MOLECULAR ROADBLOCKS TO MAMMALIAN HAIR CELL REGENERATION: NOTCH SIGNALING IN THE NORMAL AND TRAUMATIZED ORGAN OF CORTI

by

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Dedication

For my family and especially my parents who always supported me.

For Luke, who waited so long.

For Irene, who paved the way. For 'The Badgers,' who shared my triumphs, cried with me on doorsteps, followed me around the world, and stood up for me when no one else would.

And, for my grandfather, Raymond Batts, who worked for "the telephone company" all his life, repairing devices that were initially intended to help the deaf while stubbornly refusing to use a hearing aid until his death in the summer of 2007.

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List of Abbreviations

NICD	Notch intracellular domain
Atoh1	Atonal1
Hes1	Hairy enhancer of split1
Hes5	Hairy enhancer of split5
MAML1-3	Mastermind-like 1-3
DAPT	*N*-^{1}-S-phenylgly cine t-butyl ester
IHC	inner hair cell
OHC	outer hair cell
CSL	CBF1/RBP-jk, Su(H), Lag-1
CBF-1	Centromere-binding factor 1
Dll1	Delta-like1
bHLH	basic-helix-loop-helix
GFP	green fluorescent protein
SEM	scanning electron micrograph
TACE	TNF-alpha converting enzyme
ADAM	alpha disintegrin-metalloproteinase
RPE	retinal pigmented epithelium
Dn	dominant negative
DAPI	4', 6-diamidino-2-phenylindole
EGF	epidermal growth factor
Sox2	(sex determining region Y)-box 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
HRP	horseradish peroxidase
APP	amyloid precursor protein
PFA	paraformaldehyde
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
DMEM	Dulbecco's modified essential medium
cDNA	complementary DNA
cos7	*Cercopithecus aethiops* kidney cells

ABSTRACT

The organ of Corti is an organized mosaic of nonsensory supporting cells and sensory hair cells; the latter are vulnerable to damage from ototoxic drugs, loud noise and sound, aging, or infection. Once lost, hair cells are spontaneously regenerated in non-mammalian species, but auditory hair cell loss in mammals results in permanent hearing loss because these cells are not replaced. Avian hair cell regeneration by direct and indirect transdifferentiation is known to rely on the reinitiation of Notch signaling and subsequent transcription of the basic-helix-loop-helix (bHLH) gene *Atoh1* in areas of hair cell loss. In mammals, *Atoh1* is necessary and sufficient for hair cell development and can also force mature supporting cells to change fate to a sensory hair cell if over-expressed. *Atoh1* is antagonized in cochlear development by the Notch-dependent bHLH genes *Hes1* and *Hes5*, which direct cells to a supporting cell fate.

This dissertation includes molecular descriptions of Notch signaling proteins in the organ of Corti in late development, maturity, and trauma. The studies were aimed at elucidating whether mammals preserve the ability to reinitiate Notch signaling following trauma and to learn as many details of the response as possible. Immunohistochemistry and Western blotting of cochlear proteins demonstrate low level Notch signaling in the normal mature epithelium, and an up-regulation in signaling that occurs within twenty-

four hours of ototoxic drug administration. The response is characterized by increases in Jagged1 in the pillar cells, Notch1 in supporting cell nuclei, and Hes1 and Hes5 in the nuclei and cytoplasm of supporting cells which is reflective of their developmental locations. No Atoh1, Jagged2 or Delta1 were observed at any time following a lesion. Signaling peaks at two weeks, and diminishes to below baseline levels by two months. This observation about the up-regulation of Atoh1-repressors presents an opportunity and ideal timeframe for therapeutic Notch knockdown following hair cell loss. To develop a future tool to examine Notch knockdown *in vivo*, we engineered a recombinant adenoviral vector which expresses a dominant-negative version of the Notch signaling binding protein, Mastermind-like (MAML). This vector, Ad.dnMAML1-GFP (GFP is green fluorescent protein), presents an improvement over the traditional means of Notch knockdown by offering stable expression of a specific transcription-deficient Notch substrate fused with a GFP reporter.

Chapter 1

Introduction

1.1 Why Study Hair Cells?

Auditory hair cells are the sensory cells of the cochlea, which transduce environmental sound waves into neural information. The loss of hair cells predisposes humans to a loss in hearing sensitivity, and because hair cells are not replaced, hearing loss increases with age or after other hair cell trauma. Hearing loss is a major human health concern, especially because improvements in modern medicine continue to extend our lifespan. Lowered hearing sensitivity can significantly reduce the quality of life, since it impacts the ability to communicate and interact, appreciate music, to be aware of one's environment, or to perform everyday tasks. Finally, the loss of hair cells affects us all indiscriminately. Most people, regardless of gender, age, race, or nationality, have lost, are losing, and will continue to lose hair cells and suffer some degree of hearing loss.

Studies on hair cell protection and regeneration may help relieve one of the most prevalent and widespread disabilities in the world. Additionally, the study of the inner ear affords us knowledge of one of the most intricate and amazing mechanical devices known, the cochlea, which occupies a space of only two cubic centimeters and has over a million moving parts [2].

1.2 Auditory Structures and the Detection of Sound

Sound detection occurs when vibrations in the audible spectrum are collected by the pinnae of the outer ear and are focused down the ear canal towards the eardrum (Figure 1). Vibrations are transmitted through the eardrum to the middle ear via the ossicles, the malleus, incus, and stapes. The footplate of the stapes fits in a cochlear opening called the oval window, and transmits sound vibrations to the cochlear fluids contacting the sensory cells of the inner ear.

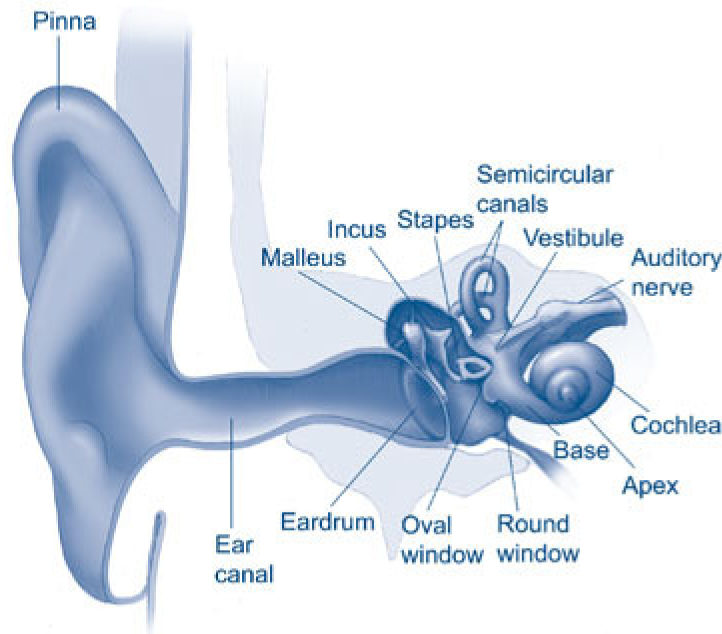

Figure 1. Cross section of the outer, middle, and inner ear structures. Source: open source Wikipedia, http://en.wikipedia.org/wiki/Cochlea.

The cochlea is a snail-shaped organ comprised of three chambers: the scala vestibuli, the scala tympani, and the scala media (Figure 2). The scala media is the membranous cochlear duct containing the sensory epithelium, the organ of Corti. The scala vestibuli is superior to the cochlear duct and ends at the oval window and the scala tympani is inferior to the scala media and ends at the round window. While the scala tympani and scala vestibuli are filled with the sodium-rich fluid perilymph, the scala media is filled with the potassium-rich fluid, the endolymph which bathes the organ of Corti. Endolymph and perilymph are separated by physical barriers, preserving their different ionic makeup. Reissner's membrane separates the scala media from the scala vestibuli and the basilar membrane separates the scala media from the scala tympani.

Sound vibrations relayed to the oval window by the stapes enter perilymph, travel through the scala vestibuli across Reissner's membrane to the basilar membrane of the scala media. Lower frequency vibrations produce maximal displacement at the distal region of the basilar membrane at the cochlea's apex, while higher frequency vibrations produce maximal displacement in the proximal region at the base of the cochlea. The vibrations exit the cochlea via the scala tympani and the round window.

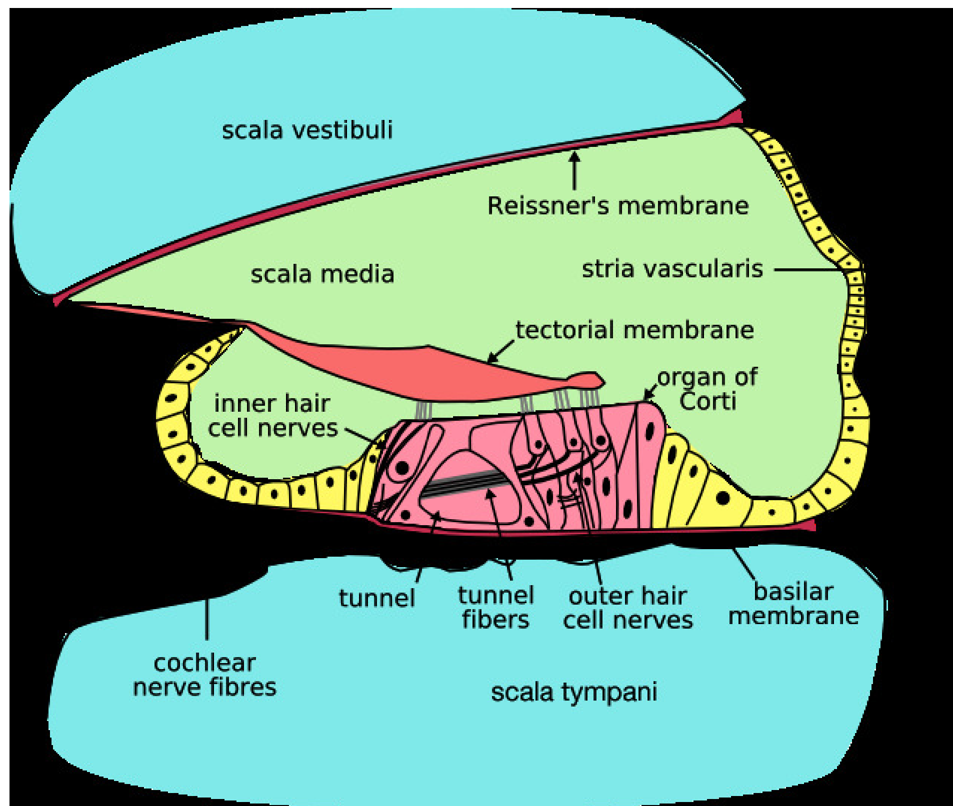

Figure 2. The chambers and microarchitecture of the cochlea. Source: open source Wikipedia, http://en.wikipedia.org/wiki/Cochlea.

The organ of Corti consists of a heterogeneous population of two types of sensory hair cells (inner and outer hair cells) and several types of non-sensory supporting cells (Figure 3, left), and sits atop the basilar membrane in the scala media.

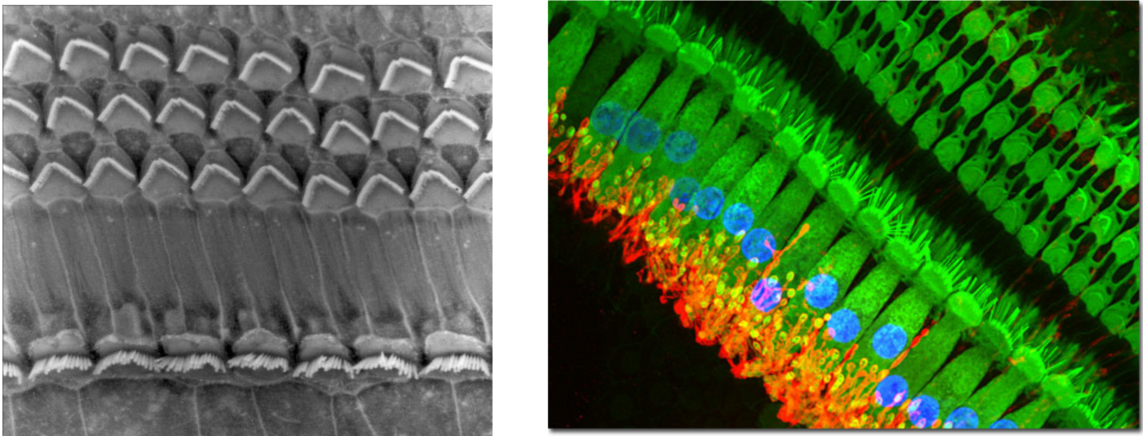

Figure 3: Scanning electron micrograph (SEM) and fluorescently-stained whole mounts of the organ of Corti. Left: A SEM illustrating the three rows of outer hair cells (top) and one row of inner hair cells (bottom), surrounded by nonsensory supporting cells. Source: Remy Pujol, Promenade 'Round the Cochlea, www.cochlea.org. Right: Confocal z-stack of a guinea pig organ of Corti whole mount stained with phalloidin to label actin (in green), DAPI to label nuclei (in blue), neurofilament to label neuronal processes (red). Source: Sonja Pyott, Department of Biology and Marine Biology, University of North Carolina, Wilmington, Wilmington, NC, USA. From Olympus BioScapes Digital Imaging Competition, http://www.olympusbioscapes.com/staticgallery/2007/4.html.

The hair cells are (mis)named for their hair-like actin-containing stereocilia (including a single long kinocilium) which protrude from the apical surface of the cell into the endolymph. The stereocilia contain mechanically-gated ion channels, which open when they are deflected towards the kinocilium and have an increased probability of being closed when deflected away from the kinocilium [3]. Endolymphatic fluid in the lumen

containing the hair cells is rich in potassium, and when these ion channels are opened, potassium as well as calcium enters the hair cell causing depolarization. Inner hair cell depolarization results in the opening of voltage-gated calcium channels, which in turn triggers neurotransmitter release into the synaptic space of afferent (spiral ganglion) nerve terminals. The neurotransmitters traverse the synaptic cleft and bind to the nerve terminals, increasing the probability of an action potential in the afferent nerve (Figure 3, right). These afferents collectively form the auditory component of the vestibulocochlear nerve (cranial nerve VIII), and transmit sound information coded as electrical stimulation to higher brain areas for processing.

Hair cells are aligned in rows, with three rows of outer hair cells and one row of inner hair cells. Hair cells are separated by supporting cells so that no hair cells are directly in contact with each other. Inner hair cells receive primarily afferent innervation from spiral ganglion neurites and are responsible for sending electrical signals to the brain via the auditory nerve [4]. The outer hair cells receive primarily efferent innervation. The stereocilia of outer hair cells are embedded in the tectorial membrane, an acellular matrix which is continuous along the length of the organ of Corti. The outer hair cells respond to stereocilia deflection similarly to inner hair cells (depolarization) but instead of neurotransmitter release, the OHCs elongate their cell bodies (somatic electromotility) using a motor protein in the membrane called prestin[3,5,6]. This serves to either amplify or dampen the vibrations which the inner hair cell stereocilia receive by exerting force on the tectorial and basilar membrane.

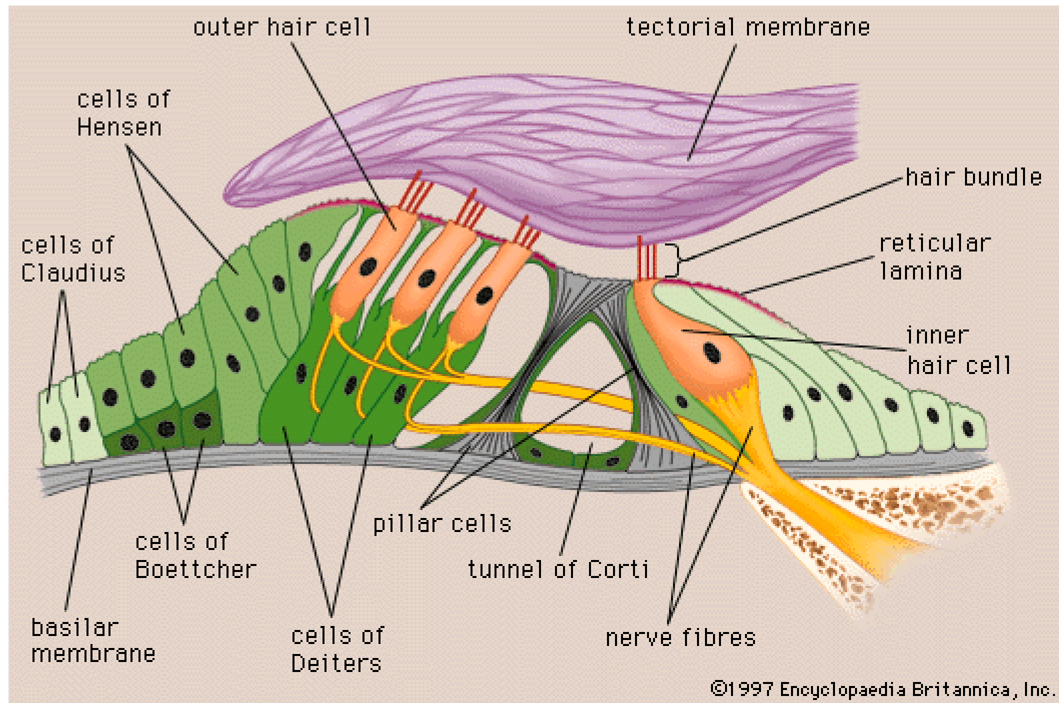

Figure 4. Sensory and non-sensory cells types comprising the organ of Corti. The left side of the figure represents the lateral (outer) edge of the epithelium while the right side represents the medial edge within the modiolus. Source: Encyclopedia Brittanica.

The supporting cells inside of the organ of Corti are the inner and outer pillar cells and the Deiters cells (Figure 4). Immediately outside the organ of Corti, towards the lateral wall, are the Hensen cells and Claudius cells, and medial to the inner hair cells are the interdental cells. Generally, the supporting cells provide structural support as well as ionic support to the hair cells, by recycling potassium back into endolymph and maintaining the fluid potential [7,8]. They are joined to one another by actin-rich gap

junctions and while they are believed to play a glial-like function, much is still unknown about their specific roles [9].

1.3 Sensorineural Hearing Loss, Ototoxicity, and Hair Cell Damage

Over 30 million people in America alone are affected by hearing loss, characterized as the full or partial decrease in the ability to detect auditory stimuli [10]. One of the most common forms of hearing impairment is sensorineural hearing loss, which refers to a defect in the cochlea. The congenital, sudden, or progressive loss of hair cells is a common cause of sensorineural hearing loss since unlike other mammalian epithelia, the organ of Corti is non-regenerating and any loss of the sensory cells is permanent [11].

Hair cells are vulnerable to damage from loud noise, environmental factors, infection, aging, and ototoxic drugs. Many drugs—such as the cancer chemotherapy drug cisplatin, aminoglycoside antibiotics, and heavy metals—are known as "ototoxic drugs" for their ability to damage hair cells[12-17]. Over the course of the lifespan of humans, as we age we are left with fewer and fewer hair cells and ever-worsening hearing. Hearing impairment is not just restricted to the elderly and appears to be increasing in incidence in young people. Reports of hearing loss increased 26% from 1971 to 1990 in those between 18 and 44 years old [18]. This places hearing loss resultant from hair cell death as an important public concern.

Exactly how hair cells die after trauma has only recently been elucidated. For many years, it was unknown whether hair cells underwent apoptosis or necrosis, were phagocytosed by lymphocytes or other cells, or were ejected from the organ of Corti into the lumen. Recent work has pointed to several avenues for hair cell death, which mainly depend on the type of insult to the hair cells. For example, following noise exposure, outer hair cells were reported to die by both apoptosis and necrosis, but louder noise resulted in more incidence of apoptosis as opposed to necrosis [19]. Conversely, following aminoglycoside ototoxicity, it is believed that the avenue for cell death is not limited to apoptosis [20]. The presence of the hair-cell specific protein prestin in supporting cells following an aminoglycoside insult points to a mechanism where supporting cells phagocytose the damaged hair cells, internalizing prestin [21]. As hair cells die, the remaining supporting cells expand to fill their empty spaces, preserving the physical integrity of the ionic barrier between the scala media and the organ of Corti (Figure 5). Expanded supporting cells which create this type of epithelium are often called a phalangeal scar to reflect its recovery from damage, but no traditional scarring from a fibroblast source is thought to occur.

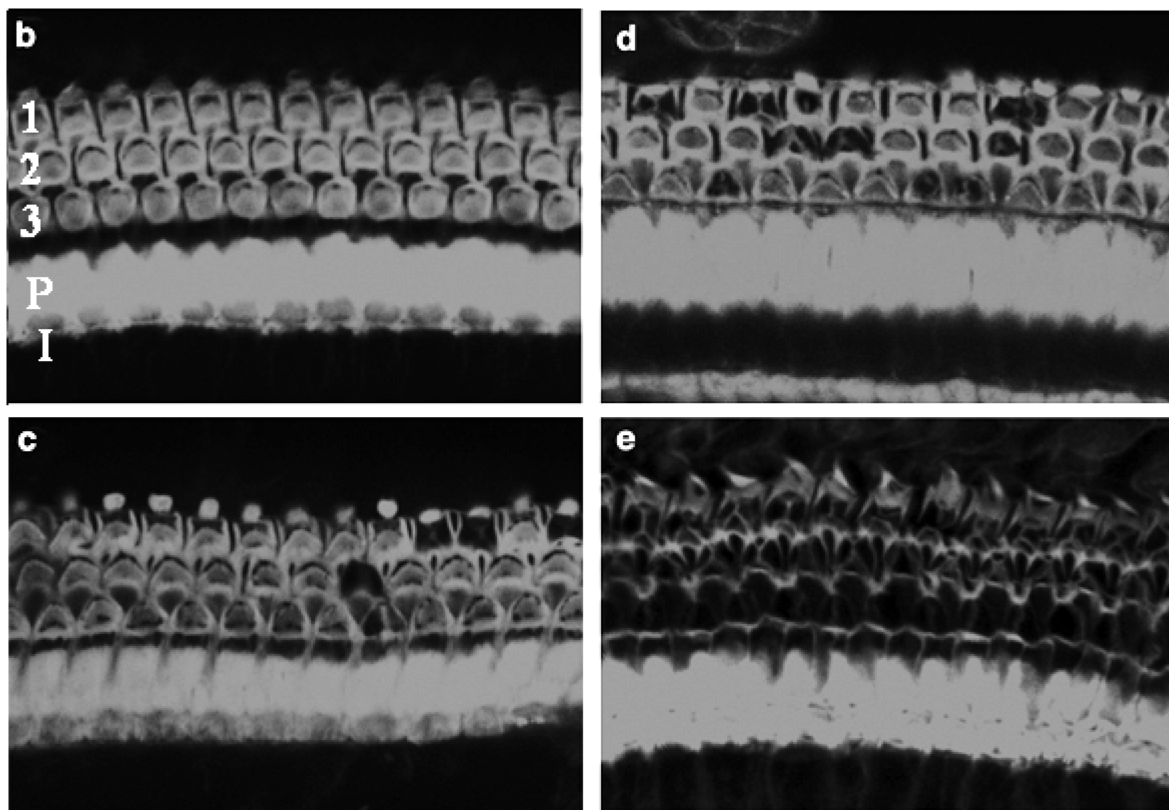

Figure 5: Progressive hair cell loss following an aminoglycoside insult in the normal guinea pig organ of Corti. Phalloidin labels actin delineating the junctions between hair cells and supporting cells. B: Normal organ of Corti with inner (I) and outer (1-3) hair cell rows, and pillar cells (P) labeled. C-E: Increasing numbers of hair cells are lost following ototoxic drug administration. Modified from Jiang et al., 2006.

In contrast to mammals, non-mammalian species such as birds, reptiles, and fish can regenerate hair cells following hair cell death in their auditory sensory epithelia. This is achieved through two known mechanisms: cell division of supporting cells resulting in a hair cell as progeny, and the direct change in cell fate from supporting cell to sensory hair cell (called transdifferentiation)[103]. In birds, hair cell regeneration can occur by either mitotic or direct transdifferentiation, or both mechanisms can occur simultaneously after

damage to the basilar papilla. The mechanism that defines the ability of non-mammalian species to regenerate hair cells, and the inability of mammals to do the same, is one of the most important and highly-debated questions of hearing research due to the repercussions such a finding might have on clinical therapies. Current treatments for sensorineural hearing loss—sound amplification via a hearing aid or direct auditory nerve stimulation with a cochlear implant—circumvent rather than mend the organic problem within the cochlea, and can be insufficient, expensive, invasive, or all of these. A great need exists for the examination of the molecular and genetic mechanisms that promote a regenerative auditory epithelium in the case of non-mammals and discourage this same process in mammals. Research into the molecular mechanism for this lack of regeneration in mammals has come to focus on gene cascades that regulate or are regulated by Notch signaling. The Notch pathway controls hair cell specification in mammalian and non-mammalian development, and avian hair cell regeneration after trauma to the auditory epithelium.

1.4 The Notch Cell Signaling Pathway

1.4.1 Signaling and Lateral Inhibition

The Notch cell signaling pathway is a highly conserved receptor-ligand signaling system present in many multicellular organisms, regardless of complexity. Notch

signaling is known to play a role in regulating a variety of stem cell types by maintaining pluripotency, directing asymmetrical cell division, and directing cell fate choices later in differentiation [22,23]. During the development of complex tissues, differentiating cells may use Notch protein signals to communicate their intended fates to surrounding cells in order to prevent homogenous cell fate choices. This is lateral inhibition, a process whereby initially equivalent cells that produce a ligand force neighboring cells to produce less of that ligand [24,25]. This in turn enables the ligand-producing cells to increase production even more significantly. In this way, initially small differences in Notch proteins between adjacent cells are gradually increased as development progresses, affecting gene transcription and ultimately cell fate (Figure 6).

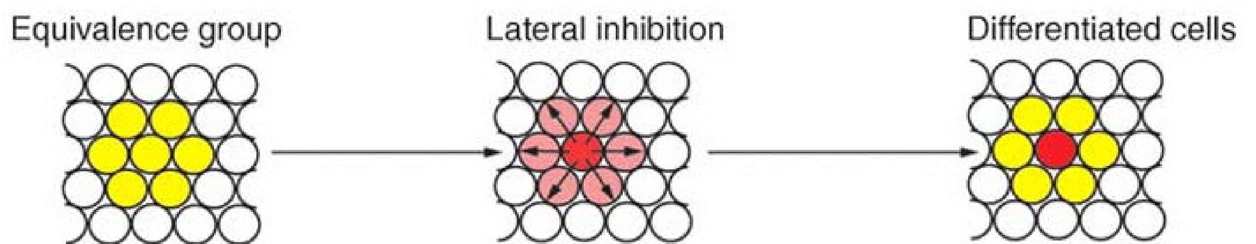

Figure 6: Model of lateral inhibition. From: Ehebauer et al. 2006[25].

Vertebrates possess four different types of Notch receptors (Notch1, Notch2, Notch3, and Notch4) as well as several types of ligands (Jagged1 and 2, Delta-like 1-4) [24]. The receptor is a single-pass transmembrane receptor with a large extracellular domain composed primarily of cysteine knot motifs and epidermal growth factor (EGF)-like

repeats [26]. Both the interior and exterior portions of the receptor are capable of being cleaved. The interaction of a Notch receptor in one cell and the ligand in an adjacent cell leads to two cleavage events in the membrane (Figure 7)[27]. The first cleavage event mediated by an alpha disintegrin-metalloproteinase (ADAM) called TNF-alpha converting enzyme (TACE) occurs in the extracellular domain of the Notch receptor near the transmembrane region [28]. This releases the extracellular portion of the receptor, and generates a substrate for the next cleavage event by γ-secretase (also called the presenilin complex) in the transmembrane domain. This second cleavage event results in the liberation of the Notch intracellular domain (NICD) and is the crucial signaling event in the Notch cascade. NICD travels to the nucleus to form an 'activator complex' with CSL and Mastermind (MAML), and it is this unified complex that facilitates Notch-activated gene transcription [29-36].

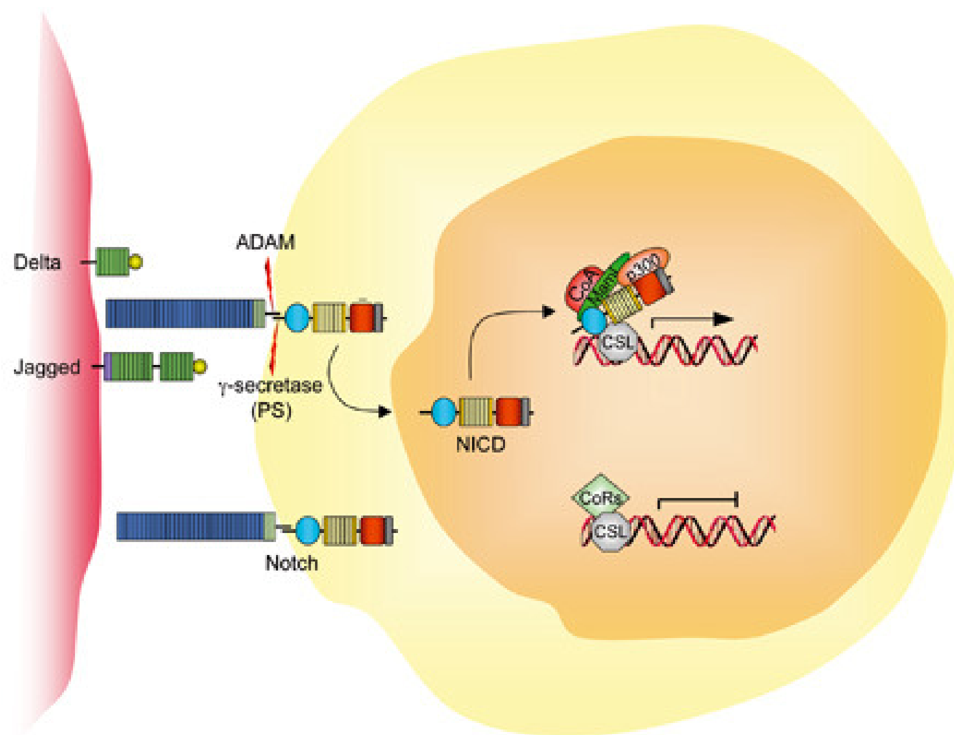

Figure 7: Canonical Notch signaling model. From: Radtke et al, 2006[27].

This activator complex positively affects the transcription of the basic-helix-loop-helix (bHLH) genes in the Hairy-enhancer-of-split family (Hes genes) while in the absence of the NICD signal, this same complex represses Hes transcription [37,38]. In this way the presence of NICD can drastically affect the production of proteins that influence one of two possible fates. Notch signaling through the expression of Hes can also up-regulate the expression of Notch itself [39]. There is continuous recycling and manufacturing of the Notch ligands and receptors during signaling; the process is not static. During degradation, the receptor becomes mono-ubiquitynilated, targeting it to the

lysosome for degradation. NICD in the nucleus is degraded in a proteasome-dependent manner. The exact site of NICD degradation is not known but is believed to be outside the nucleus[24,25].

The Notch system was first identified in 1917 by Thomas Hunt Morgan in *Drosophila*, and was so named for the abnormally "notched" wings in flies where the signal had been disrupted[23,40]. The sequencing and molecular analysis of Notch proteins was undertaken in the 1980s, with rapid advances in knowledge of the system occurring in the last 20 years. Due to the well-conserved nature of the system and its component proteins (e.g., flies have *Serrate* while mammals have two *Jagged* ligand equivalents), examples in fate choices directed by Notch in flies have provided good evidence for what happens in higher order animals[41-43]. In the *Drosophila* nervous system, Notch is involved in switching epidermal progenitor cells between two alternative fates: non-neural versus the neuroblast fate, and between two types of neurons [44,45]. By lateral inhibition mediated by Notch and its ligand Delta, the number and placement of fly neuroblasts in the neuroepithelial sheet are determined and disruptions in this patterning yield either too many or too few of a particularly cell type.

In the developing vertebrate, Notch receptors and ligands are expressed broadly, in varying concentrations, and are essential to proper cell patterning and developmental progression. Because of the widespread dependence on Notch, disruptions in Notch signaling can be devastating and often fatal in earlier stages of development [46,47]. During nervous system development, *Notch1* and its ligands *Delta* and *Jagged* are widely

expressed in the germinal zones during development [48,49]. Constitutive expression of *Notch* in an embryonic cell line suggests that its activation might make progenitor cells differentiate into glial cells rather than neurons [50,51]. Over-expression of the ligand *Delta1* (resulting in Notch activation) suppresses neurogenesis, whereas over-expression of a dominant negative inhibitor of Delta1 leads to premature commitment of stem cells to the neuronal fate [52].

In maturity, Notch signaling is generally found to limit cell proliferation at around adolescence and to either down-regulate drastically or serve to maintain adult stem cell populations. In the majority of differentiated cells types, Notch signaling is quiescent. However, in regenerating tissues such as the olfactory epithelium, the tastebuds of the tongue or the intestinal crypt cells lining the gut, Notch-mediated gene transcription plays a vital role in maintaining and directing the continual turnover of cells [53-55]. Other tissues, like the liver, can be prompted to engage in regeneration and repair when they suffer significant cellular trauma and can be rejuvenated when Notch is up-regulated [56,57]. In the adult central nervous system, Notch is present in the neurogenic zones in both ependymal and subependymal areas [58,59]. Notch is also expressed on cells which are not stem cells per se, but have stem cell-like properties. For example, Notch is found on the long-lived adult oligodendrocyte O-2A progenitors that have stem cell-like properties, and Jagged1 is expressed by mature oligodendrocytes and neurons [60]. Activation of Notch on the adult O-2A cells may maintain them in an immature state. Another example of this is dental pulp cells, which express Notch after development and have stem cell-like qualities and

require the reinitiation of Delta1-Notch signaling in order to regenerate [61]. Disruptions in Notch signaling in maturity are responsible for at least three human diseases, T cell acute lymphoblastic leukemia, Alagille syndrome, and cerebral autosomal dominant arteriopathy, as well as a variety of cancerous tumors [62-67].

1.4.2 Notch in Cochlear Development

In the developing mammalian cochlea, the Notch1 receptor and the ligands Jagged1, Jagged2, and Delta-like1 (Dll1) have been identified [68]. Notch-Jagged1 signaling is required for the initial specification of the prosensory area which will eventually become the organ of Corti [69]. As development progresses, a diverse matrix of sensory and non-sensory cell fates is generated through lateral inhibition and the restriction of the prosensory gene *Atonal1* (*Atoh1)* to hair cells and Hes to supporting cells (Figure 8). This is achieved as nascent hair cells increase Dll1 ligand production, causing the surrounding cells to accumulate the cleaved intracellular portion of the Notch1 receptor (NICD) and to increase the transcription of *Hairy enhancer of split1* (*Hes1)* or *Hes5*. Notch signaling down-regulates Atoh1 by up-regulating its repressors (Hes1 and Hes5), and in the absence of Notch signaling, Atoh1 is up-regulated [70,71]. Sufficient repression of Atoh1 by Hes directs a cellular subset to a supporting cell fate [72].

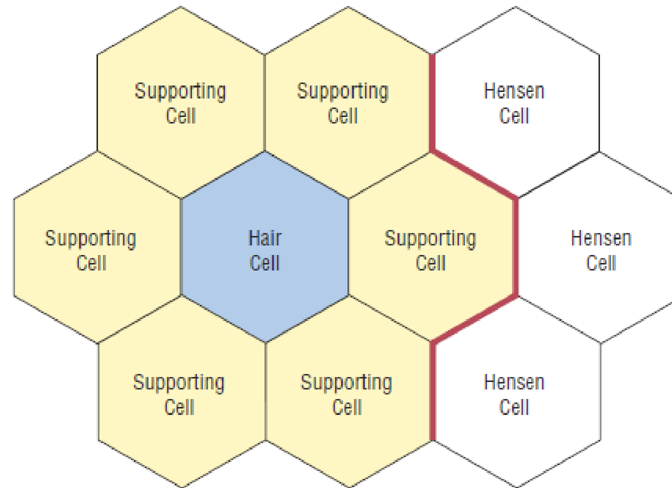

Figure 8: Lateral inhibition generates diverse cell fates in the organ of Corti. Atoh1 becomes restricted to hair cells (blue), and the surrounding cells are directed to a nonsensory fate through Notch-mediated Hes expression. From Woods et al., 2004[76].

In mice, at embryonic day 8.5 (E8.5) the otic placode gives rise to the otocyst and around E11 there is a ventral outgrowth which will become the cochlear duct. Hair cell development begins first in the vestibular system around E12 and follows in the cochlea one day later [73]. Hair cell development takes several weeks to complete and it is not until postnatal day 14 (P14) that murine hair cells are functional[a]. Before E12, the cochlear duct contains proliferating cells which give rise to three separate structures: the prosensory (sex determining region Y)-box 2 (Sox2)-positive sensory area (where hair cells and supporting cells will arise in the organ of Corti), and the nonsensory inner and

[a] This is in contrast to other mammals such as guinea pigs and humans who are born hearing, with fully functional hair cells.

outer sulci (which will contain only supporting cells) [74]. Through the activity of $p27^{kip1}$, progenitors in the sensory area exit the cell cycle around E13-14 first at the apical region of the cochlea and last at the base [75]. As the wave of mitosis ends, the transcription of the prosensory bHLH gene *Atoh1* begins in cells in the prosensory domain [73,76]. Atoh1 is required for a cell to assume a hair cell fate, and its deletion results in an absence of hair cells [76,77]. Conversely, over-expression of Atoh1 in the developing organ of Corti or in the greater epithelial ridge leads to nearly all those cells assuming a hair cell fate [78 76]. This prosensory role continues to exert an effect even in a differentiated cell: Atoh1 can force mature supporting cells in the adult guinea pig to transdifferentiate into hair cells [79,80].

The Notch1 receptor is initially widely distributed throughout the cochlear epithelium following the specification of the prosensory domain. As hair cell commitment continues, the ligands Delta1 and Jagged2 become restricted to nascent hair cells while the ligand Jagged1 becomes restricted to supporting cells by postnatal day three[70,74]. Similar investigations into the developmental patterns of Notch gene expression in the chick basilar papilla (the avian equivalent of the organ of Corti) have shown similar down-regulations of *Serrate1* (homolog to *Jagged1*) and *Notch1* in hair cells as development progresses [81].

Multiple mouse mutant studies have been performed to confirm the role of Notch in cochlear development, knocking out specific components of the Notch pathway. In *Notch1*$^{-/-}$ mutants supernumerary inner hair cells (IHCs) and outer hair cells (OHCs) were

generated, suggesting that Notch activation in later cochlear development serves to restrict hair cell number [68]. Mutants that lacked *Hes1* or *Hes5* expression, the product of Notch activation, exhibited an extra row of IHCs and OHCs, respectively [71]. The population of supporting cells was markedly decreased in these mutants, suggesting that cells that normally would have been destined for a nonsensory fate did not receive proper lateral inhibition of prosensory genes, and developed into hair cells. A similar phenomenon was observed when anti-sense to the Notch ligand Jagged1 was applied to developing mouse organ of Corti *in vitro*. Supernumerary hair cells were observed and there were extra rows of both IHCs and OHC [69,70]. Additionally, over-expression of Hes1 was sufficient to inhibit Atoh1-induced hair cell formation in the greater epithelial ridge [72]. These results are strong evidence that Notch signaling and downstream bHLH gene transcription together play a major role in determining the right amount of sensory and nonsensory cells in the organ of Corti and that disruption to either the signaling or to the downstream targets can sway the outcome.

1.5 Differentiated Cell Fate Conversion: Transdifferentiation

1.5.1 Spontaneous Transdifferentiation

After development, with few exceptions, cell fate is permanently fixed. However, in some cell types, fate conversion does occur under special circumstances via

transdifferentiation. Transdifferentiation is the direct conversion of a differentiated cell type from one fate to another fate and may occur with or without cell division. Direct transdifferentiation without cell division requires that drastic and permanent changes in gene transcription occur in a differentiated cell, sufficient for a change in fate to occur [82-84]. Indirect transdifferentiation describes a process whereby a progenitor-like differentiated cell divides into two daughter cells, and one or both of the daughter cells assume a different fate from the parent cell. Transdifferentiation by either means is uncommon in nature, but experimentally induced transdifferentiation has been documented in several tissue types[79,84,85] [86]— including the mature nonsensory cells of the organ of Corti following *Atoh1* over-expression. A few examples have been observed in which transdifferentiation occurs spontaneously as part of a reparative process, one of which is the avian basilar papilla during hair cell regeneration [87]. Although a full understanding of the molecular mechanism guiding the process of transdifferentiation has yet to be achieved, the study of transdifferentiation is worthwhile as it may provide clues into how nonsensory cells of the inner ear might be coaxed into sensory fates.

The most common occurrence of transdifferentiation is probably metaplasia. In these cases, gene expression patterns in a specific cell type change and as a result the cell attains a different morphology and behavior, oftentimes contributing to tumorigenesis [88,89]. For example, Barrett's metaplasia is an example of a pathological condition where the stratified squamous epithelium that normally lines the lower part of the esophagus transdifferentiates into an epithelium with features of the intestinal lining. Several genes

have been shown to have altered expression in cells of Barrett's metaplasia [90,91] but the initial trigger for the transdifferentiation is yet to be identified. Transdifferentiation has also been shown in the muscularis layer of the esophagus. Specifically, smooth muscle cells can transdifferentiate to skeletal muscle cells in the developing mouse esophagus. It was shown that transdifferentiation is the fate of all smooth muscle cells in normal development of the upper esophagus [92]. Spontaneous transdifferentiation can, in some cases, be found in response to an experimentally- induced lesion. In fish and in developing chick embryos, if the retinal sensory receptor cells (rods and cones) are traumatized, retinal pigment epithelial (RPE) cells can transdifferentiate to become new sensory cells [93,94]. However, RPE cells transdifferentiate without division, resulting in the eventual depletion of the cell population, pathological eye disease, and the inability to continuously regenerate photoreceptors.

1.5.2 Transdifferentiation in Avian Hair Cell Regeneration

In terms of a therapy for cochlear hair cell loss, the most pertinent example of robust trauma repair based on spontaneous transdifferentiation is found in the basilar papilla of birds. While the inability to replace auditory hair cells is typical of all mammals, other vertebrates do have the ability to regenerate hair cells [95,96]. In the absence of stem cells or undifferentiated basal cells, regeneration in the inner ear sensory epithelium must depend on direct or indirect transdifferentiation. Studies in birds, using

noise overstimulation or ototoxic drugs (both of which eliminate many of the original hair cells), have shown that in response to the lesion, supporting cells can divide and the daughter cells become new hair cells [97-101]. Indirect avian supporting cell to hair cell transdifferentiation includes a de-differentiation step where the cell rounds up near the luminal surface and divides [100,102]. After division is complete, daughter cells start to differentiate toward their phenotypic fate. In other cases, avian transdifferentiation from the supporting cell to the hair cell phenotype takes place without mitosis [82,103,104].

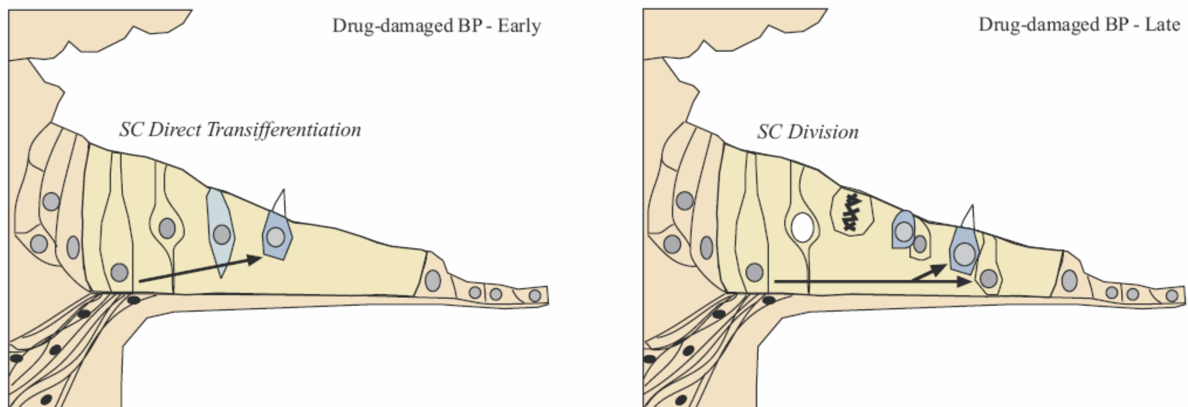

Figure 9: Avian hair cell regeneration occurs through direct (nonmitotic, left panel) and indirect (mitotic, right panel) transdifferentiation. Following hair cell loss, the reinitiation of Delta-Notch signaling induces some nonsensory cells begin to sequester Atoh1 (blue) and transdifferentiate to hair cells. From Stone, 2007 [105].

In the mature avian basilar papilla, Atoh1 is not detectable but it becomes reactivated in supporting cells engaging in either direct or indirect transdifferentiation in response to trauma [105-108] (Figure 9). This increase in nuclear *Atoh1* occurs within a few hours of the insult, while hair cells are still intact, and persists in supporting cells which

change fate. Important changes in Notch signaling occur in the regenerating basilar papilla simultaneous with changes in Atoh1 expression. In the normal, quiescent basilar papilla, supporting cells actively transcribe Notch and its ligand Serrate (homolog of Jagged), but no transcription of the ligand Delta is seen [87]. However, by the third day following gentamycin administration, dividing supporting cells in the basilar papilla begin to transcribe Delta and eventually high levels of Delta are seen in daughter cells which differentiate into hair cells. By 10 days post gentamycin, Delta is highly down-regulated and regeneration is nearly complete [87]. Other components of the avian Notch signaling system, like Serrate, were not reported to change in response to trauma. This suggests that supporting cells in the avian basilar papilla can respond to trauma and hair cell death by up-regulating Notch-Delta signaling events which positively affect prosensory *Atoh1* transcription. Furthermore, it suggests that Notch-Serrate signaling persists in the basilar papilla post-developmentally and that this signaling in maturity may serve to maintain cell fate and prevent transdifferentiation until hair cells are lost. During the regenerative process in the basilar papilla, Serrate-Notch signaling is not seen to change and may be out-weighed by the large increase in Delta-Notch interaction.

1.5.3 Transdifferentiation in the Mature Organ of Corti

The observation that non-sensory cells of the avian inner ear can spontaneously undergo transdifferentiation to produce new hair cells provided a possible means to

induce such transdifferentiation in mammals. The discovery of genes, particularly *Atoh1,* that induce hair cell development was instrumental for prompting experiments into the forced transdifferentiation of mammalian non-sensory cells into hair cells. However, while mitotic cells are seen in birds following hair cell loss, this is not the case following hair cell loss in mammals [100]. This means that *Atoh1*-induced transdifferentiation in mammals would likely be direct as opposed to indirect. Re-expression of *Atoh1* in injured adult organ of Corti appears to force cells into the hair cell phenotype. The first proof for this principle was demonstrated by expressing *Atoh1* in cultured explants of the rat organ of Corti, and observing an increase in hair cells [78, 138]. *Atoh1* over-expression could also induce transdifferentiation of cultured dissociated supporting cells into the hair cell phenotype [109]. The appearance of ectopic hair cells following *Atoh1*-over-expression in normal mature guinea pigs illustrated that nonsensory cells outside of the organ of Corti proper could transdifferentiate in to hair cells [80]. Follow-up experiments using guinea pigs with aminoglycoside-traumatized organs of Corti showed that over-expression of *Atoh1* could generate new hair cells in the organ of Corti itself, resulting in improved auditory thresholds as compared to deaf controls [79]. Some of the cells that were found in deafened ears treated with *Atoh1* appeared as nearly normal hair cells in their proper location and orientation, whereas other cells appear as supporting cells with irregular stereocilia, providing evidence of incomplete transdifferentiation. A report describing the delivery of *N*-[1]-S-phenylgly cine t-butyl ester (DAPT), an inhibitor of gamma-secretase cleavage of Notch, to the traumatized guinea pig organ of Corti showed myosinVIIa-

positive cells in the organ of Corti proper [110]. However, these myosin positive cells did not look like normal hair cells, suggesting that transdifferentiation may have begun but was not successfully concluded.

While true transdifferentiation of mammalian supporting cells to fully functional, perfect hair cells remains to be shown, these experiments using *Atoh1* over-expression and DAPT delivery demonstrate that developmental genes can induce a partial and permanent change of a nonsensory cell to a hair cell-like cell in the mature organ of Corti. Perhaps more importantly, they illustrate that the fate of differentiated supporting cells is malleable given the right stimulus.

1.6 Summary

Sensorineural deafness is the most common sensory disorder in the Western world, representing a prevalent disability negatively impacting both the quality of life and individual productivity and communicative ability of many people. Rapid progress has been made in the past few decades towards discovering genes and signaling events involved in the development of the auditory system in mammals, and these studies have prompted attempts to recapitulate pro-hair cell events in the mature deaf ear. This approach makes sense, as hair cell regeneration in the avian basilar papilla is more or less a recapitulation of the series of events in development which lead to initial hair cell

specification. These events are controlled by the reinitiation of Notch-Delta signaling and are coupled with *Atoh1* expression, and supporting cell transdifferentiation in areas of hair cell loss.

Studies in mammals, which force the expression of the prosensory gene *Atoh1,* illustrate that supporting cell to hair cell transdifferentiation in the mature traumatized organ of Corti is possible given the right molecular cues and timing. But the incompleteness of transdifferentiation in *Atoh1*-over-expression studies paired with the lack of robust and successful regeneration in animals given a gamma-secretase inhibitor following hair cell trauma suggests that there may be powerful inhibition of transdifferentiation in the sensory epithelium preventing regeneration from occurring spontaneously. However, that signal has not been described, leaving a large gap between what we know happens (transdifferentiation does not occur spontaneously) and what we know is possible (*Atoh1* can force transdifferentiation in mature supporting cells). In order to design human therapies that repair the natural ability of the cochlea to transduce sound by regenerating hair cells, it is important to first determine why hair cell regeneration does not occur. The molecular response of the mammalian auditory epithelium to trauma and hair cell loss is not well characterized. In particular, determining the activity of the Notch signaling system following a hair cell lesion has fundamental clinical implications because it may provide a mechanism for converting existing supporting cells into hair cells.

1.7 Scientific Aims and Hypotheses

This dissertation describes three scientific aims. The first aim was to determine whether Notch signaling persists in the mature mammalian auditory epithelium and to determine which components of the signaling cascade are present. This study also served as a baseline for the second aim: to examine Notch signaling following ototoxic trauma resulting in hair cell loss. Particularly important was the determination of parallels and differences between the molecular response to trauma in the organ of Corti versus the response in the birds basilar papilla, and whether proteins whose transcription is known to be regulated by Notch (the bHLH genes Hes1, Hes5, and indirectly Atoh1) changed in parallel with signaling. The third and last aim, built upon the previous two, involved the design of a substrate-specific and long-lasting inhibitor of Notch signaling which can be used as a future tool to study the effects of Notch signaling reduction following hair cell loss in the organ of Corti.

We hypothesized that low-level Notch may persist in the mature organ of Corti, providing signals to supporting cells to maintain their fate. This was based on the knowledge that supporting cell fate is malleable and capable of at least partial transdifferentiation under the right circumstances. We also hypothesized that Notch signaling would be reinitiated in the traumatized organ of Corti, but that the signaling components and downstream transcription targets would be different from what had been reported during avian hair cell regeneration. Specifically, we hypothesized that the

reactivation of Notch signaling in supporting cells would not involve the ligand Delta1as in birds, and would ultimately result in the transcription of the bHLH genes *Hes1* and/or *Hes5* as opposed to the prosensory bHLH gene *Atoh1*. *Atoh1* was hypothesized to be absent as its presence in mature cells is associated with transdifferentiation to a hair cell fate, which is known not to occur in mammals. However, the presence of Hes proteins after ototoxicity would provide a logical explanation behind why transdifferentiation did not occur in mammals.

Chapter 2

Notch Signaling in the Late Developmental and Mature Normal Organ of Corti

2. 1 Introduction

Over the past 15 years, the important role of Notch signaling in mammalian cochlear development has emerged. Notch1 and Jagged1 are critical for the initial specification of the prosensory domain which will become the organ of Corti, and signaling events involving Jagged1, Jagged2, and Delta1 direct fate choices between sensory and nonsensory cells types [74,111,112]. Cell fate heterogeneity is accomplished via lateral inhibition of adjacent cells— Notch signaling positively regulates the expression of the bHLH genes *Hes1* and *Hes5*, which antagonize the expression of the prosensory bHLH gene *Atoh1* [69,73,74,76,109,113-115] . However, the status of Notch signaling in the late developmental and completely mature mammalian organ of Corti has not been well

described. Notch signaling does persist postnatally in many epithelia with long-lived cell types or rapid cell turnover, and is reported to persist as Serrate-Notch signaling in the bird basilar papilla [87]. So, it is possible that Notch signaling also persists in the adult mammalian ear as potential means for maintaining supporting cell fate.

Quite a few examples of postnatal Notch signaling exist in vertebrates. Particularly, Notch signaling continues to be active to maintain populations of adult stem cells or in epithelia with high turnover rate and frequent repair. One such system is the mammalian brain, which must constantly form new connections (i.e., for learning and memory) and repair or prune older neurons and glia. While Notch signaling is not essential for the generation of neuronal progenitors, it is essential for their maintenance. Notch signaling regulates the differentiation of neurons and glia from adult neuronal stem cells produced postnatally in a few locations (for example, the subventricular zone); such cells migrate to other locales as needed [49,116,117]. The ablation of Notch signaling leads to cell cycle exit by neuronal progenitors and the beginning of migration, while over-expression of Jagged1-Notch activity induces re-entry into the cell cycle and ectopic neurogenesis [48]. In adult animals deficient in the bHLH transcription factor *Hes1*, the adult neuronal progenitor population is severely limited, reinforcing that Notch-dependent Hes activity controls the cell cycle of progenitors [118]. Thus, in the adult mammalian brain, Notch signaling and subsequent bHLH gene transcription persists to provide the signal for progenitors which can either enter or exit the cell cycle, required for ongoing neuronal plasticity.

Notch also regulates cell fate choices in some types of epithelia. Notch-Delta signaling promotes the differentiation of astrocytes, radial glia, and Muller cells in the zebrafish retina[119-121]. In the epidermis, Notch promotes the differentiation of hair follicles while Notch inhibition causes proliferation [122]. The mature intestine requires the simultaneous activity of Wnt and Notch signaling and subsequent high levels of Math1/Atoh1 to maintain cell turnover of the epithelium. Intestinal stem cells reside near the base of the "crypts" in the intestine, and there, four distinct cell types are constantly being produced to replace the sloughed-off old intestinal lining cells [123,124]. Delta-Notch signaling occurs at the base of the crypts and can be activated by Wnt signaling [125]. Hematopoietic stem cells are also regulated by the dual activity of Wnt and Notch signaling; Wnt expression activates Notch, which in turn signals for self-renewal [126]. Notch activation prevents B-cell differentiation, but has been shown to promote the T-cell lineage [127] [128] [129]. Notch signaling is also essential for proper maintenance of muscle tissue in adults. Following trauma, Notch signaling must be up-regulated via the increased production of the ligand Delta1 for efficient activation of muscle stem satellite cells; interference with Notch signaling reduces the ability of muscle to heal [130,131]. Furthermore, the ability of Notch to regulate healing diminishes as we age. Aged muscle fails to up-regulate Delta following injury, although proper muscle healing was restored by activating Notch signaling with Delta [132].

While the organ of Corti is not spontaneously regenerative, it has been shown that mature supporting cells can switch their fate via transdifferentiation if *Atoh1* is over-

expressed [79]. Therefore mammalian supporting cells, while differentiated and mature, have a malleable fate similar to "progenitor-like" cells in other epithelia which continue to express Notch into adulthood. Additionally, expression of the Notch1 receptor and ligand Serrate1 (homolog of Jagged1), persist in the normal adult avian basilar papilla [87]. In order to provide a better picture of the molecular differences between the regenerative basilar papilla and the non-regenerative organ of Corti, the initial status of the Notch signaling system in healthy tissue needs to be investigated. Prior work has varied widely in the methods used to examine the question. Conflicting findings have been reported—Jagged1 was reported to be expressed in postnatal guinea pig supporting cells in one brief report, [110] while all Notch molecules were reported to be absent in mature mouse organ of Corti by another set of authors [133].

The need exists to investigate the presence or absence of members of the Notch signaling system in the normal organ of Corti, with particular focus on the known downstream targets of active signaling, the bHLH proteins Hes1 and Hes5. These findings would shed light on the molecular mechanisms known to define cell fate in the organ of Corti in development and known to persist in many regenerative epithelia including the avian basilar papilla.

Guinea pigs are an ideal animal model for studies involving ototoxicity, as they are born with completely mature, functional inner ears. Therefore, to observe late developmental Notch signaling in the inner ear, we performed immunohistochemistry with antibodies to Notch molecules (Jagged1, Jagged2, Delta1, Notch1, Hes1, Hes5, and

Atoh1) on postnatal day seven mouse cochleae and performed identical immunohistochemistry on guinea pig adult organ of Corti. Specificity of immunohistochemical data was verified by examining the presence of the Notch proteins in dissected cochlear lysates separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2 Materials and Methods

Animals

All animal experiments were approved by the University of Michigan Institutional Committee on Care and Use of Animals (UCUCA) and performed using acceptable veterinary standards. To examine Notch signaling in the mature organ of Corti, we used 10 pigmented adult male guinea pigs (Elm Hill Breeding Laboratory) with a normal Preyer's reflex which weighed between 250-400g (about 2 months old) at the beginning of the experiments. To observe the late developing postnatal ear, we used 15 P7 male mice. Animals were housed at the Animal Care facility at the University of Michigan. Prior to all sacrifice, animals were anesthetized with a subcutaneous injection of xylazine (10 mg/kg) and ketamine HCl (40 mg/kg).

Immunocytochemistry

Animals were deeply anesthetized with xylazine and ketamine as above, decapitated, and the temporal bones were removed. The apical tip of the otic capsule was removed, the oval window opened by dislodging the stapedial footplate, and the round window opened with forceps. Paraformaldehyde (PFA) 4% in phosphate buffered saline (PBS) was locally perfused through the cochlea. The temporal bone with cochlea *in situ* was allowed to fix for two hours, at which time the bony capsule was dissected away and the modiolus with surrounding cochlear duct removed. These cochleae were stripped of the stria vascularis, tectorial membrane, and Reissner's membrane to fully expose the organ of Corti. Specimens were washed three times for ten minutes in PBS and permeabilized using 0.1% Triton X-100 in PBS for ten minutes. Tissue was then blocked using 10% normal goat serum (Jackson Immunoresearch, West Grove, PA) in PBS for thirty minutes at room temperature. Following blocking, specimens were incubated overnight at 4°C in a 1:100 primary antibody solution in PBS. Primary antibodies used were raised in rabbit and are as follows: a) an antibody against the C-terminus of Notch1 (Abcam, ab27526, lot# 181697; whole receptor = ~280 kDa, cleaved portion NICD = 120 kDa), b) Jagged1 (Santa Cruz, H-114, sc8303, lot # E0604; 150 kDa), c) Jagged2 (Santa Cruz, H-143, sc5604, lot# K1904; 150 kDa), d) Dll1 (Santa Cruz, T-20, sc9932, lot# C1505; 75 kDa), e) Atoh1/Math1 (Abcam, ab22270-100, lot# 151411; 38 kDa), f) Hes1 (Chemicon, Ab5702, lot# LV1438380; ~30 kDa), and g) Hes5 (Chemicon, Ab5708, lot# 0511016244; ~20 kDa). The following morning, samples were washed three times in

PBS and incubated in a Rhodamine (TRITC)-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, 111-025-144) for thirty minutes at room temperature. All samples were additionally incubated with Alexa Fluor 488 conjugated phalloidin (Molecular Probes Invitrogen, Carlsbad, CA) for twenty minutes. If visualization of the nucleus was desired, samples were incubated in the DNA-binding fluorescent stain 4', 6-diamidino-2-phenylindole (DAPI) diluted 1:600, for three minutes. The cochleae were then washed three times for five minutes in PBS, dissected into four pieces (apex, 3rd turn, 2nd turn, base) and each segment mounted onto a slide with Gel/Mount mounting media (Biomeda, Foster City, CA). The second and third turns were used for analysis.

Confocal Microscopy

Immunohistochemically-processed cochlear whole mounts were examined with a Zeiss LSM 510-META Laser Scanning Confocal Microscope mounted on a Zeiss Axiovert 100M inverted microscope, equipped with four lasers: a Coherent Enterprise laser for UV (351,364 nm), an Argon laser for FITC/GFP (458, 488, 514 nm), a Helium Neon 1 laser for Rhodamine, Texas Red, and Cy3 (543 nm), and a Helium Neon 2 laser for Cy5 (633 nm). Wavelengths utilized in this study include 364 nm (for DAPI), 488 nm (for Alexa 488-conjugated phalloidin), and 543 nm (for Rhodamine). Scanning was performed with all lasers simultaneously in the multi-track setting and META-analysis

provided fine separation of wavelengths and the ability to track all dyes in the sample simultaneously. The Linear Unmixing function was used to separate mixed signals pixel by pixel, using the entire emission spectrum of each dye in the examined specimen and eliminating broadband autofluorescence. Scans were recorded in 1µm single images or 1µm z-stack series at 1.5-sec intervals, at 40x or 60x magnification with zoom. Confocal images were cropped and labeled in Adobe Photoshop.

Western Protein Analysis of Dissected Cochlear Lysates

Cochlear lysates of postnatal mouse and adult guinea pigs were examined for the presence of Notch proteins. Cochleae were dissected from the temporal bone and the auditory nerve and as much bone and tissue as possible were removed from the organ of Corti with a fine needle under a stereoscope. Remaining tissue was incubated in cold T-Per Lysis Buffer (Pierce Biotechnology, Thermo Fischer, Rockford, IL) for 30 minutes then homogenized with a small plastic pestle. The homogenate was kept on ice a further 30 minutes then spun in a centrifuge until a visible pellet formed. The supernatant was retained and the amounts of protein standardized (30 µg). Separation of proteins was achieved using Nu-Page SDS-PAGE with X-Cell SureLock system and Novex 4-12% Bris-Tris gels with MOPS buffers (Invitrogen, Carlsbad, CA). Separated proteins were transferred to a PVDF membrane by electrophoresis. Afterwards, the membrane was blocked with 5% dry milk in PBS for an hour and then incubated in 1% milk in PBS plus

the primary antibody (1:5000) overnight at 4°C. Following three brief washes in TBS-T, the membranes were incubated for 30 minutes in a complementary horseradish peroxidase (HRP)-conjugated secondary antibody solution (1:8000) in 5% dry milk in TBS. The membranes were again washed in TBS-T three times for 10 minutes each. Developing fluid (Pierce Biotechnology, Thermo Fischer, Rockford, IL) was applied to the membrane, and film was quickly placed on the membrane in the dark and developed. Average film development time was one minute in a sealed cassette. Film was then processed in the dark and the film was digitally scanned. The scan was inverted and sharpened in Adobe Photoshop. Cell lysates RAW 264.7 and ECV304 (Santa Cruz Biotechnology, Santa Cruz, CA) were used as positive controls of the proteins Jagged1 and Dll1 respectively, and both contained the Notch1 receptor.

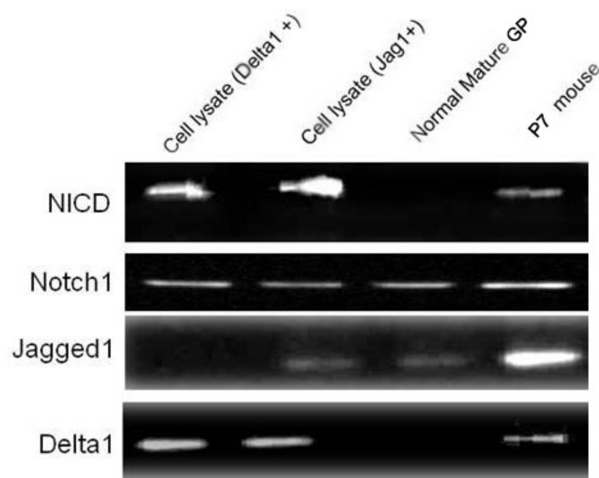

Figure 10: Confirmation of specificity of Notch molecules from postnatal mouse and adult guinea pig cochlear lysates, separated by SDS-PAGE. Normal guinea pig cochleae were positive for Notch1 and Jagged1 while P7 mouse was positive for NICD, Notch1, Jagged1, and Delta1. Our blot indicated that the RAW 264.7 lysate (second lane) was

positive for both Jagged1 and Dll1 while ECV304 (first lane) produced a positive band only in the case of Dll1.

2.3 Results

Notch molecules and Hes activity in the late postnatal (P7) mouse organ of Corti

At postnatal day seven, the mouse organ of Corti continues to express elements of the Notch signaling system, although each one is well restricted to a particular cell type. The ligand Delta1 was highly expressed and restricted to the inner and outer hair cells (Figure 11, left panel, with outer hair cells in focus). The ligand Jagged1 was also highly expressed and was located in the inner and outer pillar cells, which sit between the inner hair cells and the first row of outer hair cells (inner pillars) and between the first and second row of outer hair cells (outer pillar cells) (Figure 11, right panel). The Notch1 receptor was ubiquitously expressed in all supporting cells. Jagged2 was not observed (not shown).

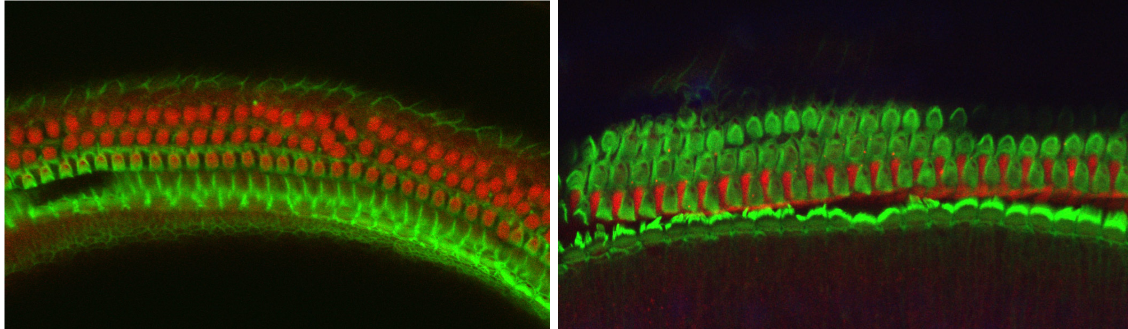

Figure 11: The ligands Delta1 (red, left) and Jagged1 (red, right) in the late postnatal mouse ear (P7). While Delta1 is concentrated in the hair cells, Jagged1 is found mainly in the pillar cells. Actin is labeled by fluorescent phalloidin (green).

The bHLH proteins Hes1 and Hes5 were also expressed in the postnatal organ of Corti. Hes1 was observed in a tight band of expression in supporting cells medial to the inner hair cells, as well as more diffusely in the Hensen cells in the outer sulcus (Figure 12, left panel). Hes5 was observed in nearly all the supporting cells in the outer hair cell area (pillars, Deiters) as well as the Hensen cell bodies and nuclei (Figure 12, right panel). Atoh1 was not observed in this tissue (not shown).

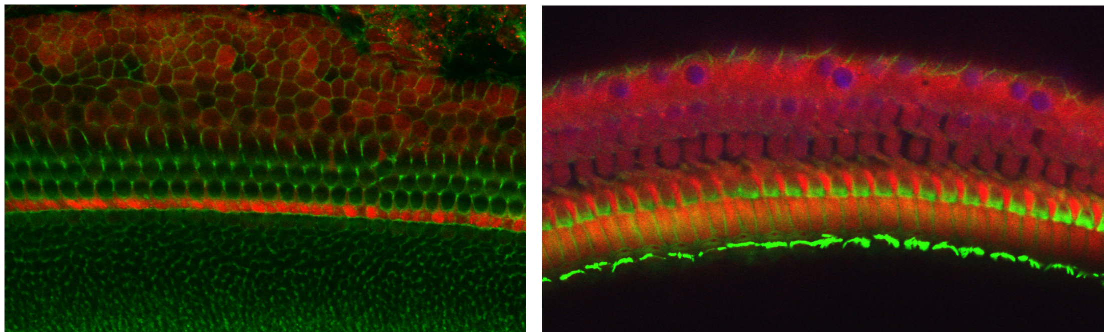

Figure 12. The bHLH proteins Hes1 (red, left) and Hes5 (red, right) in the P7 mouse ear. Hes1 is concentrated in the Hensen cells and supporting cells medial to the inner hair

cells. Hes5 is found in supporting cells in the outer hair cell area and Hensen cells. DAPI (blue) labels nuclei in the right panel. Actin is labeled by fluorescent phalloidin (green).

<u>Notch molecules and Hes activity in the mature guinea pig organ of Corti</u>

In the mature guinea pig organ of Corti, we did not observe Notch1 signal in the nuclei of the normal cells in the organ of Corti (Figure 13 A), but more apical to the nuclei we did observe punctate membranous and cytoplasmic Notch1 signal in Deiters cell bodies (Figure 13 B). Higher magnification of Notch1-positive Deiters cells also revealed non-nuclear cytoplasmic Notch1 signal which appeared to outline the cell body (Figure 13, C). This predominantly non-nuclear location indicates that the Notch1 receptor is not actively being cleaved following ligand interaction.

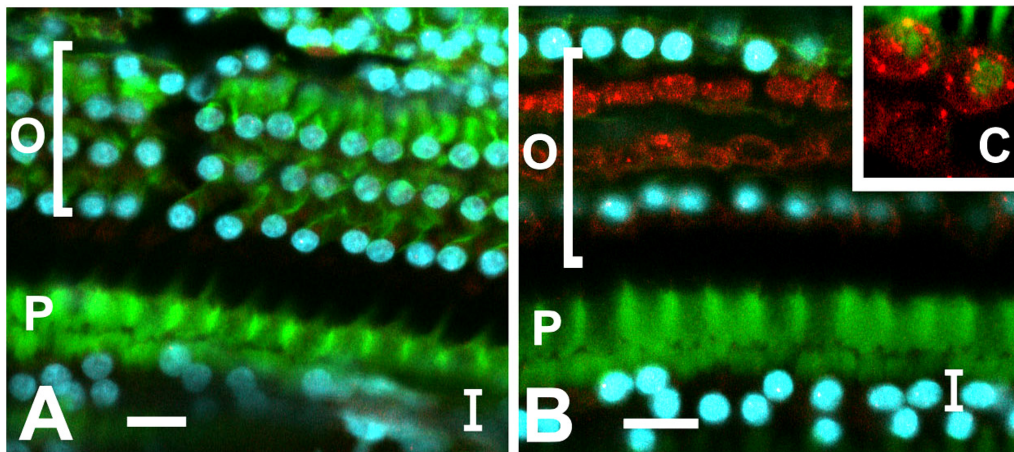

Figure 13: Notch1 is located in the membranes but not nuclei of normal supporting cells. Notch1 (red) is located in the Deiters cell bodies (B) and membranes (C). DAPI (blue) labels nuclei. Actin is labeled by fluorescent phalloidin (green). Scale bar = 2 μm.

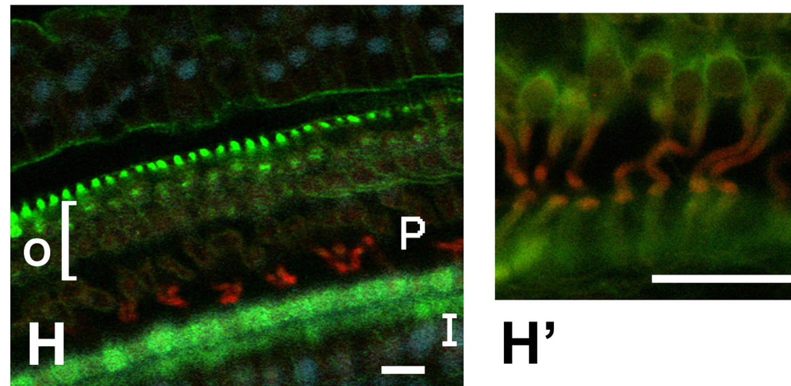

Figure 14: Jagged1 is located at low levels in the cell bodies of the outer pillar cells in the normal organ of Corti. Jagged1 (red) is shown in low (H) and high (H’) magnification. Actin is labeled by fluorescent phalloidin (green). 'O' indicated the outer hair cell region, 'I' indicates the inner hair cell region, and 'P' labels the area of the pillar cells. Scale bar = 2 µm

The ligand Jagged1 was weakly expressed in the outer pillar cells (Figure 14 H). Higher magnification of the Jagged1-positive outer pillar cells suggested that only the middle region of the cell body was positive for Jagged1 (Figure 14 H’). The ligands for Dll1 or Jagged2 were not detected (not shown). Hes1 signal was faintly observed only in some of the nuclei of the supporting cells flanking the inner hair cell area, as illustrated by DAPI co-label (Figure 15 D, and E shows same field without DAPI). Hes5 signal was not observed in the nuclei of any sensory or nonsensory cells in the outer (Figure 15 F) or inner (Figure 15 G) hair cell area. However, diffuse low-level cytoplasmic Hes5 label was observed in some cells in the inner hair cell area.

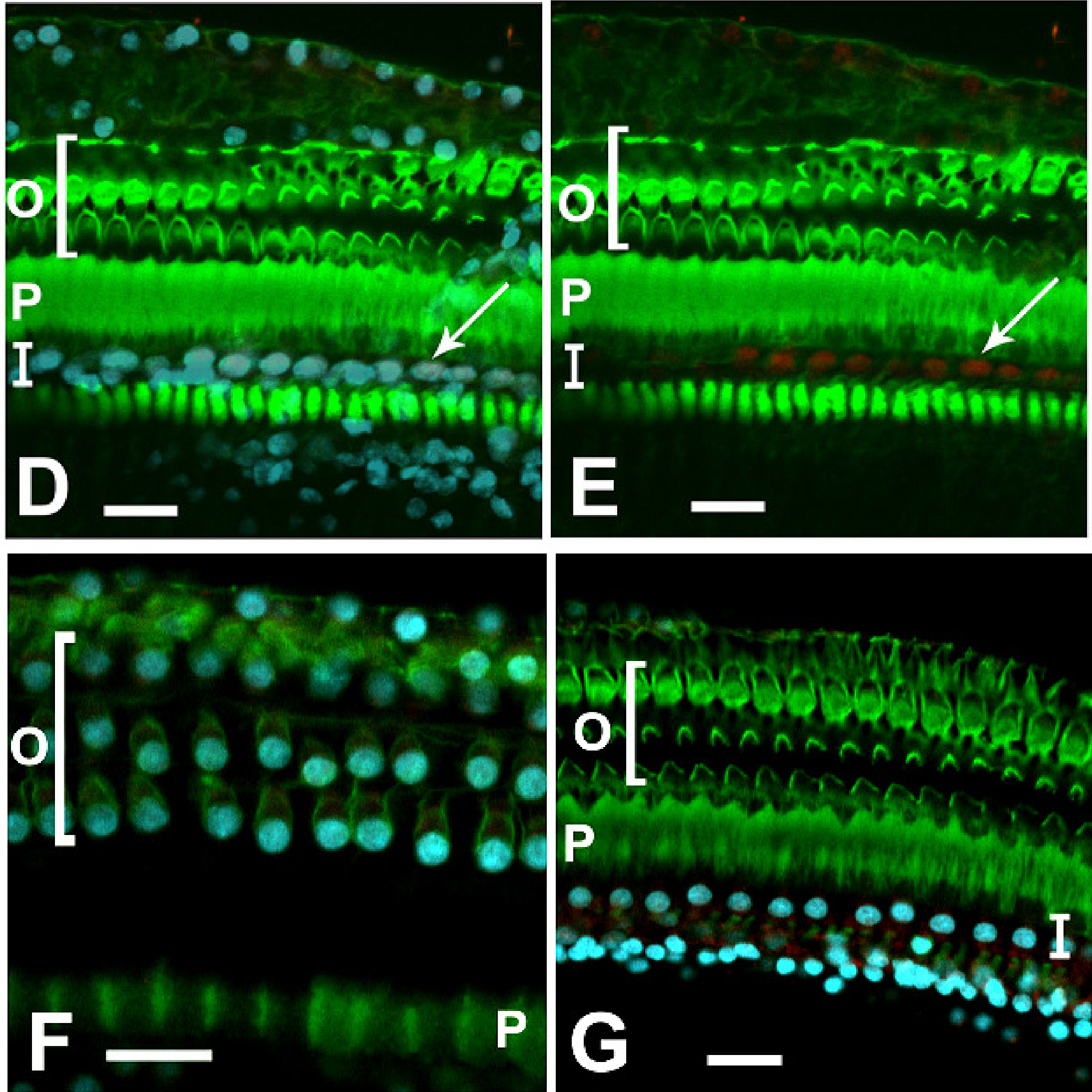

Figure 15: Hes1 and Hes5 in the mature guinea pig organ of Corti. D, E: Hes1 (red) is located in the nuclei of supporting cells medial to the inner hair cells, indicated by an arrow. F, G: Hes5 (red) is absent from the cells of the mature organ of Corti. Actin is labeled by fluorescent phalloidin (green). DAPI (blue) labels nuclei. 'O' indicated the outer hair cell region, 'I' indicates the inner hair cell region, and 'P' labels the area of the pillar cells. Scale bar = 2 μm

2.4 Discussion

We report that the presence of some components of the Notch pathway persist in to late development in the mouse (P7) organ of Corti as well as in the fully mature guinea pig organ of Corti. In the P7 mouse organ of Corti, Jagged1 is highly expressed and is restricted to the pillar cells, and Delta1 is also highly expressed but restricted to the hair cells. Hes1 is located mainly in a tight band of expression medial to the inner hair cells while Hes5 is much more widely expressed throughout the supporting cells of the outer hair cell area. Notch1 is also widely and diffusely expressed in the supporting cells in P7 mouse organ of Corti.

In the fully mature guinea pig organ of Corti, the presence of these same molecules is much lower and Delta1 is completely absent. Similar to P7 mouse, Jagged1 is found in the pillar cells in the mature ear but at much lower levels. Similarly, the Notch1 receptor which was widely expressed at P7 became restricted to the cytoplasm of Deiters cells and at low levels. Hes5 was not observed in the mature cells of the organ of Corti. Low level nuclear Hes1 label was observed in supporting cells medial to the inner hair cells, similar in location to P7 but also at much lower levels.

These data suggest that in the time between late postnatal (P7) and full maturation of the organ of Corti, there is a down-regulation of Notch activity and bHLH expression. By P7, no Atoh1 was observed (this was also true of the adult ear) indicating that hair cells do not need Atoh1 to maintain their differentiated state. However, the hair-cell

specific ligand Delta1 was still found in the late postnatal hair cells. This could be interpreted in several ways. It is possible that the sensory fate of the hair cell- and concurrent nonsensory fate of the surrounding cells-may need to be reassured through continued lateral inhibition into later postnatal stages. It is also possible that the ligand is no longer playing a role in signaling, but has not yet been degraded. However, low levels of Notch1, Jagged1, and Hes1 all persist in the fully mature guinea pig ear, suggesting that the nonsensory fate of supporting cells may be also be continually reinforced via low level Notch signaling and Hes1 transcription. Additionally, future experiments might determine whether interspecies differences between guinea pig and mouse ear development might be a confounding factor, perhaps through the use of prenatal guinea pigs and fully adult mice.

In the avian basilar papilla, Serrate1-Notch signaling persists in the normal mature epithelium although the function of this continued signaling is not known[87]. Following trauma, Notch-Serrate signaling is replaced by Notch-Delta signaling in areas of hair cell loss, then *Atoh1* begins to be expressed in some supporting cells which go on to transdifferentiate to hair cells. Serrate1 is the avian homolog of mammalian Jagged1. As we have reported that Jagged1-Notch1 signaling persists in the fully mature organ of Corti, the next step is to examine whether this signaling changes following hair cell loss. The result of auditory hair cell loss in mammals is never spontaneous hair cell regeneration or transdifferentiation. Therefore, if an increase in Notch signaling—and

Hes activity— occurs following hair cell loss, this signaling may present a mechanism by which regeneration is repressed.

Chapter 3

Notch Signaling and Hes Activity in the Traumatized Organ of Corti

3. 1 Introduction

Auditory hair cells are vulnerable to damage from acoustic over-stimulation, ototoxic drugs such as aminoglycosides, cisplatin, or aging, while their non-sensory supporting cell counterparts are substantially more resistant to these factors. Non-mammalian vertebrates can replace lost hair cells through direct and indirect transdifferentiation of surviving supporting cells [82,95,96,103,134] Mammals, including humans, do not spontaneously replace auditory hair cells when lost [11,135-138]. However, at least one proof of concept exists showing that in mammals surviving supporting cells can be forced to transdifferentiate into new hair cells given the right stimulus. Over-

expression of the pro-hair cell gene *Atoh1,* which is normally only present during fetal development, provides such a stimulus [79,80,139,140]. This suggests that although the mammalian cochlear sensory epithelium has lost the ability to spontaneously initiate the events needed to replace hair cells, the molecular activity required for inducing a hair cell fate is still present and functional in mature supporting cells. Furthermore, it suggests that unlike many other mature cell types, mammalian supporting cell fate may be altered if appropriate signals are provided.

While the Notch pathway has been implicated in hair cell regeneration following trauma to the basilar papilla in birds [87], the status of Notch molecules in the damaged mammalian auditory epithelium is not well characterized. We have reported in Chapter 2 that low-level Jagged1, Notch1, and Hes1 persist in the mature organ of Corti, providing a baseline to which post-ototoxic changes can be compared. It is important to determine whether and how the activity of Notch-dependent proteins (such as the Hes) changes following a lesion as it is the case in some other epithelia. The results of such studies could help design therapies for hair cell regeneration based on the repression of Hes activity.

The bHLH genes *Hes1* and *Hes5* are well-characterized repressors of Atoh1 expression in the developing inner ear, and the repressive signals are specific to different areas of the sensory area. Interfering with the developmental expression of *Hes1* in the cochlea results in over-abundance of inner hair cells, while knocking out *Hes5* results in over-abundance of outer hair cells [71,78]. Presumably, the elimination of the repressive

(and locale-specific) signals provided by Hes activity allows for the over-expression of *Atoh1*, directing more cells than is normal to a hair cell fate. This repressive activity is crucial for producing a heterogeneous mosaic of hair cells and supporting cells during mammalian ear development, but continued Hes activity in adults may continue to promote maintenance of the supporting cell phenotype in maturity. We reported low-level Hes1 in the mature organ of Corti in areas reflective of its developmental expression as well as low levels of Jagged1 and Notch1. This suggests that Notch signaling-mediated Hes transcription is not completely down-regulated in maturity and may serve to maintain nonsensory fate. Continuation of this expression, or perhaps its up-regulation, would and pose a barrier to supporting cell-hair cell transdifferentiation in mammals following a lesion.

An important question is whether Notch-dependent *Hes* activity is present following hair cell loss in mammals and could potentially account for the failure to regenerate new hair cells after a lesion. Furthermore, it is important to determine whether Notch signaling is up-regulated after a lesion as the timing for that up-regulation may suggest intervals for effective manipulation of these signals. We report immunohistochemistry and Western blot protein analysis of Notch molecules and downstream bHLH proteins in the lesioned guinea pig organ of Corti at acute and chronic time points (24 hours, 3 days, 5 days, 7 days, 14 days, 30 days, and 60 days after drug administration). The mature organ of Corti exhibited low level cytoplasmic Notch1 signal in the Deiters cells, very low Jagged1 signal in the pillar cell bodies, and low level

nuclear Hes1 signal in the supporting cells in the inner sulcus. By 24 hours following ototoxic drug delivery, we observed a large increase of nuclear Notch1 signal in the Deiters cells, high Jagged1 signal in the inner and outer pillar cells as well as nuclear Hes1 and Hes5 activity in the surviving supporting cells in the former inner and outer hair cell areas, respectively. The increase in Notch proteins appears to peak at 14 days post-ototoxicity, before declining below baseline levels by 60 days.

3.2 Materials and Methods

Animals

All animal experiments were approved by the University of Michigan Institutional Committee on Care and Use of Animals and performed using acceptable veterinary standards. We used 78 pigmented adult male guinea pigs (Elm Hill Breeding Laboratory) with a normal Preyer's reflex and weighing between 250-400g at the beginning of the experiments. Animals were housed at the Animal Care facility at the University of Michigan. Seventy-four animals were systemically deafened with kanamycin and ethacrynic acid (see below). Four animals received sham saline injections to provide a control for the deafening surgery (no difference was observed between saline and un-injected controls). Prior to all surgical procedures, guinea pigs were anesthetized with a subcutaneous injection of xylazine (10 mg/kg) and ketamine HCl (40 mg/kg).

Deafening surgery and progression of hair cell loss

Seventy-four adult guinea pigs were bilaterally chemically deafened through the combination of a single subcutaneous dose of kanamycin (500 mg/kg) followed two hours later by an intravenous dose of ethacrynic acid (50 mg/kg) in the jugular vein. The pairing of these two drugs systemically is designed to consistently eliminate all outer hair cells and most or all inner hair cells while initially sparing supporting cells, in the lower three turns of the cochlea [79,141]. Hair cell elimination begins to be evident at about 24 hours following drug administration, with a few missing hair cells in the organ of Corti. A complete lesion of all hair cells in the lower three turns is consistently achieved by 3 days following this ototoxicity protocol. Therefore, figures of lesioned tissue from animals sacrificed at 24 hours following drug administration will contain some residual hair cells. Animals were sacrificed and prepared for analysis (immunocytochemistry or protein extraction from lysates prepared from dissected cochleae) at the following time points: 1 hour (n=5), 4 hours (n=5), 24 hours (n=8), 3 days (n=8), 5 days (n=8), 7 days (n=8), 2 weeks (n=8), 1 month (n=8), or 2 months (n=6). Ten guinea pigs were sacrificed without undergoing deafening in order to serve as normal controls. Results from that cohort are reported in Chapter 2 and were used as a basis for comparison to post-lesion data.

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Immunocytochemistry

Animals were deeply anesthetized with xylazine and ketamine as above, decapitated, and the temporal bones were removed. The apical tip of the otic capsule was removed, the oval window opened by dislodging the stapedial footplate, and the round window opened with forceps. Paraformaldehyde (PFA) 4% in phosphate buffered saline (PBS) was locally perfused through the cochlea. The temporal bone with cochlea *in situ* was allowed to fix for two hours, at which time the bony capsule was dissected away and the modiolus with surrounding cochlear duct removed. These cochleae were stripped of the stria vacularis, tectorial membrane, and Reissner's membrane to fully expose the organ of Corti. Specimens were washed three times for ten minutes in PBS and permeabilized using 0.1% Triton X-100 in PBS for ten minutes. Tissue was then blocked using 10% normal goat serum (Jackson Immunoresearch, West Grove, PA) in PBS for thirty minutes at room temperature. Following blocking, specimens were incubated overnight at 4°C in a 1:100 primary antibody solution in PBS. Primary antibodies used were raised in rabbit and are as follows: a) an antibody against the C-terminus of Notch1 (Abcam, ab27526, lot# 181697; whole receptor = ~280 kDa, cleaved portion NICD = 120 kDa), b) Jagged1 (Santa Cruz, H-114, sc8303, lot # E0604; 150 kDa), c) Jagged2 (Santa Cruz, H-143, sc5604, lot# K1904; 150 kDa), d) Dll1 (Santa Cruz, T-20, sc9932, lot# C1505; 75 kDa), e) Atoh1/Math1 (Abcam, ab22270-100, lot# 151411; 38 kDa), f) Hes1 (Chemicon, Ab5702, lot# LV1438380; ~30 kDa), and g) Hes5 (Chemicon, Ab5708, lot#

0511016244; ~20 kDa). The following morning, samples were washed three times in PBS and incubated in a Rhodamine (TRITC)-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, 111-025-144) for thirty minutes at room temperature. All samples were additionally incubated with Alexa Fluor 488 conjugated phalloidin (Molecular Probes Invitrogen, Carlsbad, CA) for twenty minutes. If visualization of the nucleus was desired, samples were incubated in the DNA-binding fluorescent stain 4', 6-diamidino-2-phenylindole (DAPI) diluted 1:600, for three minutes. The cochleae were then washed three times for five minutes in PBS, dissected into four separate pieces (apex, 3rd turn, 2nd turn, base) and mounted onto a slide with Gel/Mount mounting media (Biomeda, Foster City, CA). The second and third turns were used for analysis.

Because the Notch1 antibody used recognizes the c-terminal fragment of the Notch1 receptor, it labels membrane-bound and cytoplasmic epitopes when un-cleaved, and the nuclear domain (NICD) when cleaved and activated. To rule out background fluorescence, we incubated normal and lesioned (1 day post drug administration) guinea pig organ of Corti in just primary or just secondary antibody. We observed no signal in controls incubated in primary alone. A very low level of uniform background immunofluorescence was seen in controls incubated in just secondary antibody, which did not correspond to the location, intensity, and pattern of fluorescence reported here.

<u>Confocal Microscopy</u>

Immunohistochemically-processed cochlear whole mounts were examined with a Zeiss LSM 510-META Laser Scanning Confocal Microscope mounted on a Zeiss Axiovert 100M inverted microscope, equipped with four lasers: a Coherent Enterprise laser for UV (351,364 nm), an Argon laser for FITC/GFP (458, 488, 514 nm), a Helium Neon 1 laser for Rhodamine, Texas Red, and Cy3 (543 nm), and a Helium Neon 2 laser for Cy5 (633 nm). Wavelengths utilized in this study include 364 nm (for DAPI), 488 nm (for Alexa 488-conjugated phalloidin), and 543 nm (for Rhodamine). Scanning was performed with all lasers simultaneously in the multi-track setting and META-analysis provided fine separation of wavelengths and the ability to track all dyes in the sample simultaneously. The Linear Unmixing function was used to separate mixed signals pixel by pixel, using the entire emission spectrum of each dye in the examined specimen and eliminating broadband autofluorescence. Scans were recorded in 1µm single images or 1µm z-stack series at 1.5-sec intervals, at 40x or 60x magnification with zoom. Confocal images were cropped, labeled, and spaced in Adobe Photoshop.

Western Protein Analysis of Lysates from Dissected Cochleae

Cochlear lysates of control and hair cell-lesioned guinea pigs were examined for the presence of Notch proteins before and after a lesion at several time-points. Cochleae were dissected from the temporal bone and the auditory nerve and as much bone and

connective tissue as possible removed from the organ of Corti with a fine needle under a stereoscope. The remaining tissue was incubated in cold T-Per Lysis Buffer (Pierce Biotechnology, Thermo Fischer, Rockford, IL) for 30 minutes then homogenized with a small plastic pestle. The homogenate was kept on ice a further 30 minutes then spun in a centrifuge until a visible pellet formed. The supernatant was retained and the amounts of protein standardized (30 µg). Separation of proteins was achieved using Nu-Page SDS-PAGE with X-Cell SureLock system and Novex 4-12% Bris-Tris gels with MOPS buffers (Invitrogen, Carlsbad, CA). Separated proteins were transferred to PVDF membrane by electrophoresis. Afterwards, the membrane was blocked with 5% dry milk in PBS for an hour and then incubated in 1% milk in PBS plus the primary antibody (1:5000) overnight at 4°C. Following three brief washes in TBS-T, the membranes were incubated for 30 minutes in a complementary HRP-conjugated secondary antibody solution (1:8000) in 5% dry milk in TBS. The membranes were again washed in TBS-T three times for 10 minutes each. Developing fluid (Pierce Biotechnology, Thermo Fischer, Rockford, IL) was applied to the membrane, and film was quickly placed on the membrane in the dark and developed. Average film development time was one minute in a sealed cassette. Film was then processed in the dark and the film was digitally scanned. The scan was inverted and sharpened in Adobe Photoshop. Cell lysates RAW 264.7 and ECV304 (Santa Cruz Biotechnology, Santa Cruz, CA) were used as positive controls of the proteins Jagged1 and Dll1 respectively, and both contained the Notch1 receptor. Mouse embryo tissue extract (from embryonic day 17-19) were used as positive controls

for Hes1 and Hes5. Our blot indicated that the RAW 264.7 lysate was positive for both Jagged1 and Dll1 while ECV304 produced a positive band only in the case of Dll1. A lane with buffer and no protein served as a negative control. In addition to the predicted bands (for mouse) referenced above, the Hes5 blot had a faint non-specific band at ~40 kDa, and the Notch1 blot had a faint nonspecific band at ~250 kDa. The Hes1 blot was clean of non-specific bands. Glyceraldehyde 3-phosphate dehydrogenase (GapdH), a common cellular housekeeping protein (37 kDa), was used as a loading control to quantify relative protein amount using "ImageJ" analysis software.

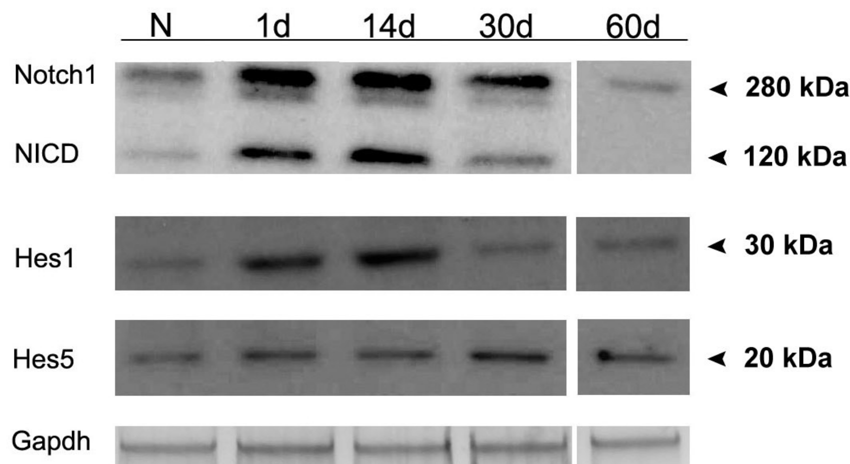

Figure 16: Western blot of dissected whole cochlear lysates from normal and hair cell lesioned guinea pigs, separated by SDS-PAGE and blotted against antibodies to Notch proteins. N = normal mature guinea pig, 1d = 1 day after the lesion, 14d = 14 days after, 30d = 30 days after, 60d = 60 days after. Predicted weights of each molecule are listed at right, confirming specificity.

3.3 Results

Notch molecules and Hes activity are up-regulated in the traumatized organ of Corti

Notch1-Jagged1 signaling in the guinea pig organ of Corti appeared to be drastically up-regulated within one day after the administration of ototoxic drugs. Total protein amounts of Notch1 receptor, the cleaved Notch1 receptor (NICD), Jagged1, Hes1 and Hes5 all appeared to be significantly increased in response to ototoxicity, with striking increases apparent by 24 hours following the ototoxic drug administration (Fig. 16 shows blot and Fig. 17 shows trend quantification).

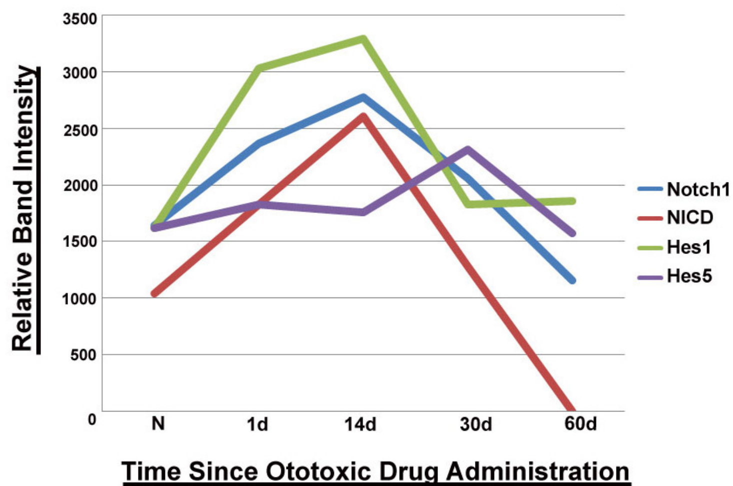

Figure 17: Quantified relative band intensity of Notch proteins from SDS-PAGE separated dissected whole cochlear lysates, as a function of time since kanamycin and ethacrynic acid delivery. Western blot is shown in Figure 16. The total amount of protein of Notch1, NICD, Hes1 and Hes5 all increased following a hair cell lesion, peaking at about 14 days and returning to baseline or below at 60 days. N = normal mature guinea pig, 1d = 1 day after the lesion, 14d = 14 days after, 30d = 30 days after, 60d = 60 days after.

Immunohistochemical results showed that Notch1 signal was widespread in the surviving supporting cells of the lesioned organ of Corti (Fig. 18, right upper and lower panels). DAPI staining of this tissue demonstrated that the nuclei of many remaining supporting cells in the organ of Corti were now positive for Notch1 signal, indicating the intracellular portion of the receptor had traveled to the nucleus following cleavage (Fig. 18 A and A'). Some non-nuclear, cytoplasmic or membranous Notch1 signal was also evident in some of the supporting cells.

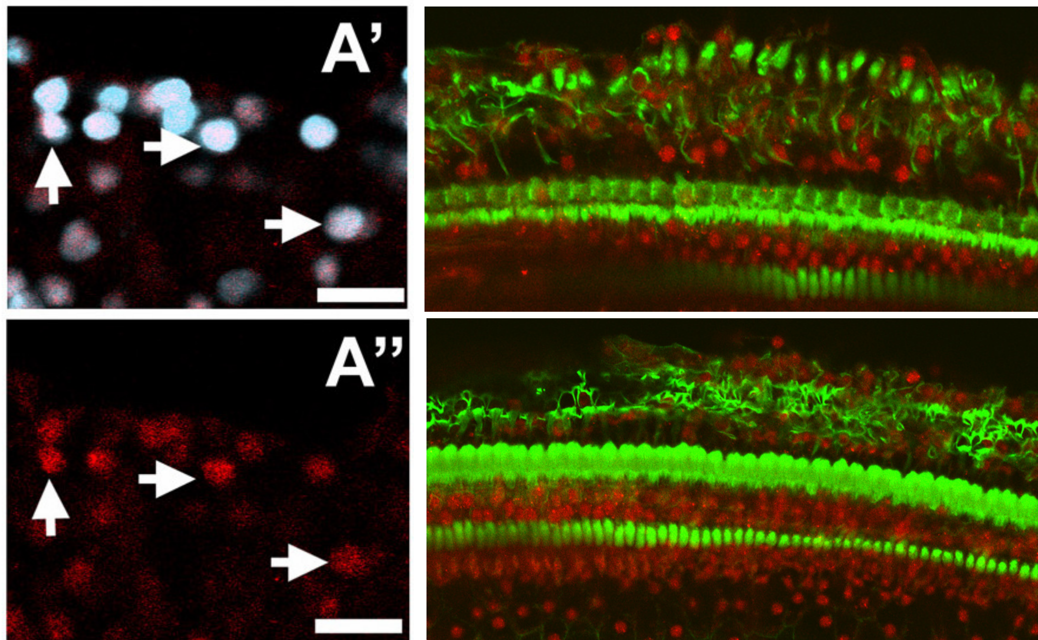

Figure 18: Notch1 signal becomes nuclear within 24 hours of ototoxic drug delivery. A' and A'': DAPI (blue) and Notch1 (red) are co-localized in surviving supporting cells. Lower magnification images of organ of Corti whole mounts show widespread nuclear location throughout many surviving supporting cells. Right panels: Upper panel and lower panels illustrate two focal planes (lower panel is towards the apical surface) of the same tissue. Actin is labeled by fluorescent phalloidin (green) in the right panels. Scale bars = 2 μm.

Hes1 and Hes5 also appear to increase in the nuclei of supporting cells, however their locations did not generally overlap. At 24 hours following the ototoxic drug delivery, many supporting cell nuclei in the outer hair cell area were labeled with Hes5 (Figure 19 B, B' and B'') while supporting cells which lie medial to the inner hair cells were now highly positive for Hes1 in both the nuclei and cytoplasm (Figure 19 C, C'). Very few nuclei of cells in the inner hair cell area or the inner sulcus were positive for Hes5, and no Hes1 signal was observed in the outer hair cell area. The nuclei of cells immediately adjacent to the inner hair cells (shown by left arrow in Figure 19 C and C') all appeared to be more brightly positive for Hes1 signal than cell nuclei positioned more medially towards the inner sulcus (shown by right arrow in the same panels). Cytoplasmic Hes1 labeling was strongly observed in these cells, yet nuclear label was significantly brighter. Hes1 signal was also observed in most Hensen cell nuclei and in the cytoplasm (Fig. 19 D and D').

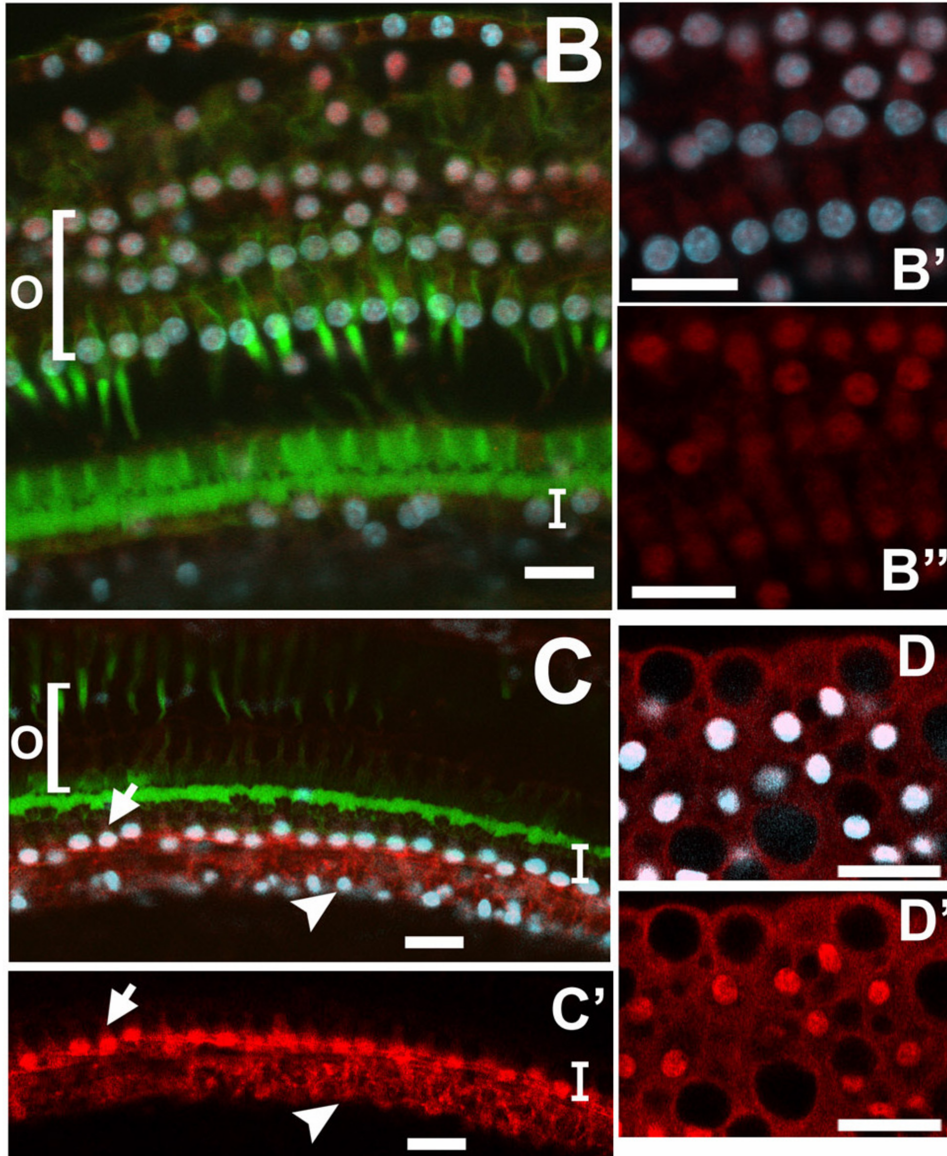

Figure 19: Hes1 and Hes5 signal are located in the nuclei of supporting cells at 24 hours following an ototoxic lesion. B, B' and B'': DAPI (blue) and Hes5 (red) are co-localized in many surviving supporting cells in the outer hair cell area. C, C': Hes1 (red) is brightly positive in the nuclei and cytoplasm of supporting cells medial to the inner hair cell area. D, D': Hensen cell bodies and nuclei are positive for Hes1. Actin is labeled by fluorescent phalloidin (green). The outer hair cell area is indicated by O, and the inner hair cell area is indicated by I. Scale bars = 2 μm.

The ligand Jagged1 appeared to be very highly up-regulated 24 hours following the onset of ototoxicity. Specifically, Jagged1 signal was highly positive in the inner (Figure 20 E) and outer (Figure 20 E') pillar cell bodies and in the Deiters cells. Delta1 and Jagged2 were not observed (not shown).

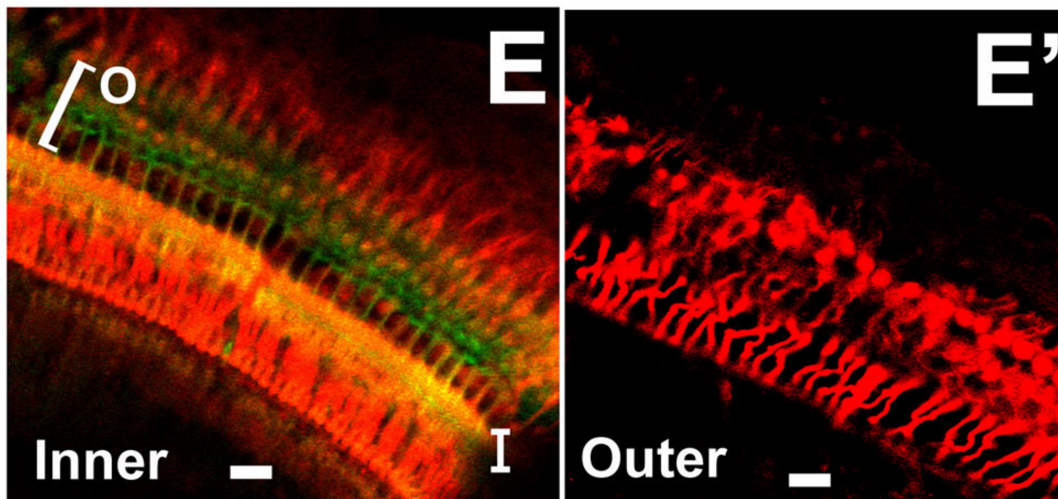

Figure 20: Twenty-four hours following ototoxic treatment, Jagged1 is up-regulated in the inner and outer pillar cells. Jagged1 signal was localized to both sets of pillar cell bodies and some signal was observed in the Deiters cells. Actin was labeled by fluorescent phalloidin in E. The outer hair cell area is indicated by O, and the inner hair cell area is indicated by I. Scale bars = 2 μm.

The traumatized organ of Corti remained positive for Notch1, Jagged1, Hes1, and Hes5 for at least one month following ototoxic drug administration and the presence of these molecules reached baseline levels by two months. According to Western blot analysis of protein from traumatized cochlear lysates at several time points following ototoxicity, the highest levels of Notch1, NICD, and Hes1 was 14 days later (Figure 16).

Immunohistochemistry for Notch1 at two weeks demonstrated widespread nuclear Notch1 signal in most of the remaining supporting cells of the traumatized organ of Corti (Figure 21 A). Higher magnification showed that the majority of Notch1 signal was localized to nuclei of surviving supporting cells (Figure 21 B and B'). The inner and outer pillar cells of the 14-day post traumatized organ of Corti were still brightly positive for Jagged1 signal (Figure 21 C). Hes1 signal was also localized in supporting cell nuclei medial to the former inner hair cell area and nuclei in the inner sulcus, with high concentrations of Hes1 protein visible in scars where inner hair cells were absent (arrows in Figure 21 D and D'). Some cytoplasmic Hes1 signal was also visible in this area. Hensen cell nuclei as well as cytoplasm were still positive for Hes1 (data not shown), similar to their appearance at 24 hours following ototoxicity (Figure 19 D and 3D'). Hes5 signal was observed in the DAPI-stained nuclei of the remaining supporting cells in the outer hair cell area (Figs. 21 E and E'), but not in the nuclei of the remaining cells in the inner hair cell area. Some cytoplasmic Hes5 expression was observed in these cells at low levels. At no observed time point following ototoxicity did the expression of Hes1 and Hes5 appear to overlap.

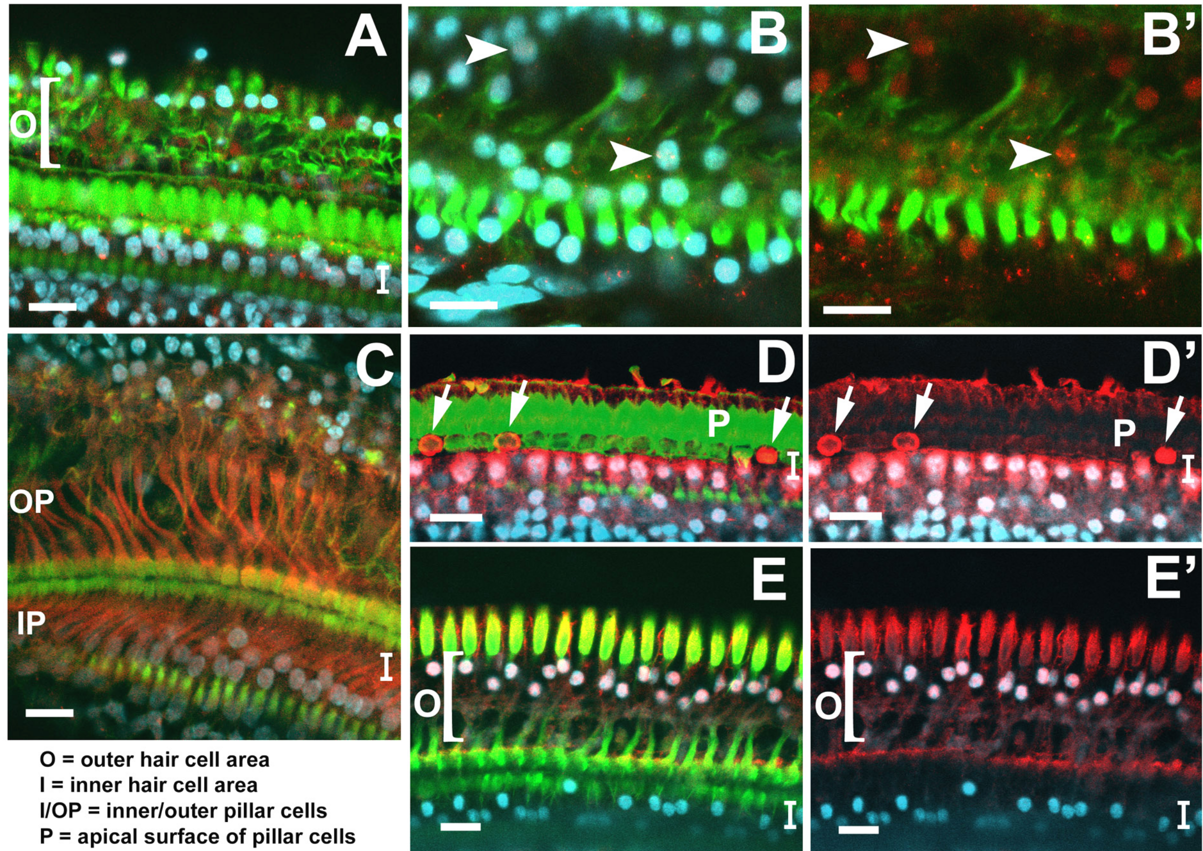

Figure 21: Immunohistochemistry of guinea pig organ of Corti whole mounts two weeks following ototoxic drug administration. Stained with fluorescent phalloidin (green), DAPI (blue), and antibodies to Notch signaling molecules (red: A-B, Notch1; C, Jagged1; D-D', Hes1; E-E', Hes5). A, B: Two weeks following drug administration, nearly all DAPI-labeled nuclei (blue) of remaining supporting cells were also positive for Notch1 (red). In B', DAPI signal is omitted to more clearly show Notch1. Arrows in B and B' point to two double-labeled nuclei. C: All the inner and outer pillar cells and most of the Deiters cells were still brightly positive for Jagged1 signal (red). D, D': Many supporting cell nuclei medial to the inner hair cell area (I) were co-labeled with DAPI and Hes1 (red). Additionally, there were high concentrations of non-nuclear Hes1 protein in some scars where inner hair cells had died (arrows in D, D' which shows DAPI and Hes1 only). E, E': All supporting cell nuclei in the outer hair cell area were co-labeled with DAPI and Hes5 signal, while all nuclei in the inner hair cell area were negative for Hes5. Labels: 'O' refers to the outer hair cell area; 'I' refers to the inner hair cell area; 'P' refers to the apical surface of the inner and outer pillar cells with 'IP' and 'OP' referring to inner and outer pillar cells, respectively. Scale bars represent 2μm.

Western blot analysis of protein from lysates prepared from traumatized cochleae demonstrated the increased presence of Hes1, Hes5, NICD, and Notch1 at 2 weeks and one month following ototoxicity, as compared with normal baseline levels of these proteins (Figure 16). However, at one month, with the exception of Hes5 in which changes could not definitively be demonstrated, there was a decrease in these protein amounts as compared to 24 hours and 14 days following drug administration. This decrease in protein amount at 30 days was reflected in decreased immunohistochemical signal as well (not shown). At two months following ototoxic drug administration, the blot indicated that levels of all observed proteins were at or below normal baseline levels (Figure 16 and 17). No perceptible immunohistochemical signal was observed at 2 months following ototoxicity for any of the proteins mentioned (Figure 22 shows Notch1 (A) and Jagged1 (B), others not shown). Immunohistochemical analysis to Dll1, Atoh1, and Jagged2 were all negative at all time points observed (not shown).

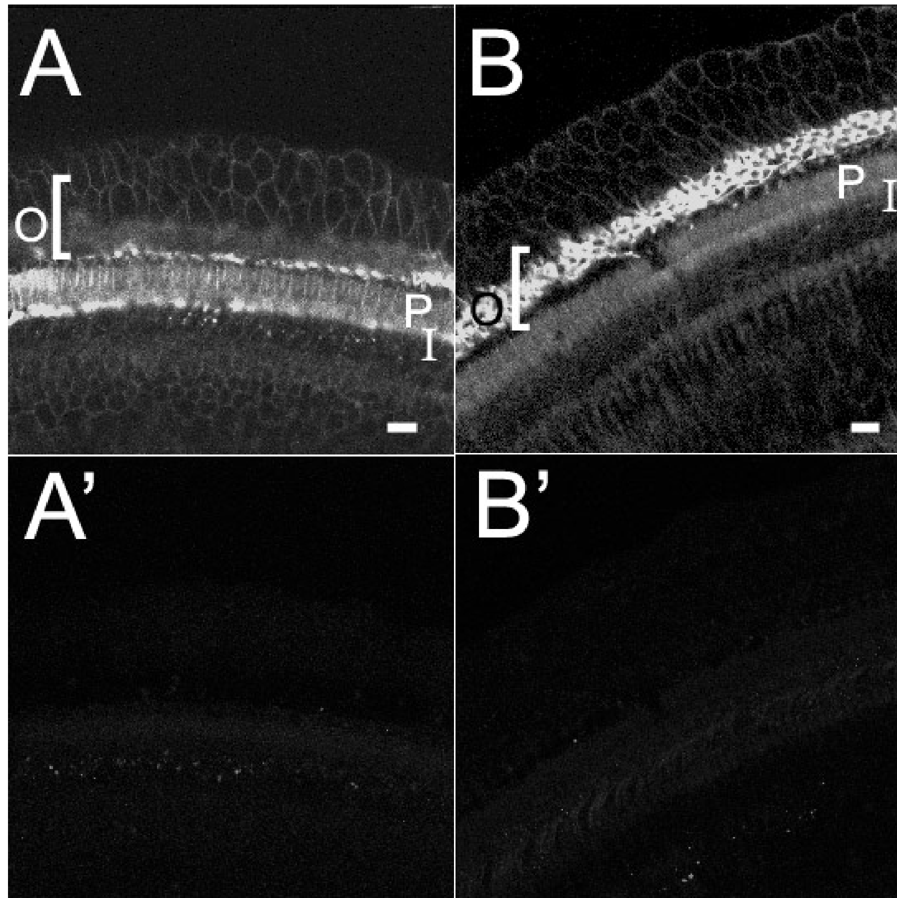

Figure 22: Grey scale confocal images of two month deafened guinea pig organ of Corti whole mounts stained for fluorescent phalloidin (A and B) and Notch1 (A’) or Jagged1 (B’). Two months following ototoxic drug administration, immunohistochemistry to Notch1 and Jagged1 was negative. Actin labeling in the upper panels illustrates a mature scar in the organ of Corti, devoid of inner and outer hair cells. Actin junctions are compressed between supporting cells and the areas where hair cells once were. Scale bars = 2 μm.

3.4 Discussion

Summary of results

In Chapter Two we examined baseline levels of Notch signaling proteins in the normal guinea pig ear, and observed that signaling is active at low levels in normal animals. We report here that Notch signaling dramatically increases and becomes active (through the appearance of the cleaved portion of the Notch receptor) in surviving supporting cell nuclei in response to trauma induced by the delivery of ototoxic chemicals. Baseline expression of Notch molecules consists of non-nuclear Notch1, low level Jagged1 in pillar cells, and low level nuclear Hes1 in supporting cells medial to the inner hair cells. Post-lesion signaling, which is characterized by an increase in Jagged1 and nuclear Notch1 signaling, is coupled with increases in Notch-dependent proteins Hes1 and Hes5 in supporting cell nuclei. Hes1 and Hes5 expression following a lesion appeared to be location-specific and non-overlapping, with Hes1 located in the nuclei and cytoplasm of remaining supporting cells in the inner hair cell area and Hes5 located in the nuclei and cytoplasm of supporting cells in the outer hair cell area. The intensity of the signaling response varies over time: Notch1, Jagged1, Hes1, and Hes5 appeared to peak at between 14 and 30 days following the lesion (Figures 16, 17) and were absent by 2 months (Figure 22).

Initiation of Notch signaling following trauma

After cell fates are determined during development, Notch signaling is static in tissues which do not have active cell turnover. However, in some tissues trauma triggers Notch signaling as part of the process of cellular replacement, transdifferentiation, or repair. Post-developmental initiation of Notch signaling has been reported to accompany changes in cell fate in mouse endothelial cells [53], mammalian muscle precursor cells [57,132], mammalian dental pulp cells [61], intestinal crypt cells in the fruit fly, zebrafish, rodents, and humans [142], adult neural stem cells in the mammalian subventricular zone [116], Barrett's metaplasia in humans [143], as well as the avian basilar papilla during trauma and hair cell regeneration [87]. One factor in post-traumatic Notch up-regulation may be the severity of the lesion and cell damage in the epithelium. This would help explain why the few studies reporting on the status of the organ of Corti following a lesion have produced varied results. In one recent study, both Sox2 and Jagged1 were reportedly unchanged following a partial lesion of the organ of Corti in mouse [133]. This particular study used a single high-dose injection of kanamycin (1000 mg/kg) followed 30 minutes later by a dose of furosemide (400 mg/kg) which was reported to lesion outer hair cells almost exclusively, sparing inner hair cells. Our lesion model in guinea pigs achieves a more complete lesion, yielding a nearly complete loss of both outer hair cells and inner hair cells [21,79].

It is possible that in addition to loss of hair cells, injury to supporting cells is also required for drastic up-regulation of Notch signaling in the auditory epithelium. This notion is supported by the observation that in the mouse, where the organ of Corti is more

resistant to ototoxic drug regimens, the loss of outer hair cell was not accompanied by a substantial increase in Notch family molecules [133]. In contrast, in the guinea pig ear, both outer and inner hair cells are eliminated, supporting cells are also likely to be affected and the increase in Notch signaling in the supporting cells is drastic. The change in two Notch molecules in the guinea pig is corroborated by another study [110].

Hes activity is increased in surviving supporting cell nuclei

Notch-dependent Hes1 and Hes5 were observed to increase in the nuclei of supporting cells in response to ototoxicity, hypothetically as a result of increased Notch signaling in the damaged organ of Corti. During development of the organ of Corti, Hes gene expression represses *Atoh1* transcription and thus has a deleterious effect on prosensory fate (hair cell fate)[140]. We reported that Hes expression persists at low levels in the organ of Corti, and hypothesized that the function may be to maintain non-sensory fate in mature supporting cells. Following an aminoglycoside-induced hair cell lesion, the cellular and structural integrity of the organ of Corti is compromised and the epithelium undergoes reorganization, perhaps to preserve any remaining function. We further speculate that the increased Notch signaling may be due to the initiation of massive apoptotic and other cell death pathways in the hair cells, as well as the formation of increased supporting cell-supporting cell junctions as hair cells die. It is unclear at present

whether the changes in supporting cells that up-regulate Notch are the direct result of drug toxicity, or a response to the loss of the neighboring hair cells, or both.

Hes activity may serve to discourage or prevent transdifferentiation of supporting cells to hair cells. Transdifferentiation in the absence of mitosis would rapidly result in supporting cell depletion in and the eventual destruction of the remaining portions of the organ of Corti. It is possible that transdifferentiation does not occur spontaneously to prevent this eventuality and to preserve the cellular integrity of the organ of Corti. We observed Hes1 and Hes5 in non-overlapping areas of the organ of Corti, with Hes1 restricted to the former inner hair cell area and the Hensen cells, and Hes5 located in the former outer hair cell area. This suggests that the response of mature supporting cells in response to post-lesion Notch signaling depends on their prior developmental history and their location in the mature organ of Corti. It further suggests that positional information that impacts cell fate and protein production in response to signaling events is retained in the mature organ of Corti.

We show the Notch1 receptor and Jagged1 ligand are increased in surviving supporting cells in response to cellular trauma and hair cell loss. We further hypothesize that the remaining supporting cells engage in Notch1-Jagged1 signaling, resulting in NICD cleavage from the Notch1 receptor and its translocation to the nucleus. We believe that the increases seen in Hes1 and Hes5 in the damaged organ of Corti are due to promotion of their transcription in response to nuclear NICD in supporting cells. This in turn results in accumulation of Hes protein in the supporting cells, creating an

antagonistic environment for supporting cell to hair cell transdifferentiation to occur. This pattern of Notch activation is fundamentally different from what has been reported in avian hair cell regeneration following trauma, which is characterized by Delta1-Notch1 signaling and the presence of Atoh1 in areas of regeneration [87]. We did not observe the Delta1 homolog Dll1, or Jagged2, or Atoh1 at any time point following a lesion in mammals, although at 24 hours following the lesion Jagged1 and Notch1 were highly up-regulated in supporting cells.

Conclusions

We demonstrate that mammals preserve the ability to reinitiate Notch signaling following hair cell loss. A severe lesion to the organ of Corti leads to up-regulation of Jagged1-Notch1 signaling and the nuclear presence of the bHLH proteins Hes1 and Hes5. The changes in Notch signaling following a lesion to the mammalian auditory epithelium are inherently different from the response preceding hair cell regeneration in birds, where Delta1-Notch1 signaling is increased after a lesion, paired with the production of prosensory bHLH protein Atoh1 [87]. Our data present a potential mechanism by which mammalian supporting cell transdifferentiation may be repressed by Notch-dependent Hes activity after hair cell loss, and outlines a window of time in which Notch signals are up-regulated, peak, and begin to decline. These data provide the impetus to design a substrate-specific inhibitor of Notch signaling that could be used to interfere in Notch

signaling in a permanent, specific, and measureable way for the purpose of inducing supporting cell to hair cell transdifferentiation.

Chapter 4

Development of a Tool for Notch Signaling Knockdown: Ad.dnMAML1-GFP

4.1 Introduction

Notch signaling reinitiation following hair cell loss occurs in the avian basilar papilla, and the response is characterized by Delta ligand expression, Atoh1 production, and hair cell regeneration by direct or indirect transdifferentiation. Conversely, we have described that the reinitiation of Notch in the organ of Corti is characterized by Jagged1 ligand expression, Hes1 and Hes5 production, and a lack of supporting cell transdifferentiation. This difference is interesting to ponder given the role of Notch-dependent Hes activity in the development of both epithelia. Hes1 and Hes5 antagonize Atoh1 and hair cell fate during initial cell fate specification, while manipulations which decrease Hes activity increase Atoh1 and prosensory cell fates [71,72]. We hypothesize that

Hes1 and *Hes5* expression levels pose a powerful molecular roadblock to the differentiation of supporting cells in the mammalian organ of Corti by suppressing Atoh1. In order to regenerate hair cells in mammals it may be necessary to prevent this post-traumatic signaling response from occurring, by developing therapies which block Notch-mediated gene transcription. To test this, it is necessary to develop specific local inhibitors of Notch signaling that could be delivered to the supporting cells of the traumatized inner ear.

The influence of Notch inhibition on the fate of cells in the organ of Corti has been examined before. Small molecule inhibitors of γ-secretase, an enzyme which cleaves the Notch receptor to release NICD, have been used as a means of reducing Notch signaling experimentally. If γ-secretase activity is blocked, in theory NICD would not be formed and Hes gene transcription in the nucleus would not occur. It has been shown that DAPT application interfered with Notch signaling in *Drosophila* development and negatively affected the expression of proteins that were dependent on Notch signaling. The expression of *wingless* was lost in the wing margins where expression depended on Notch, but not in Notch-independent areas [144]. Also, DAPT treatment in zebrafish embryos led to disordered somite formation and induced ectopic neurogenesis of motor neurons. These effects were identical to previously observed Notch signaling deficiencies and could be reversed by the administration of recombinant NICD [145].

Notch signaling inhibition in the cochlea via the application of DAPT could be hypothesized to remove the repression which Hes exerts on *Atoh1* transcription, and

direct supporting cells to a hair cell fate. In fact, application of DAPT to the immature mouse cochlea *in vitro* results in an increased number of hair cells, indicating that DAPT blocks Hes transcripts by blocking Notch processing, allowing the unchecked accumulation of Atoh1 in more cells than normal [74]. Mammalian *in vivo* applications have been less encouraging and raise questions as to whether the *in vitro* and non-mammalian results are applicable to mammals. In particular, DAPT application to the mature lesioned guinea pig organ of Corti has had less success in achieving supporting cell to hair cell transdifferentiation. In a study which examined osmotic pump delivery of DAPT to the scala tympani following an aminoglycoside lesion, although some abnormal expression of myosinVIIa was observed in the nonsensory cells of the organ of Corti, no true or functional hair cells were seen [110] Furthermore, it is not clear if this myosinVIIa may have merely appeared in the supporting cells following the phagocytosis of dying hair cells during the lesion, as has been reported to occur [20]. Future studies focusing on DAPT-mediated Notch knockdown in the cells of the inner ear are warranted before making judgments on its efficacy.

While DAPT application may be a logical method for Notch interference, some problems are involved with the use of γ-secretase inhibitors. γ- secretase is a promiscuous enzyme involved in the cleavage processing of several other substrates unrelated to Notch [146]. An important Notch-independent substrate for γ-secretase cleavage is the amyloid

precursor protein (APP)[b]. γ-secretase cleaves APP at several points, and while improper cleavage results in the neurotoxic amyloid β42 protein, this is rare and pathologic [29]. APP is an integral membrane protein in mammals, widely expressed in many tissues, particularly in synapses. Although the primary function of APP is not yet fully known, it has been implicated in regulation of synapse formation and neural plasticity [147]. APP expression is up regulated during neuronal differentiation and after neuronal injury and has a proposed role in synaptic formation and repair [148,149]. Therefore, interfering with the proper function of APP by blocking γ-secretase may have a negative effect on the ability of any newly regenerated hair cells to form synapses with afferent nerve terminals. It may also impart global deficits on neuronal function if DAPT were to leave the perilymph and enter the cerebrospinal fluid (with which it is patent). This concern is substantiated by studies looking at mice with improper levels of APP, where the regulation appeared to require a delicate balance. Either too much or too little APP resulted in impaired long-term potentiation and memory loss similar to dementia in humans [150,151]. This suggests that the infusion of DAPT, and other inhibitors of γ-secretase, may be non-specific to Notch, confounding, and perhaps counterproductive to hair cell regeneration in mammals.

One way to advance Notch inhibition therapy is to develop a tool for highly specific interference of Notch signaling that would be supporting-cell specific as well as

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[b] In fact, DAPT was initially developed as a clinical therapy for Alzheimer's disease to prevent the improper cleavage of the amyloid precursor which generates amyloid β.

traceable. It would also be highly beneficial to produce stable, sustained knockdown of Notch, since we have shown (in Chapter 3) that Notch signaling up-regulation continues in the traumatized organ of Corti for at least one month following ototoxicity. If this sustained Notch1-Jagged1 signaling induces Hes expression following a lesion, this would repress any Atoh1 transcription and prevent supporting cell to hair cell transdifferentiation. Adenoviral vectors delivered to the mature organ of Corti have already been shown to preferentially transfect the nonsensory cells of the inner ear, and stably express reporter genes for more than one month [80,152,153]. While human gene therapy may require the use of more sophisticated or vetted vectors, adenoviral gene therapy is an ideal choice for testing in animal models whether the over-expression of certain genes may result in Notch knockdown.

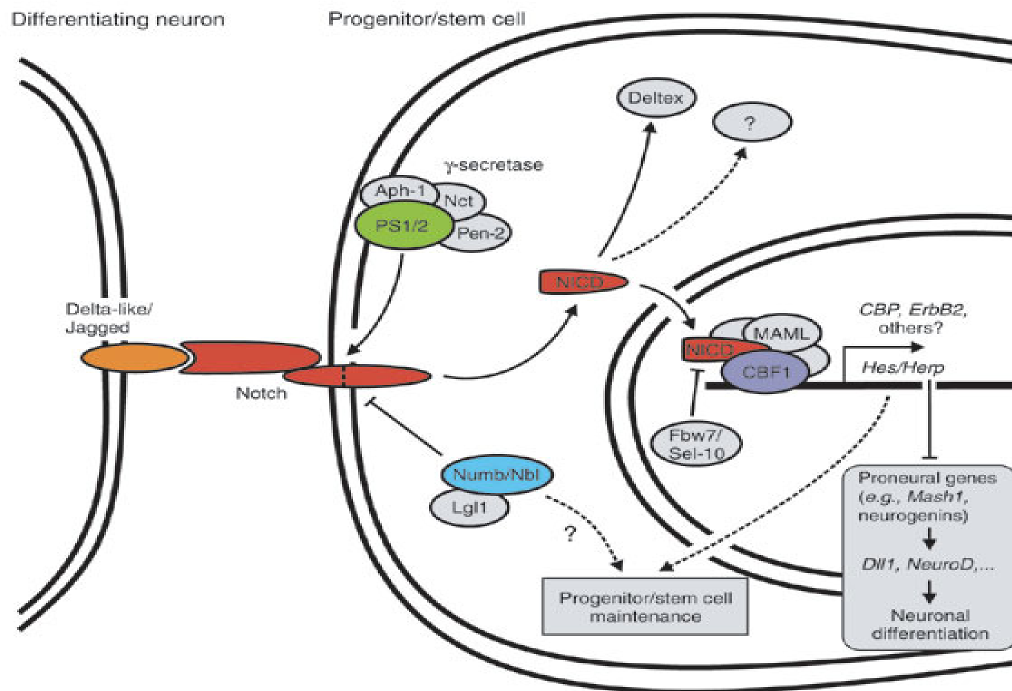

Figure 23: MAML forms a transcription activator complex with NICD and CBF1 in the nucleus, resulting in Hes gene transcription. From Zine et al 2003.[154].

Mastermind-like (MAML) is a family of genes which encode critical transcriptional activators for Notch dependent Hes gene transcription, making it an excellent target for engineering experimental Notch knockdown [31]. MAML forms a functional DNA-binding transcription complex with NICD and CSL (CBF1/RBP-Jk in mammals, SuH in flies, and Lag-1 in *C. elegans*) in the nuclei of cells engaging in Notch signaling (Figure 23) [155-157]. The presence of NICD results in the displacement of CSL's repressors and the recruitment of transcriptional co-activators, including MAML. The

new complex, whose core is NICD, MAML and CSL, is then able to activate target gene expression, particularly the bHLH genes in the Hes family [158].

Three mammalian MAML proteins have been identified, MAML1, 2, and 3, all which have significant sequence and structure homology [159]. All contain an N-terminal basic domain responsible for NICD binding and transcriptional domains required for transcriptional activation to occur [158]. The three MAMLs exhibit distinct expression patterns and of the three, MAML1 alone has been identified as expressed in the developing cochlea of both mice and humans [160,161]. Furthermore, MAML1 and MAML2 have a high affinity to the intracellular domain of the Notch1 receptor, which is the Notch receptor in the inner ear, and highly amplify *Hes1* transcription, while MAML3 was found to bind less efficiently to Notch and to not affect Hes.

Additionally, our preliminary immunohistochemistry verified that MAML1 (Abcam, ab#17019; paired with a rhodamine-conjugated secondary antibody) was observed in the developing mouse organ of Corti (Figure 24) and in the nuclei of some cells in the mature mouse utricle (Figure 25), while negative controls had no signal. These data along with previous reports suggest that MAML, and particularly MAML1, is an ideal target for manipulation of Notch-mediated transcription in the traumatized inner ear during the reinitiation of Notch signaling[160,161].

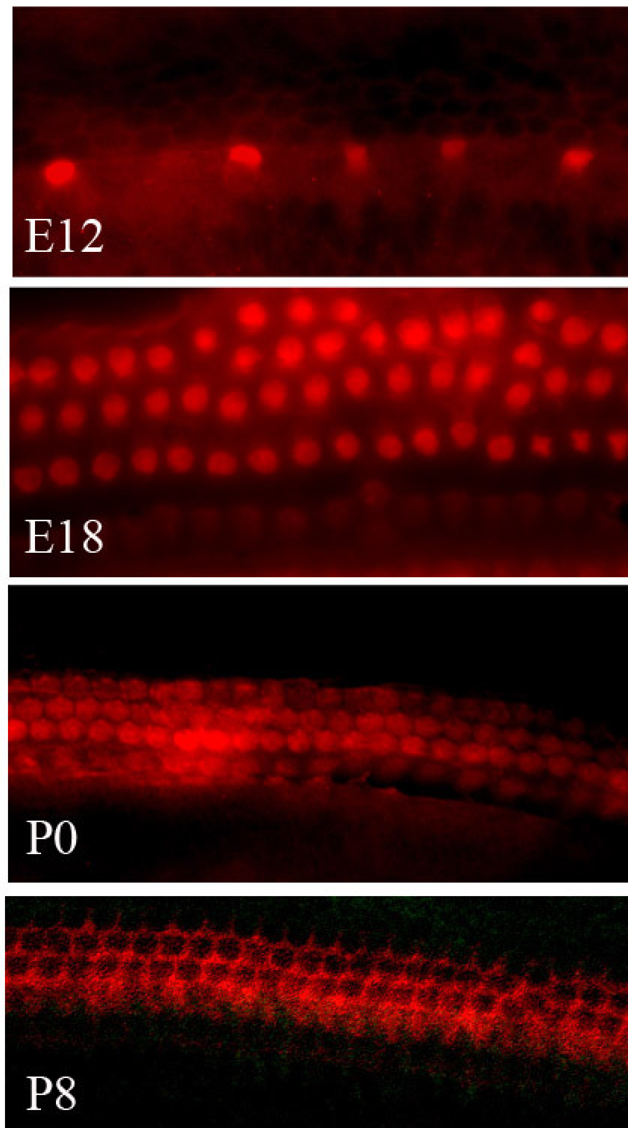

Figure 24. MAML1 is present in the developing and postnatal mouse organ of Corti. At embryonic day 12 (E12) MAML1 (red) signal is restricted to a few cells in the inner sulcus but by E18 MAML1 signal is widespread. This signal appears to persist in the postnatal organ of Corti and is first found in all cell types and then is restricted to supporting cells by P8, but the location does not appear to be tightly nuclear as in embryogenesis.

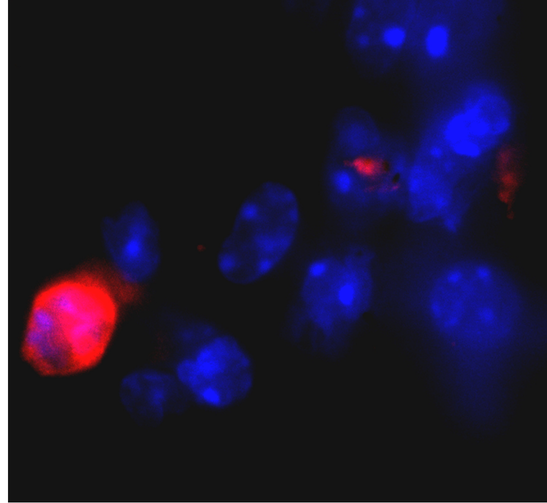

Figure 25. 40x magnification of DAPI (blue) labeled nuclei in the adult mouse utricle. One nucleus is highly positive for MAML1 (red).

Mutations in the MAML1 gene which change the essential DNA binding domains, rendering the protein deficient in transcriptional activities but still capable of binding NICD, interfere with the ability of MAML1 to facilitate Notch signaling and downstream gene transcription [158]. A particularly valuable tool is a dominant-negative mutation of MAML1 (dnMAML1), which consists of a 62 amino-acid peptide N-terminus of mouse MAML1 (structurally shown to interact with the Notch1-CSL transcriptional complex) but has the DNA transcriptional site removed [155]. Due to the high homology of the three MAML family members, dnMAML1 is a Pan-Notch inhibitor and interferes with the endogenous function of all MAML protein transcriptional activation of all four Notch receptors [162]. dnMAML1 (under the transcription of a tamoxifen-inducible Cre-lox) knock-in mouse models have very recently been generated

to investigate in vivo functional consequences of inhibiting endogenous MAML function. Inducible mouse models were developed as true knockouts of MAML function were early embryonic lethal, further substantiating the role of MAML in mediating Notch signaling. Preliminary studies following the induction of dnMAML1-cre mice have reported defects in several systems including T-helper cell development, proper skin cell turnover and vascular smooth muscle, and cardiovascular development and maintenance, all of which are Notch-signaling dependent processes[163,164].

The roles of MAML in other systems including the inner ear have not been well characterized, but the generation of a tool to do so could prove valuable to inner ear research. Taken together with the expression of MAML1 and Notch1 in the embryonic mammalian cochlea, the known coactivation of the Notch1 receptor by MAML1 and 2, and the deficits in Notch-dependent processes in other systems following dnMAML1 induction, it is logical to assume that disruption of MAML in the developing or lesioned inner ear should inhibit Notch signaling and subsequent Hes transcription.

We report here the generation of an adenoviral vector with an insert of the dnMAML1 gene fused with a green fluorescent protein (GFP) reporter, Ad.dnMAML1-GFP. The experimental virus stably transfects two tested mammalian cell types—cos7 and guinea pig fibroblast cells (from David Corey, Massachusetts Eye and Ear Infirmary)— and produces the fluorescent fusion protein at levels detectable without immunohistochemistry.

4.2 Methods and Results

Overview

To create a recombinant adenoviral vector with a dnMAML1-GFP insert, first the dnMAML1-GFP complementary DNA (cDNA) was isolated and subcloned into a pACCMV2 shuttle vector with a CMV promoter in front of the cloning site. Next, a recombinant adenoviral plasmid was generated between the *EcoRI* and *XbaI* restriction sites in competent *E coli* cells using antibiotic selection with ampicillin, and then linearized with *PmeI*. Last the recombinant adenoviral DNA was transfected into 293 cells by the UM Vector Core with viral plaques forming about 10 days later. Ad.MAML1-GFP was verified by immunohistochemistry and Western blot of infected cell lysates to generate MAML1 and GFP.

Immunohistochemistry

To test for the presence of MAML1 at embryonic day (E) 12, E18, P0, and P8 normal mice were deeply anesthetized with xylazine and ketamine as described in the methods of Chapters 2 and 3, decapitated, and the temporal bones were removed. The apical tip of the otic capsule was removed, the oval window opened by dislodging the

stapedial footplate, and the round window opened with forceps. Paraformaldehyde (PFA) 4% in phosphate buffered saline (PBS) was locally perfused through the cochlea. The temporal bone with cochlea *in situ* was allowed to fix for two hours, at which time the bony capsule was dissected away and the modiolus with surrounding cochlear duct removed. These cochleae were stripped of the stria vacularis, tectorial membrane, and Reissner's membrane to fully expose the organ of Corti. Specimens were washed three times for ten minutes in PBS and permeabilized using 0.1% Triton X-100 in PBS for ten minutes. Tissue was then blocked using 10% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in PBS for thirty minutes at room temperature. Following blocking, specimens were incubated overnight at 4°C in a 1:100 primary antibody solution in PBS. The MAML1 primary antibody used was raised in goat (Abcam, ab17019). The following morning, samples were washed three times in PBS and incubated in a Rhodamine (TRITC)-conjugated AffiniPure donkey anti-goat IgG secondary antibody (Jackson ImmunoResearch, 111-025-144) for thirty minutes at room temperature. The cochleae were then washed three times for five minutes in PBS, dissected into four pieces (apex, 3rd turn, 2nd turn, base) and whole mounted onto slides with Gel/Mount mounting media (Biomeda, Foster City, CA). For the *in vitro* experiments described, cells were allowed to grow on to coverslips which were the exposed to the above fixation and staining protocol. Visualization of the nucleus was achieved by incubation in the DNA-binding fluorescent stain 4', 6-diamidino-2-phenylindole (DAPI) diluted 1:600, for three minutes.

Adenoviral Shuttle Plasmid

The adenoviral shuttle plasmid pACCMV2 was acquired from Tom Lanigan in the University of Michigan Vector Core. This 4769 base pair (bp) plasmid had a packaging capacity of 7.4 kb (kilobases). It contained the unique restriction tags *SwaI*, *SfiI*, and *PmeI* and contained a CMV promoter, an ampicillin resistance cassette, a pUC19 polylinker, SV40 splice polyA site, and a loxP site (Figure 26).

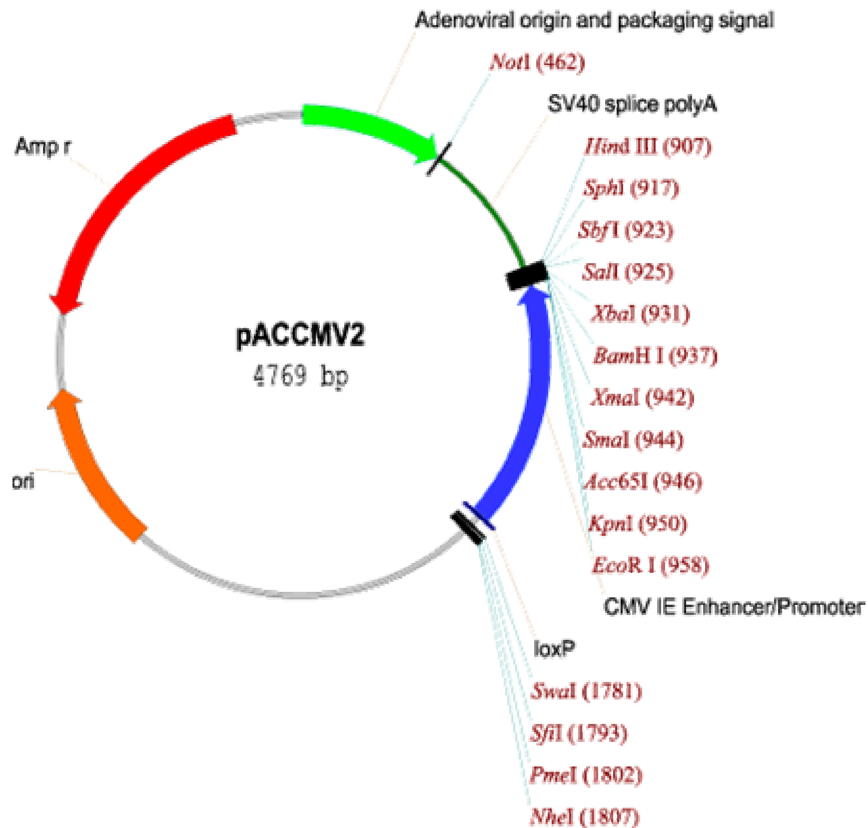

Figure 26. Restriction map of pACCMV2 shuttle vector. The cassette conferring ampicillin resistance is in red, and the splice site is the dark green area between the adenoviral packaging signal (bright green) and the CMV promoter (blue). Source: UM Vector Core.

dnMAML1-GFP

A construct encoding the pan-Notch inhibitor DNMAML1 had been fused with a GFP reporter gene [162]. This construct had been developed to express a dominant negative version of MAML1 which had its NICD binding domain intact but its DNA

transcriptional region deleted, providing a binding substrate whose transcriptional activities were completely compromised.

Restriction Digest and Ligation

To prepare the plasmid DNA for ligation and cloning, pdnMAML1-GFP was digested with *BamHI* and *XbaI* to excise the dnMAML1-GFP cDNA. The pACCMV2 plasmid was also restricted with the enzymes *BamHI* and *XbaI* to release the ligation site. Digestion occurred in individual plastic tubes in the presence of the appropriate buffer solutions, placed in a 37º C water-bath overnight. The restriction products were visualized with 1% agarose gel electrophoresis (140 volts for one hour) and expected weights (~1 kb for the dnMAML1-GFP cDNA and ~5 Kb for the pACCMV2) were compared against a 10 Kb ladder (Figure 27, upper panels). The cDNA was then excised and purified of agarose with Qiagen QIAQuick Elution Kit (Figure 27, lower panels).

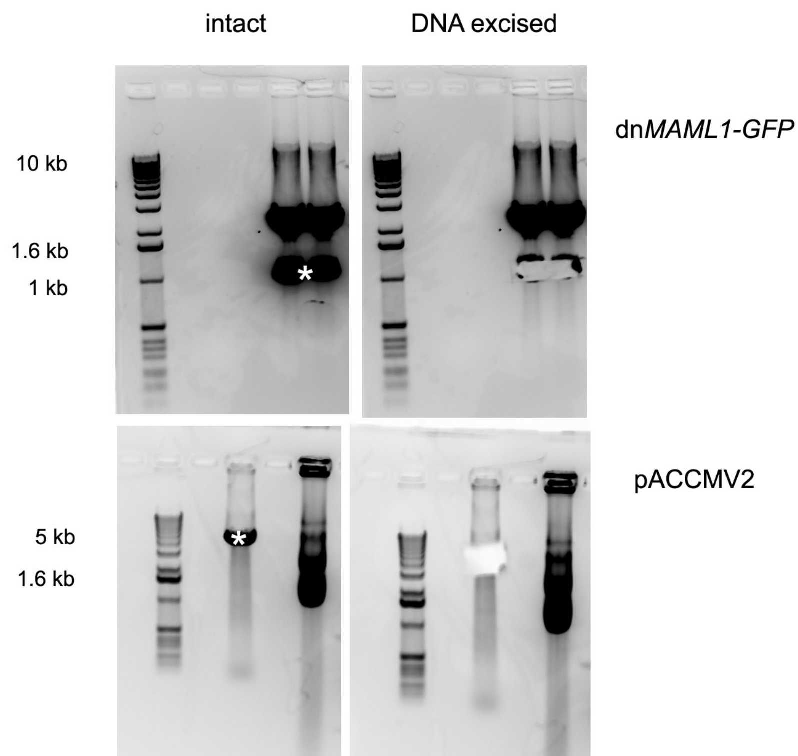

Figure 27. Visualization and excision of cDNA. DNA Restricted from pdnMAML1-GFP (upper panels, ~ 1 kb) and pACCMV2 plasmids (lower panels, ~ 4.8 kb) against a 10 kb ladder (left-most lane in each gel). cDNA from pACCMV2 was compared to uncut plasmid (the right-most lane in the lower two gels) to confirm restriction.

The shuttle and insert were ligated together with a T4 DNA ligase reaction in a 1:3 vector to insert concentration. The ligation reaction was performed at overnight at 16 degrees C water bath.

<u>Bacterial Cells and Transformation</u>

The recombinant ligated DNA was cloned and propagated in One Shot MAX Efficiency DH5α T1R *E. col*i competent cells (Invitrogen), a strain resistant to T1 and T5 bacteriophages and have a high transfection efficiency rate (>$1x10^9$). One microliter of ligated DNA was added to 100 µl of competent cells and allowed to mix for 10 minutes on ice in a plastic tube. The tube was then placed in a 42 degree C water bath for 45 seconds to heat shock the cells and transform the DNA into the cells. 900 µl media was added and the cells were allowed to recover in a 37 degree shaking environment. The transformed cells were spread onto LB-agarose-ampicillin (100 µl per 1 mL of media) plates and allowed to grow overnight at 37 degrees. This ensured conditions that would selectively allow bacteria which had incorporated the ampicillin resistance cassette in the adenoviral shuttle vector to survive and propagate. The next morning, ten isolated colonies were chosen and propagated in ten separate 10 mL cultures of LB media for 4 hours in a 37 degree shaking environment.

DNA Extraction from Transformed Bacteria and Large Scale Prep

Bacterial DNA was extracted from the ten small scale liquid cultures with a Qiagen Mini-Prep DNA Extraction Kit and quantified to about 1 µg/µl. To confirm correct ligation in each culture, the DNA preps from each was digested with the restriction enzymes XbaI and EcoRI, or EcoRI alone. EcoRI will cut the substrate twice- once to separate dnMAML1 and GFP- and once in beginning of the splice site. XbaI will

cut on the other end of the splice site. Double restriction of the correctly ligated DNA would results in three fragments (~750 bp GFP fragment, 250 bp dnMAML1 fragment, and 4.7 kb plasmid backbone) while the single restriction with EcoRI alone would yield a two fragments (5 kb plasmid backbone and a 250 bp fragment) . All 10 samples exhibited the proper size and correct insertion of the digested insert (Figure 28).

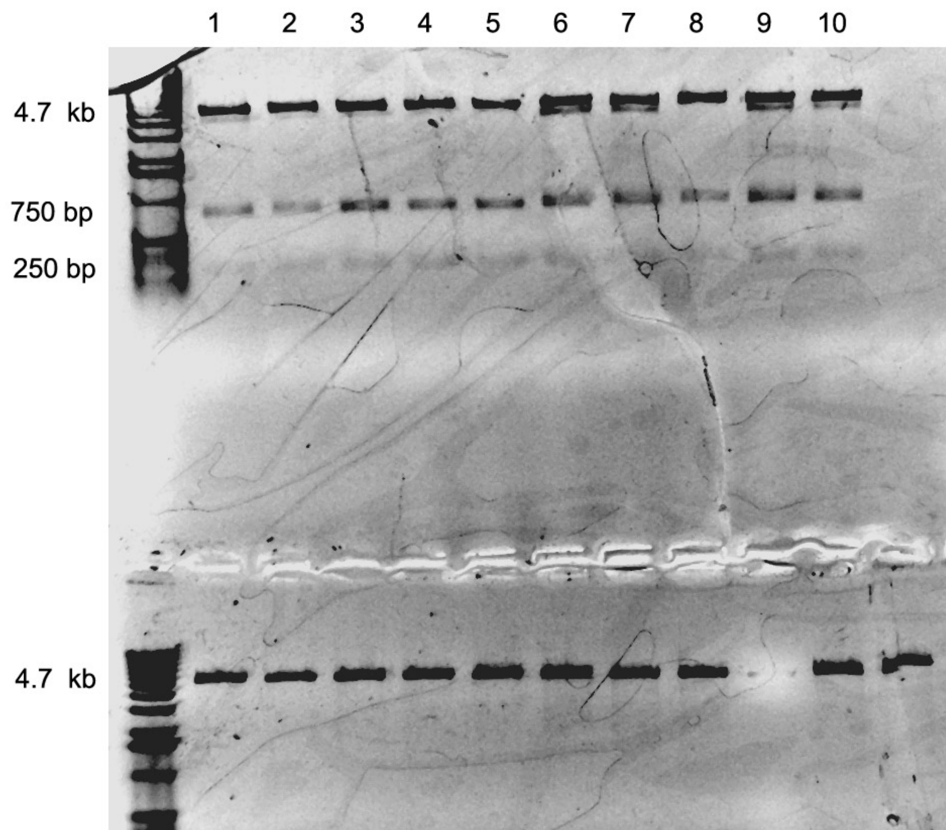

Figure 28: Visualization of ten bacterial DNA preps. Restricted with either EcoRI and XbaI (top) or EcoRI alone (bottom), compared against a 10 kb ladder (first lane). One lane was skipped in the bottom gel due to damage to the well (9 and 10 are moved one lane to the right).

We chose 100 μl of the bacterial colony #5 (which yielded #5 restriction product visualized in Figure 28) and inoculated it into three 300 mL LB with ampicillin cultures, and allowed the colony to propagate overnight at 37 degrees C in a shaking environment. Resultant bacterial DNA was extracted with a Qiagen Maxi Prep kit, which yielded about 75 mg of DNA. A sample of this DNA was digested with the enzymes XbaI and EcoRI to verify the insert, and the correct fragments and weights was observed (EcoRI digestion = 4.7 kb + 250 bp, and EcoRI + XbaI digestion = 4.7 kb + 750 bp + 250 bp) (Figure 29 left panel).

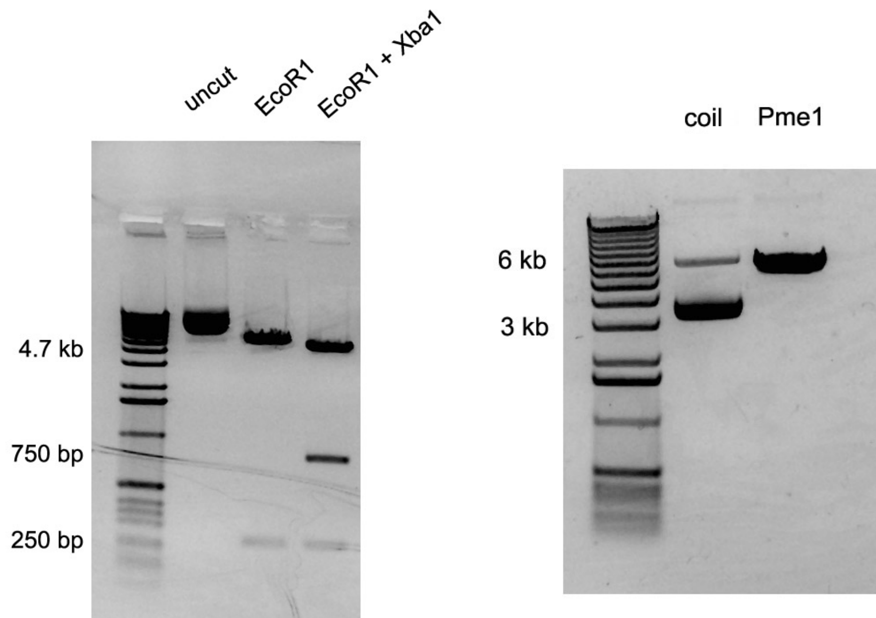

Figure 29: Visualization gels of restricted and uncut large-scale bacterial prep and linearization of DNA with the enzyme PmeI. Left: Restricted DNA was either cut with XbaI and EcoRI, or just EcoRI. Right: Supercoiled DNA (coil) and DNA linearized with PmeI (PmeI) is visualized. Expected weight of linearized recombinant DNA is ~5.8 kb.

DNA Sequencing and Linearization

The correct sequence and orientation of the recombinant DNA was confirmed by having 2 μl sequenced (this was done by the UM Sequencing Core). The information from the sequencing was compared to the known sequence of both GFP and MAML1 using BLAST, and was found to be correct. The DNA required linearization before it could be used for generating a recombinant adenovirus. A small amount of the DNA was linearized in a restriction digest with the enzyme PmeI, chosen because it would not cleave at the splice site. Supercoiled (nonlinearized) DNA was then compared to the PmeI linearized DNA (~6 kb) via electrophoresis in a 1% agarose gel. After the cleavage was confirmed to be correct, all 75 mg of DNA was linearized with the enzyme PmeI in a restriction digest reaction at 37 degrees (Figure 29 right panel). The linearized DNA was then eluted from the agarose and the resultant 60 mg of DNA was quantified to 1 mg/μl in spectrophotometer. 50 mg of linearized DNA was given to the UM Vector Core for viral plaque formation.

Testing Ad.*dnMAML1-GFP in vitro*

The ability of recombinant Ad.dnMAML1-GFP to transfect cells and produce the recombinant fusion protein was tested in two cultured mammalian cells lines: guinea pig fibroblast cells and cos7 cells (*Cercopithecus aethiops* kidney cells) in varying

concentrations (1 or 10 µl virus, titer 2 x 10^{12} particles per milliliter.) Cells cultures in Dulbecco's modified essential medium (DMEM) media at 37 degrees were grown to 60% confluence on glass coverslips and then either 1 µl, 10 µl, or no virus was placed in the media for four hours. Media was then changed, and every 24 hours after that. After three days, coverslips were stained for DAPI, and the slips were examined for the presence of the GFP reporter (which was fused to the dnMAML1 protein). While control cos7 and guinea pig fibroblast cells did not exhibit GFP signal, both cell types treated with either the 1 µl or 10 µl of virus exhibited a large amount of GFP fluorescence (Figure 30 and control guinea pig cells not shown).

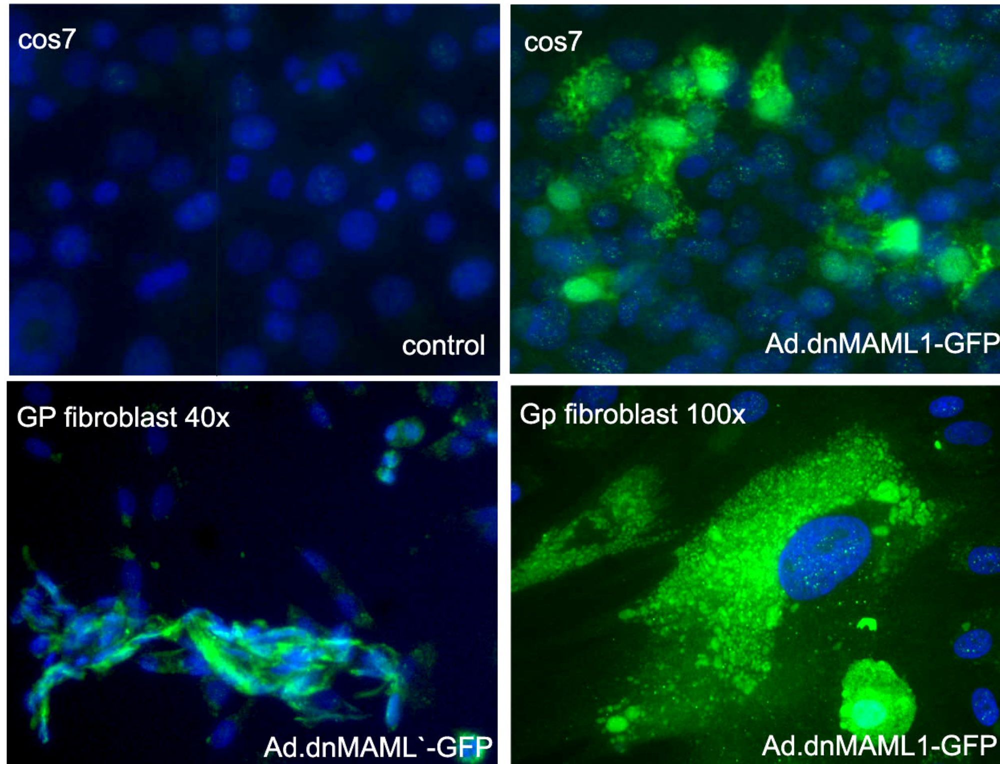

Figure 30: Ad.dnMAML1-GFP transfected both cos7 and guinea pig fibroblast cells and produced GFP fusion protein (green). Blue is the nuclear label DAPI.

4.3 Discussion and Future Work

The family of Mastermind (MAML) genes encode transcriptional co-activators required for Notch signaling and transcription of its downstream gene targets, including bHLH repressor genes *Hes1* and *Hes5*.We have described the generation and testing of a novel recombinant adenoviral vector, Ad.dnMAML1-GFP, which is capable of transfecting cells and over-expressing a dominant-negative version of MAML1, fused with a GFP reporter. We anticipate that this tool for the stable, specific, and traceable knockdown of the Notch signaling pathway will prove a valuable tool for studying the pathway in the inner ear and beyond. In regard to the traumatized inner ear, by creating an inhibitor of Notch which is specific to the formation of the repressor transcriptional complex, and sparing the CSL activator transcriptional complex, the transcription of prosensory genes such as Atoh1 will not be negatively affected. This is essential if hair cell regeneration through supporting cell transdifferentiation is the goal. Futhermore, the addition of a GFP reporter protein to the dominant-negative protein allows the experimenter to compare differences in morphology between GFP+ and GFP- cells, perhaps allowing new hair cells to be distinguished from surviving hair cells by the presence of GFP.

This vector might be paired *in vivo* or *in vitro* with other vectors known to over-express prosensory genes. It would be useful to compare the action of Notch knockdown alone with the paired effect of Ad.Atoh1 over-expression combined with Notch knockdown. It may be possible to determine whether *Atoh1* over-expression yields the same result as Notch knockdown and whether their dual use perhaps exacerbates the regenerative effect by further promoting Atoh1 transcriptional expression and Hes transcriptional repression. It might also be beneficial to study whether virally-mediated Notch knockdown could rescue Hes over-expression in transfected cells or mice models. That would provide powerful evidence that the dominant negative version of MAML1 does interfere with Notch signaling at the level of gene transcription.

This vector could have a wide array of uses outside of its initial intended target, the ear. Many other tissues or systems which uses Mastermind-mediated Notch signaling, such as bone marrow, neurogenesis, muscle maintenance, and skin (just to name a few) could be manipulated through transfection and observed by following the GFP signal. Additionally, many cancers and tumorogenic processes are misregulations of Notch signaling. It is possible that targeted and stable Notch knockdown with gene therapy might prove useful in arresting processes where abnormal signaling provokes cells to reenter the cell cycle and pathologically divide.

Chapter 5

Conclusions and Future Directions

5.1 Summary

As Notch signaling is crucial for both the development of the vertebrate ear and hair cell regeneration in non-mammalian species, it is important to gain a better understanding of the processes of Notch signaling in the mammalian ear to build hypotheses as to why our hair cells are not replaced and to design regenerative therapies. Transdifferentiation from a nonsensory supporting cell fate to a sensory hair cell fate can be forced by over-expression of Atoh1, proving that the fate of mature differentiated supporting cells is malleable [79]. However, until now the state of Notch in mature cochleae both the normal and traumatized ear were unknown. In Chapter 2 we reported that low

level Notch signaling and Hes1 persists into maturity in the normal mammalian ear, providing a potential mechanism by which supporting cell fate may be maintained. In Chapter 3 we report that Notch-Jagged1 signaling resulting in nuclear Hes1 and Hes5 is substantially up-regulated following trauma to the inner ear, and that the non-overlapping presence of Hes1 and Hes5 are reflective of their developmental locations in the early postnatal ear. This up-regulation is apparent by 24 hours, remains at high levels for two weeks, and is absent by 2 months. This finding presents a likely molecular mechanism by which supporting cell to hair cell transdifferentiation is suppressed by the increase in Hes transcription. In Chapter 4 we reported that MAML1 is present in the developing cochlea and presented a recombinant tool for the prevention of post-traumatic Hes up-regulation—Ad.dnMAML1-GFP—which can transfect cells resulting in production a dominant negative version of the Notch transcription co-activator Mastermind. Together, these data contribute knowledge to both mature and post-traumatic Notch signaling in the organ of Corti and present a therapeutic tool to examine the role of Notch knockdown in the inner ear as well as in a variety of other Notch dependent systems.

5.2 Conclusions and Future Directions

Prior work has demonstrated that the traumatized mammalian auditory epithelium can be forced to regenerate hair cells via *Atoh1* over-expression, suggesting that the molecular machinery still exists for mammals to replace the sensory cells of the inner ear.

We theorize that the up-regulation of Notch signaling and Hes activity following hair cell loss in the organ of Corti may be a major roadblock to the initiation of spontaneous hair cell regeneration via transdifferentiation. Also, that Hes activity serves to discourage a cell fate change in supporting cells that remain following a lesion which they survive. The effect of the repression of Notch signaling, and thus Hes expression and the antagonism of Atoh1, might be tested by delivering Ad.dnMAML1-GFP to the traumatized inner ear and observing later the incidence of new hair cells (which may or may not be GFP+). While it is an empirical question as to whether any transdifferentiated hair cells would continue to sequester GFP for long, if so, this would be a valuable tool to discriminate newly arisen hair cells from hair cells which may have survived a lesion.

In light of our data reporting Hes activity in the lesioned organ of Corti, it is reasonable to assume that forced mammalian hair cell regeneration via transdifferentiation may be accomplished by Hes repression. However, it may also be necessary to increase Atoh1 levels to change the fate of the remaining cells in the auditory epithelium. Future studies should explore whether Notch inhibition following a lesion has a direct effect on decreasing Hes activity in the organ of Corti. One important consideration for any therapy which is intended to block unwanted cell signaling is the determination of when such signaling begins. Because Notch signaling and Hes activity peak at around 14 days following a lesion, it likely would be beneficial to deliver therapies which relied on Notch interference before this time point. Even better

supporting cell to hair cell transdifferentiation may be expected if signaling intervention therapy was given right after the insult, bypassing the Notch cascade entirely.

It is also important to explore the possibility of enhancing hair cell regeneration via simultaneous delivery of prosensory gene therapy and inhibitors of Notch signaling, as well as determining whether over-expression of *Atoh1* in supporting cells is overriding the repressive effect Hes may have on transdifferentiation. Our data indicated that following a lesion, Hes5 was predominantly located in the nuclei of supporting cells in the outer hair cell area, and Hes1 was located in nuclei of cells in the inner hair cell area. If Hes1 and Hes5 partially recapitulate their developmental role of *Atoh1* antagonism in locale-specific ways following a lesion, specifically blocking Hes1 after a lesion may increase the likelihood of hair cell regeneration of supporting cells to inner hair cells and blocking Hes5 may provide a similar effect for supporting cell to outer hair transdifferentiation. Future work may address whether it may be possible to selectively induce regeneration of an inner or outer hair cell solely by eliminating the expression of one of the Hes genes (for example, through siRNA).

Transdifferentiation without the addition of new cells is unlikely to restore normal hearing, so future work should focus on either cell replacement or the reinitiation of mitosis in supporting cells. This is another area where the study of avian hair cell regeneration might inform studies in mammals, and lead to comparative studies of genes which prompt the reentry into (and out of) the cell cycle. Co-expression of mitogens with prosensory bHLH genes might be a valuable line of research. As mitosis and constant cell

turnover in other systems (such as the gut lining) is orchestrated via the reinitiation of both the Notch and Wnt signaling pathways, Wnt signaling may also need to be manipulated in the inner ear in concert with Notch. Future studies should also explore how to attract proper innervation to any newly regenerated hair cells, perhaps through the addition of BDNF or NT-3 into viral vectors. Additionally, if gene therapy for the inner ear is to become viable in humans, sophisticated vectors which selectively target supporting cells and have limited cross-infection will be required. This is necessary to prevent the transfection of cells outside of the organ of Corti thus limiting ectopic hair cell regeneration, which may be detrimental to hearing.

5.3 Final Remarks

It was during Alexander Graham Bell's studies of the physiology of the ear that the telephone was initially invented in 1876. However this invention's initial purpose was not long-range real time communication, but was rather intended as a device to aid the hard of hearing through the electrical transmission and amplification of a person's voice. Bell's dream was realized years later. The ideas behind the hearing aid, and to some degree the cochlear implant, have grown out of similar technology, that is, the amplification or electrical processing of sound. While these types of devices have been invaluable, they also ignore the major cause of sensorineural deafness: the loss of hair cells over time and their inability to be spontaneously replaced. The next generation of

therapies for the hard of hearing may come to focus on the regeneration or replacement of these cells to repair, rather than circumvent, the cochlea's amazing ability to transduce sound into neural information. Our best bet may be to take clues from species that retain or can replace what has been lost, and to apply knowledge of their regenerative process to comparative studies of the mammalian inner ear.

It is my hope that the studies that I have undertaken examining the status of Notch signaling components in the mammalian inner ear will contribute knowledge to that endeavor. I have attempted to gauge the status of Notch signaling events in maturity and following trauma to the organ of Corti, to observe the response of this epithelium to hair cell loss. What I have found was in fact quite different from what has been reported in the regenerating avian basilar papilla, however this was predicted due to the vast differences in outcomes following trauma. While the presence of Notch-dependent bHLH proteins which antagonize a sensory fate was found to be up-regulated following hair cell trauma, this response has the potential to be manipulated by drugs or gene therapy that block Notch. I have engineered an adenoviral vector which it is my hope can be used to study whether the knockdown of Notch activity following hair cell loss in the organ of Corti may relieve the repression on prosensory transdifferentiation. It is my hope that others will use the knowledge contained in this dissertation to design therapies that might one day realize Alexander Graham Bells' true goal— to aid the hard of hearing.

References

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1. **Lanz, T.A., *et al.* The gamma-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester reduces A beta levels in vivo in plasma and cerebrospinal fluid in young (plaque-free) and aged (plaque-bearing) Tg2576 mice. *J. Pharmacol. Exp. Ther.* 305, 864-871 (2003).**
2. **Hudspeth, A.J. How hearing happens. *Neuron* 19, 947-950 (1997).**
3. **Kennedy, H.J., Evans, M.G., Crawford, A.C. & Fettiplace, R. Depolarization of cochlear outer hair cells evokes active hair bundle motion by two mechanisms. *J. Neurosci.* 26, 2757-2766 (2006).**
4. **Kennedy, H.J. & Meech, R.W. Fast Ca2+ signals at mouse inner hair cell synapse: a role for Ca2+-induced Ca2+ release. *J Physiol* 539, 15-23 (2002).**
5. **Wu, X., Gao, J., Guo, Y. & Zuo, J. Hearing threshold elevation precedes hair-cell loss in prestin knockout mice. *Brain Res. Mol. Brain Res.* 126, 30-37 (2004).**
6. **Geleoc, G.S. & Holt, J.R. Auditory amplification: outer hair cells pres the issue. *Trends Neurosci.* 26, 115-117 (2003).**
7. **Spicer, S.S., Smythe, N. & Schulte, B.A. Distribution of canalicular reticulum in Deiters cells and pillar cells of gerbil cochlea. *Hear. Res.* 130, 7-18 (1999).**
8. **Tucker, J.B., Mackie, J.B., Bussoli, T.J. & Steel, K.P. Cytoskeletal integration in a highly ordered sensory epithelium in the organ of Corti: reponse to loss of cell partners in the Bronx waltzer mouse. *J. Neurocytol.* 28, 1017-1034 (1999).**
9. **Forge, A., *et al.* Gap junctions and connexin expression in the inner ear. [Review] [46 refs]. *Novartis Found. Symp.* 219, 134-150; discussion 151-136 (1999).**
10. **Patel, N.P., Mhatre, A.N. & Lalwani, A.K. Biological therapy for the inner ear. *Expert Opin Biol Ther* 4, 1811-1819 (2004).**
11. **Johnsson, L.G. & Hawkins, J.E., Jr. Degeneration patterns in human ears exposed to noise. *Ann. Otol. Rhinol. Laryngol.* 85, 725-739 (1976).**
12. **Anniko, M. & Sobin, A. Cisplatin: evaluation of its ototoxic potential. *Am. J. Otolaryngol.* 7, 276-293 (1986).**
13. **Comis, S.D., *et al.* Early morphological and chemical changes induced by cisplatin in the guinea pig organ of Corti. *J. Laryngol. Otol.* 100, 1375-1383 (1986).**
14. **Liu, W., *et al.* Caspase inhibitors prevent cisplatin-induced apoptosis of auditory sensory cells. *Neuroreport* 9, 2609-2614 (1998).**

15. **Rybak, L.P. & Somani, S. Ototoxicity. Amelioration by protective agents. *Ann. N. Y. Acad. Sci.* 884, 143-151 (1999).**
16. **Zheng, Y., Schachern, P.A., Sone, M. & Papapella, M.M. Aminoglycoside ototoxicity. *Otol Neurotol* 22, 266-268 (2001).**
17. **Wu, W.J., *et al.* Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague-Dawley rat. *Hear. Res.* 158, 165-178. (2001).**
18. **Rabinowitz, P.M., Galusha, D., Dixon-Ernst, C., Slade, M.D. & Cullen, M.R. Do ambient noise exposure levels predict hearing loss in a modern industrial cohort? *Occup. Environ. Med.* 64, 53-59 (2007).**
19. **Ping Yang, W., Henderson, D., Hua Hu, B. & Nicotera, T.M. Quantitative analysis of apoptotic and necrotic outer hair cells after exposure to different levels of continuous noise. *Hear. Res.* 196, 69-76 (2004).**
20. **Jiang, H., Sha, S.H., Forge, A. & Schacht, J. Caspase-independent pathways of hair cell death induced by kanamycin in vivo. *Cell Death Differ.* 13, 20-30 (2006).**
21. **Abrashkin, K.A., *et al.* The fate of outer hair cells after acoustic or ototoxic insults. *Hear. Res.* (2006).**
22. **Doe, C.Q. & Spana, E.P. A collection of cortical crescents: asymmetric protein localization in CNS precursor cells. *Neuron* 15, 991-995 (1995).**
23. **Lewis, J. From signals to patterns: space, time, and mathematics in developmental biology. *Science* 322, 399-403 (2008).**
24. **Ehebauer, M., Hayward, P. & Martinez-Arias, A. Notch signaling pathway. *Sci STKE* 2006, cm7 (2006).**
25. **Ehebauer, M., Hayward, P. & Arias, A.M. Notch, a universal arbiter of cell fate decisions. *Science* 314, 1414-1415 (2006).**
26. **Ma, B., Simala-Grant, J.L. & Taylor, D.E. Fucosylation in prokaryotes and eukaryotes. *Glycobiology* 16, 158R-184R (2006).**
27. **Radtke, F., Clevers, H. & Riccio, O. From gut homeostasis to cancer. *Curr Mol Med* 6, 275-289 (2006).**
28. **Hartmann, D., *et al.* The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. *Hum. Mol. Genet.* 11, 2615-2624 (2002).**
29. **Hartmann, D., Tournoy, J., Saftig, P., Annaert, W. & De Strooper, B. Implication of APP secretases in notch signaling. *J. Mol. Neurosci.* 17, 171-181 (2001).**
30. **LaVoie, M.J. & Selkoe, D.J. The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. *J. Biol. Chem.* 278, 34427-34437 (2003).**
31. **Wu, L., *et al.* Cloning and functional characterization of the murine mastermind-like 1 (Maml1) gene. *Gene* 328, 153-165 (2004).**

32. **Cave, J.W. & Caudy, M.A. Promoter-specific co-activation by Drosophila mastermind. *Biochem. Biophys. Res. Commun.* 377, 658-661 (2008).**
33. **Kovall, R.A. More complicated than it looks: assembly of Notch pathway transcription complexes. *Oncogene* 27, 5099-5109 (2008).**
34. **Fukami, M., *et al.* Mastermind-like domain-containing 1 (MAMLD1 or CXorf6) transactivates the Hes3 promoter, augments testosterone production, and contains the SF1 target sequence. *J. Biol. Chem.* 283, 5525-5532 (2008).**
35. **Wu, L., Maillard, I., Nakamura, M., Pear, W.S. & Griffin, J.D. The transcriptional coactivator Maml1 is required for Notch2-mediated marginal zone B-cell development. *Blood* 110, 3618-3623 (2007).**
36. **McElhinny, A.S., Li, J.L. & Wu, L. Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. *Oncogene* 27, 5138-5147 (2008).**
37. **Jarriault, S., *et al.* Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol. Cell. Biol.* 18, 7423-7431 (1998).**
38. **Kageyama, R. & Ohtsuka, T. The Notch-Hes pathway in mammalian neural development. *Cell Res.* 9, 179-188 (1999).**
39. **Weinmaster, G. Notch signaling: direct or what? *Curr. Opin. Genet. Dev.* 8, 436-442 (1998).**
40. **Jack, J. & DeLotto, Y. Effect of wing scalloping mutations on cut expression and sense organ differentiation in the Drosophila wing margin. *Genetics* 131, 353-363 (1992).**
41. **de Celis, J.F., Barrio, R., del Arco, A. & Garcia-Bellido, A. Genetic and molecular characterization of a Notch mutation in its Delta- and Serrate-binding domain in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* 90, 4037-4041 (1993).**
42. **de Celis, J.F. & Garcia-Bellido, A. Roles of the Notch gene in Drosophila wing morphogenesis. *Mech. Dev.* 46, 109-122 (1994).**
43. **de Celis, J.F. & Garcia-Bellido, A. Modifications of the notch function by Abruptex mutations in Drosophila melanogaster. *Genetics* 136, 183-194 (1994).**
44. **Campos-Ortega, J.A. Genetic mechanisms of early neurogenesis in Drosophila melanogaster. *Mol. Neurobiol.* 10, 75-89 (1995).**
45. **Skeath, J.B. & Doe, C.Q. Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the Drosophila CNS. *Development* 125, 1857-1865 (1998).**
46. **Ye, Y., Lukinova, N. & Fortini, M.E. Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants. *Nature* 398, 525-529 (1999).**

47. **Martin-Bermudo, M.D., Carmena, A. & Jimenez, F. Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification. *Development* 121, 219-224 (1995).**
48. **Stump, G., *et al.* Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain. *Mech. Dev.* 114, 153-159 (2002).**
49. **Irvin, D.K., Zurcher, S.D., Nguyen, T., Weinmaster, G. & Kornblum, H.I. Expression patterns of Notch1, Notch2, and Notch3 suggest multiple functional roles for the Notch-DSL signaling system during brain development. *J. Comp. Neurol.* 436, 167-181 (2001).**
50. **Shawber, C., Boulter, J., Lindsell, C.E. & Weinmaster, G. Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev. Biol.* 180, 370-376 (1996).**
51. **Lindsell, C.E., Boulter, J., diSibio, G., Gossler, A. & Weinmaster, G. Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol. Cell. Neurosci.* 8, 14-27 (1996).**
52. **Austin, C.P., Feldman, D.E., Ida, J.A., Jr. & Cepko, C.L. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* 121, 3637-3650 (1995).**
53. **Lindner, V., *et al.* Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction. *Am. J. Pathol.* 159, 875-883 (2001).**
54. **Rodriguez, S., *et al.* Notch2 is required for maintaining sustentacular cell function in the adult mouse main olfactory epithelium. *Dev. Biol.* 314, 40-58 (2008).**
55. **Seta, Y., Toyono, T., Kataoka, S. & Toyoshima, K. Regulation of taste bud cell differentiation by notch signaling pathway. *Chem. Senses* 30 Suppl 1, i48-49 (2005).**
56. **Kohler, C., *et al.* Expression of Notch-1 and its ligand Jagged-1 in rat liver during liver regeneration. *Hepatology* 39, 1056-1065 (2004).**
57. **Conboy, I.M., *et al.* Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* 433, 760-764 (2005).**
58. **Berezovska, O., Xia, M.Q. & Hyman, B.T. Notch is expressed in adult brain, is coexpressed with presenilin-1, and is altered in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 57, 738-745 (1998).**
59. **Sestan, N., Artavanis-Tsakonas, S. & Rakic, P. Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science* 286, 741-746 (1999).**
60. **Wang, S., *et al.* Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* 21, 63-75 (1998).**

61. **Mitsiadis, T.A., Fried, K. & Goridis, C. Reactivation of Delta-Notch signaling after injury: complementary expression patterns of ligand and receptor in dental pulp. *Exp. Cell Res.* 246, 312-318 (1999).**
62. **Wang, S.F., *et al.* Development of Notch-dependent T-cell leukemia by deregulated Rap1 signaling. *Blood* 111, 2878-2886 (2008).**
63. **Piccoli, D.A. & Spinner, N.B. Alagille syndrome and the Jagged1 gene. *Semin. Liver Dis.* 21, 525-534 (2001).**
64. **Ruchoux, M.M. & Maurage, C.A. CADASIL: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *J. Neuropathol. Exp. Neurol.* 56, 947-964 (1997).**
65. **Haglund, K., Rusten, T.E. & Stenmark, H. Aberrant receptor signaling and trafficking as mechanisms in oncogenesis. *Crit. Rev. Oncog.* 13, 39-74 (2007).**
66. **Hambardzumyan, D., Becher, O.J. & Holland, E.C. Cancer stem cells and survival pathways. *Cell Cycle* 7, 1371-1378 (2008).**
67. **Zhang, P., Yang, Y., Zweidler-McKay, P.A. & Hughes, D.P. Critical role of notch signaling in osteosarcoma invasion and metastasis. *Clin. Cancer Res.* 14, 2962-2969 (2008).**
68. **Zine, A., Van De Water, T.R. & de Ribaupierre, F. Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. *Development* 127, 3373-3383 (2000).**
69. **Kiernan, A.E., *et al.* The Notch ligand Jagged1 is required for inner ear sensory development. *Proc. Natl. Acad. Sci. U. S. A.* 98, 3873-3878 (2001).**
70. **Brooker, R., Hozumi, K. & Lewis, J. Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. *Development* 133, 1277-1286 (2006).**
71. **Zine, A., *et al.* Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J. Neurosci.* 21, 4712-4720 (2001).**
72. **Zheng, J.L., Shou, J., Guillemot, F., Kageyama, R. & Gao, W.Q. Hes1 is a negative regulator of inner ear hair cell differentiation. *Development* 127, 4551-4560 (2000).**
73. **Lanford, P.J., *et al.* Notch signalling pathway mediates hair cell development in mammalian cochlea [see comments]. *Nat. Genet.* 21, 289-292 (1999).**
74. **Daudet, N., Ariza-McNaughton, L. & Lewis, J. Notch signalling is needed to maintain, but not to initiate, the formation of prosensory patches in the chick inner ear. *Development* 134, 2369-2378 (2007).**
75. **Chen, P. & Segil, N. p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. *Development* 126, 1581-1590 (1999).**
76. **Woods, C., Montcouquiol, M. & Kelley, M.W. Math1 regulates development of the sensory epithelium in the mammalian cochlea. *Nat. Neurosci.* 7, 1310-1318 (2004).**

77. **Bermingham, N.A., *et al.* Math1: an essential gene for the generation of inner ear hair cells. *Science* 284, 1837-1841 (1999).**
78. **Zheng, J.L. & Gao, W.Q. Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* 3, 580-586 (2000).**
79. **Izumikawa, M., *et al.* Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nat. Med.* 11, 271-276 (2005).**
80. **Kawamoto, K., Ishimoto, S., Minoda, R., Brough, D.E. & Raphael, Y. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. *J. Neurosci.* 23, 4395-4400 (2003).**
81. **Adam, J., *et al.* Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sense-organ development. *Development* 125, 4645-4654 (1998).**
82. **Adler, H.J. & Raphael, Y. New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear [published erratum appears in Neurosci Lett 1996 May 24;210(1):73]. *Neurosci. Lett.* 205, 17-20 (1996).**
83. **Baird, R.A., Burton, M.D., Fashena, D.S. & Naeger, R.A. Hair cell recovery in mitotically blocked cultures of the bullfrog saccule. *Proc. Natl. Acad. Sci. U. S. A.* 97, 11722-11729 (2000).**
84. **Lardon, J. & Bouwens, L. Metaplasia in the pancreas. *Differentiation* 73, 278-286 (2005).**
85. **Krabbe, C., Zimmer, J. & Meyer, M. Neural transdifferentiation of mesenchymal stem cells--a critical review. *APMIS* 113, 831-844 (2005).**
86. **Thorgeirsson, S.S. & Grisham, J.W. Hematopoietic cells as hepatocyte stem cells: a critical review of the evidence. *Hepatology* 43, 2-8 (2006).**
87. **Stone, J.S. & Rubel, E.W. Delta1 expression during avian hair cell regeneration. *Development* 126, 961-973 (1999).**
88. **Horb, M.E., Shen, C.N., Tosh, D. & Slack, J.M. Experimental conversion of liver to pancreas. *Curr. Biol.* 13, 105-115 (2003).**
89. **Slack, J.M. Intestine in the lung. *J Biol* 3, 10 (2004).**
90. **Rajendra, S., *et al.* Loss of HLA class I and gain of class II expression are early events in carcinogenesis - clues from a study of Barrett's oesophagus. *J. Clin. Pathol.* (2006).**
91. **Wong, N.A., *et al.* EpCAM and gpA33 are markers of Barrett's metaplasia. *J. Clin. Pathol.* 59, 260-263 (2006).**
92. **Kablar, B., Tajbakhsh, S. & Rudnicki, M.A. Transdifferentiation of esophageal smooth to skeletal muscle is myogenic bHLH factor-dependent. *Development* 127, 1627-1639 (2000).**

93. **Mitashov, V.I. Retinal regeneration in amphibians. *Int. J. Dev. Biol.* 41, 893-905 (1997).**
94. **Stroeva, O.G. & Mitashov, V.I. Retinal pigment epithelium: proliferation and differentiation during development and regeneration. *Int. Rev. Cytol.* 83, 221-293 (1983).**
95. **Corwin, J.T. & Cotanche, D.A. Regeneration of sensory hair cells after acoustic trauma. *Science* 240, 1772-1774 (1988).**
96. **Ryals, B.M. & Rubel, E.W. Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science* 240, 1774-1776 (1988).**
97. **Cotanche, D.A. & Lee, K.H. Regeneration of hair cells in the vestibulocochlear system of birds and mammals. *Curr. Opin. Neurobiol.* 4, 509-514 (1994).**
98. **Hashino, E., Tanaka, Y., Salvi, R.J. & Sokabe, M. Hair cell regeneration in the adult budgerigar after kanamycin ototoxicity. *Hear. Res.* 59, 46-58 (1992).**
99. **Hashino, E. & Salvi, R.J. Changing spatial patterns of DNA replication in the noise-damaged chick cochlea. *J. Cell Sci.* 105, 23-31 (1993).**
100. **Raphael, Y. Evidence for supporting cell mitosis in response to acoustic trauma in the avian inner ear. *J. Neurocytol.* 21, 663-671 (1992).**
101. **Stone, J.S. & Cotanche, D.A. Identification of the timing of S phase and the patterns of cell proliferation during hair cell regeneration in the chick cochlea. *J. Comp. Neurol.* 341, 50-67 (1994).**
102. **Raphael, Y., Adler, H.J., Wang, Y. & Finger, P.A. Cell cycle of transdifferentiating supporting cells in the basilar papilla. *Hear. Res.* 80, 53-63 (1994).**
103. **Roberson, D.W., Alosi, J.A. & Cotanche, D.A. Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium. *J. Neurosci. Res.* 78, 461-471 (2004).**
104. **Roberson, D.W., Kreig, C.S. & Rubel, E.W. Light microscopic evidence that direct transdifferentiation gives rise to new hair cells in regenerating avian auditory epithelium. *Auditory Neuroscience* 2, 195-205 (1996).**
105. **Stone, J.S. & Cotanche, D.A. Hair cell regeneration in the avian auditory epithelium. *Int. J. Dev. Biol.* 51, 633-647 (2007).**
106. **Cafaro, J., Lee, G.S. & Stone, J.S. Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. *Dev. Dyn.* 236, 156-170 (2007).**
107. **Ben-Arie, N., *et al.* Functional conservation of atonal and Math1 in the CNS and PNS. *Development* 127, 1039-1048 (2000).**
108. **Jarman, A.P., Grau, Y., Jan, L.Y. & Jan, Y.N. atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. *Cell* 73, 1307-1321 (1993).**

109. **Woods, C., Montcouquiol, M. & Kelley, M.W. Math1 regulates development of the sensory epithelium in the mammalian cochlea. *Nat. Neurosci.* (2004).**
110. **Hori, R*., et al.* Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea. *Neuroreport* 18, 1911-1914 (2007).**
111. **Kelley, M.W. Cellular commitment and differentiation in the cochlea: potential advances using gene transfer. [Review] [80 refs]. *Audiol. Neurootol.* 2, 50-60 (1997).**
112. **Lewis, A.K., Frantz, G.D., Carpenter, D.A., de Sauvage, F.J. & Gao, W.Q. Distinct expression patterns of notch family receptors and ligands during development of the mammalian inner ear. *Mech. Dev.* 78, 159-163 (1998).**
113. **Daudet, N. & Lewis, J. Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* 132, 541-551 (2005).**
114. **Kiernan, A.E., Xu, J. & Gridley, T. The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. *PLoS Genet* 2, e4 (2006).**
115. **Kiernan, A.E., Cordes, R., Kopan, R., Gossler, A. & Gridley, T. The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. *Development* 132, 4353-4362 (2005).**
116. **Givogri, M.I*., et al.* Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. *Dev. Neurosci.* 28, 81-91 (2006).**
117. **Cornell, R.A. & Eisen, J.S. Notch in the pathway: the roles of Notch signaling in neural crest development. *Semin. Cell Dev. Biol.* 16, 663-672 (2005).**
118. **Hitoshi, S*., et al.* Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev.* 16, 846-858 (2002).**
119. **Nelson, B.R., Gumuscu, B., Hartman, B.H. & Reh, T.A. Notch activity is downregulated just prior to retinal ganglion cell differentiation. *Dev. Neurosci.* 28, 128-141 (2006).**
120. **Bernardos, R.L., Lentz, S.I., Wolfe, M.S. & Raymond, P.A. Notch-Delta signaling is required for spatial patterning and Muller glia differentiation in the zebrafish retina. *Dev. Biol.* 278, 381-395 (2005).**
121. **Bernardos, R.L., Barthel, L.K., Meyers, J.R. & Raymond, P.A. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. *J. Neurosci.* 27, 7028-7040 (2007).**
122. **Crosnier, C., Stamataki, D. & Lewis, J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet* 7, 349-359 (2006).**
123. **Bjerknes, M. & Cheng, H. Colossal crypts bordering colon adenomas in Apc(Min) mice express full-length Apc. *Am. J. Pathol.* 154, 1831-1834 (1999).**

124. Bjerknes, M. & Cheng, H. Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* 116, 7-14 (1999).
125. Schroder, N. & Gossler, A. Expression of Notch pathway components in fetal and adult mouse small intestine. *Gene Expr Patterns* 2, 247-250 (2002).
126. Chadwick, N.*, et al.* Notch signaling induces apoptosis in primary human CD34+ hematopoietic progenitor cells. *Stem Cells* 25, 203-210 (2007).
127. Tsukumo, S., Hirose, K., Maekawa, Y., Kishihara, K. & Yasutomo, K. Lunatic fringe controls T cell differentiation through modulating notch signaling. *J. Immunol.* 177, 8365-8371 (2006).
128. Garcia-Peydro, M., de Yebenes, V.G. & Toribio, M.L. Notch1 and IL-7 receptor interplay maintains proliferation of human thymic progenitors while suppressing non-T cell fates. *J. Immunol.* 177, 3711-3720 (2006).
129. Guidos, C.J. Synergy between the pre-T cell receptor and Notch: cementing the alphabeta lineage choice. *J. Exp. Med.* 203, 2233-2237 (2006).
130. Kuroda, K.*, et al.* Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J. Biol. Chem.* 274, 7238-7244 (1999).
131. Schuster-Gossler, K., Cordes, R. & Gossler, A. Premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy in Delta1 mutants. *Proc. Natl. Acad. Sci. U. S. A.* 104, 537-542 (2007).
132. Conboy, I.M., Conboy, M.J., Smythe, G.M. & Rando, T.A. Notch-mediated restoration of regenerative potential to aged muscle. *Science* 302, 1575-1577 (2003).
133. Oesterle, E.C., Campbell, S., Taylor, R.R., Forge, A. & Hume, C.R. Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *J Assoc Res Otolaryngol* 9, 65-89 (2008).
134. Taylor, R. & Forge, A. Developmental biology. Life after deaf for hair cells? *Science* 307, 1056-1058 (2005).
135. Bermingham-McDonogh, O. & Rubel, E.W. Hair cell regeneration: winging our way towards a sound future. *Curr. Opin. Neurobiol.* 13, 119-126 (2003).
136. Hawkins, J.E. Drug ototoxicity. in *In Handbook of Sensory Physiology*, Vol. 5 (ed. W.D., K.W.D.a.N.) 707-748 (Springer-Verlag, Berlin, 1976).
137. Hawkins, J.E., Jr. & Johnsson, L.G. Patterns of sensorineural degeneration in human ears exposed to noise. pp. 91-110. [Review] [27 refs]. *In: Henderson D, ET AL., ED. Effects of noise on hearing. New York, Raven Press* 270(1976).
138. Hawkins, J.E., Jr., Johnsson, L.G., Stebbins, W.C., Moody, D.B. & Coombs, S.L. Hearing loss and cochlear pathology in monkeys after noise exposure. *Acta Otolaryngol. (Stockh).* 81, 337-343 (1976).
139. Shou, J., Zheng, J.L. & Gao, W.Q. Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of Hath1. *Mol. Cell. Neurosci.* 23, 169-179 (2003).

140. Zheng, J.L. & Gao, W.Q. Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* 3, 580-586 (2000).

141. West, B.A., Brummett, R.E. & Himes, D.L. Interaction of kanamycin and ethacrynic acid. Severe cochlear damage in guinea pigs. *Arch. Otolaryngol.* 98, 32-37 (1973).

142. van Es, J.H. & Clevers, H. Notch and Wnt inhibitors as potential new drugs for intestinal neoplastic disease. *Trends Mol Med* 11, 496-502 (2005).

143. Liu, T., *et al.* Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. *Carcinogenesis* 28, 488-496 (2007).

144. Walker, R.G., Willingham, A.T. & Zuker, C.S. A Drosophila mechanosensory transduction channel. *Science* 287, 2229-2234 (2000).

145. Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. & Haass, C. A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep* 3, 688-694 (2002).

146. Wolfe, M.S. The gamma-secretase complex: membrane-embedded proteolytic ensemble. *Biochemistry (Mosc).* 45, 7931-7939 (2006).

147. Priller, C., *et al.* Synapse formation and function is modulated by the amyloid precursor protein. *J. Neurosci.* 26, 7212-7221 (2006).

148. Priller, C., *et al.* Excitatory synaptic transmission is depressed in cultured hippocampal neurons of APP/PS1 mice. *Neurobiol. Aging* (2007).

149. Dewachter, I., *et al.* Deregulation of NMDA-receptor function and down-stream signaling in APP[V717I] transgenic mice. *Neurobiol. Aging* (2007).

150. Phinney, A.L., *et al.* Cerebral amyloid induces aberrant axonal sprouting and ectopic terminal formation in amyloid precursor protein transgenic mice. *J. Neurosci.* 19, 8552-8559 (1999).

151. Matsuyama, S., Teraoka, R., Mori, H. & Tomiyama, T. Inverse correlation between amyloid precursor protein and synaptic plasticity in transgenic mice. *Neuroreport* 18, 1083-1087 (2007).

152. Kanzaki, S., Ogawa, K., Camper, S.A. & Raphael, Y. Transgene expression in neonatal mouse inner ear explants mediated by first and advanced generation adenovirus vectors. *Hear. Res.* 169, 112-120 (2002).

153. Ishimoto, S., Kawamoto, K., Kanzaki, S. & Raphael, Y. Gene transfer into supporting cells of the organ of Corti. *Hear. Res.* 173, 187-197 (2002).

154. Zine, A. Molecular mechanisms that regulate auditory hair-cell differentiation in the mammalian cochlea. *Mol. Neurobiol.* 27, 223-238 (2003).

155. Nam, Y., Sliz, P., Song, L., Aster, J.C. & Blacklow, S.C. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. *Cell* 124, 973-983 (2006).

156. **Smoller, D., *et al.* The Drosophila neurogenic locus mastermind encodes a nuclear protein unusually rich in amino acid homopolymers. *Genes Dev.* 4, 1688-1700 (1990).**
157. **Fortini, M.E. & Artavanis-Tsakonas, S. The suppressor of hairless protein participates in notch receptor signaling. *Cell* 79, 273-282 (1994).**
158. **Wu, L., *et al.* MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* 26, 484-489 (2000).**
159. **Lin, S.E., Oyama, T., Nagase, T., Harigaya, K. & Kitagawa, M. Identification of new human mastermind proteins defines a family that consists of positive regulators for notch signaling. *J. Biol. Chem.* 277, 50612-50620 (2002).**
160. **Sajan, S.A., Warchol, M.E. & Lovett, M. Toward a systems biology of mouse inner ear organogenesis: gene expression pathways, patterns and network analysis. *Genetics* 177, 631-653 (2007).**
161. **Robertson, N.G., Khetarpal, U., Gutierrez-Espeleta, G.A., Bieber, F.R. & Morton, C.C. Isolation of novel and known genes from a human fetal cochlear cDNA library using subtractive hybridization and differential screening. *Genomics* 23, 42-50 (1994).**
162. **Weng, A.P., *et al.* Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol. Cell. Biol.* 23, 655-664 (2003).**
163. **Tu, L., *et al.* Notch signaling is an important regulator of type 2 immunity. *J. Exp. Med.* 202, 1037-1042 (2005).**
164. **High, F.A., *et al.* An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. *J. Clin. Invest.* 117, 353-363 (2007).**