-1154.2008).
Thompson DL, Gerlach-Bank LM, Barald KF, Koenig RJ (Retinoic acid repression of bone morphogenetic protein 4 in inner ear development. Mol Cell Biol 23:2277-2286.2003).
Tissir F, Wang CE, Goffinet AM (Expression of the chemokine receptor Cxcr4 mRNA during mouse brain development. Brain Res Dev Brain Res 149:63-71.2004).
Tohyama S, Onodera S, Tohyama H, Yasuda K, Nishihira J, Mizue Y, Hamasaka A, Abe R, Koyama Y (A novel DNA vaccine-targeting macrophage migration inhibitory factor improves the survival of mice with sepsis. Gene Ther 15:1513-1522.2008).
Valles A, Grijpink-Ongering L, de Bree FM, Tuinstra T, Ronken E (Differential regulation of the CXCR2 chemokine network in rat brain trauma: implications for neuroimmune interactions and neuronal survival. Neurobiol Dis 22:312-322.2006).
Vera PL, Iczkowski KA, Wang X, Meyer-Siegler KL (Cyclophosphamide-induced cystitis increases bladder CXCR4 expression and CXCR4-macrophage migration inhibitory factor association. PLoS One 3:e3898.2008).

Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR (Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393:595-599.1998).

This chapter will be submitted for publication, but with the following authors: Fumi Ebisu[1,2] John P. Hennessy[2,4] and Kate F. Barald[1,3,4]

1. Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-2200

2. Program in Neuroscience, University of Michigan, Ann Arbor, MI

3. Department in Cellular and Molecular Biology, University of Michigan, Ann Arbor, MI

4. Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, MI