# Mechanosensory Hair Cell Regeneration in the Zebrafish Lateral Line Is Mitotic and Facilitated by Innervation

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## Abstract

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Mechanosensory hair cells in the inner ear are vulnerable to damage that can result in hearing loss and balance disorders. Exposure to excessive noise and ototoxic drugs are two of the leading causes of hair cell loss, which is irreversible in humans and other mammals. However, non-mammalian vertebrates including birds, amphibians, and fish are capable of significant hair cell regeneration that may provide insight into potential therapies for human patients.

Zebrafish and other aquatic vertebrates also express hair cells in an external sensory structure called the lateral line system, which I used to study regeneration after treatment with several commonly used ototoxins. I observed that the mechanism of hair cell regeneration is remarkably consistent, existing entirely of dividing hair cell progenitors unlike the mix of proliferative regeneration and direct transdifferentiation observed in birds and amphibians. New hair cells expressed BrdU, a marker of recent cell division, and blocking proliferation with the microtubule-disrupting drug flubendazole also prevented regeneration.

Although I observed no differences in the mechanism of regeneration, the type of ototoxin used to induce hair cell damage significantly affected the rate at which hair cells were replaced. For example, neomycin and gentamicin targeted exclusively mature, functional hair cells, which regenerated within 72 hours, but copper and cisplatin also killed non-sensory

support cells. These support cells give rise to the dividing progenitors responsible for regeneration, delaying complete hair cell replacement by several days.

I also examined how hair cell regeneration is affected by the removal of innervation, which has been previously shown to prevent tissue regeneration in the amputated amphibian limb. Although regeneration was intact in aneurogenic fish that develop hair cells without pre-existing innervation, regeneration was significantly delayed when the lateral line nerve was axotomized by laser ablation. This suggests that lateral line hair cell regeneration is at least partially nerve-dependent, though re-innervation was followed by a recovery in hair cell number. Researchers who have studied amphibian limb regeneration have implicated the role of trophic factors secreted by myelinating Schwann cells. Although my experiments indicate that Schwann cells are important for regeneration of the lateral line nerve, they do not appear to be directly responsible for mediating hair cell regeneration.

A molecular mechanism that links innervation to hair cell regeneration has not been elucidated at this time, but the identification of its components might also contribute to our understanding of how proliferative regeneration is initiated in the lateral line neuromasts. The similar responses to several different ototoxins is encouraging in that it simplifies our understanding of regeneration in the lateral line and will contribute to new genetic and chemical screens for factors that prevent hair cell damage or facilitate regeneration. With an improved understanding the mechanisms underlying replacement of lateral line hair cells, this research can help develop new approaches to hair cell regeneration in humans.

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# LIST OF ABBREVIATIONS

**ANOVA** – analysis of variance

**aLL** – anterior lateral line

**bHLH** – basic helix-loop-helix

**dpf** – days post-fertilization

**FGF** – fibroblast growth factor

**hpf** – hours post-fertilization

**hpt** – hours post-treatment

**GFP** – green fluorescent protein

**EGFP** – enhanced green fluorescent protein

**NICD** – Notch intracellular domain

**pLL** – posterior lateral line

**RT** – room temperature

**SD** – standard deviation

# LIST OF CHEMICAL ABBREVIATIONS

**BrdU** – 5-bromo-2'-deoxyuridine

**DASPEI** – 2-(4-(dimethylamino)styryl)-*N*-ethylpyridium iodide

**DAPT** – *N*-((3,5-difluorophenacetyl)-L-alanyl-2-phenyl)glycine-1,1-demethylethyl ester

**DMSO** – dimethyl sulfoxide

**FM 1-43** – *N*-(3-triethylammeniumpropyl)-4-(4-(dibutylamino)pyridinium) dibromide

**MESAB (a.k.a. MS222 or Tricane)** – ethyl-*m*-aminobenzoate methanesulfonate

**PFA** – paraformaldehyde

**Solutions**

**EM** – embryo medium (see Appendix A for composition)

**PBS** – phosphate-buffered saline

**PBSDT** – phosphate-buffered saline with 1% DMSO and 0.1% Tween-20

**PBST** – phosphate-buffered saline with 0.1% Tween-20

## ACKNOWLEDGMENTS

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## DEDICATION

This dissertation is dedicated to my fiancée, Megan, and to my parents, Don and Louise. Thank you for providing me the freedom to discover and think for myself.

## INTRODUCTION

Mechanosensory hair cells detect physical stimuli that form the basis of several sensory modalities, including the perceptions of sound, body position, and momentum. All vertebrates possess hair cells in the auditory and vestibular sensory epithelia within the inner ear. Many aquatic vertebrates also possess a structure called the lateral line system, an external sensory organ responsible for detecting water movement. Despite these varied purposes, all mechanosensory hair cells share functional and morphological properties, facilitating efforts to study their development and function in several model organisms.

Human hair cells in the inner ear are vulnerable to damage or loss through genetic predisposition, physical trauma (*e.g.*, excessive noise), and the side effects of certain pharmaceuticals. Such drugs are termed "ototoxic" and are a leading cause of hearing loss and balance disorders (Forge & Schacht, 2000; Petit *et al.*, 2001; Seidman *et al.*, 2002; Chen & Fechter, 2003; Matsui & Cotanche, 2004). The majority of hair cells do not regenerate in adult humans and other mammals (Roberson & Rubel, 1994; Matsui & Cotanche, 2004), but non-mammalian vertebrates including birds (Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Roberson *et al.*, 1996; Roberson *et al.*, 2004), amphibians (Baird *et al.*, 1996; Baird *et al.*, 2000; Taylor & Forge, 2005), and fish (Harris *et al.*, 2003; Hernández *et al.*, 2006) are capable of significant regeneration.

For my dissertation, I examined hair cell death and regeneration in the zebrafish (*Danio rerio*) lateral line after treatment with a variety of ototoxins with to determine how different treatments altered the mechanism of regeneration. There have been many reports of both proliferative regeneration and direct transdifferentiation in birds (Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Roberson *et al.*, 1996; Roberson *et al.*, 2004) and amphibians (Baird *et al.*, 1996; Baird *et al.*, 2000; Taylor & Forge, 2005), but studies in zebrafish have reported only a proliferative mechanism (Harris *et al.*, 2003; Ma *et al.*, 2008; Wibowo *et al.*, 2011) with one exception (Hernández *et al.*, 2007). I also examined the consequences of denervation on lateral line hair cell regeneration. Like the nearby support cells, the basket of axon terminals that surround hair cells and their associated glia are uniquely positioned to secrete trophic or other factors that might regulate hair cell regeneration.

I provide background on mechanosensory hair cells and their function in Chapter 1 along with a discussion of previous research on hair cell regeneration in the avian basilar papilla and the zebrafish lateral line. Additional background is presented on the organization and function of auditory hair cells in the mammalian inner ear, which may be more familiar to the reader. Chapter 2 follows with a detailed introduction to the zebrafish lateral line, its initial development, and factors known to regulate its regeneration. I also provide a comparison of the different ototoxins used in my experiments.

I describe and discuss my experiments in Chapters 3 through 5. Chapter 3 includes my comparison of the different rates of hair cell death and regeneration after treatment with neomycin or copper, two potent ototoxins that had been studied extensively yet separately using zebrafish (Harris *et al.*, 2003; Hernández *et al.*, 2006; Linbo *et al.*, 2006; López-Schier & Hudspeth, 2006; Hernández *et al.*, 2007; Ma *et al.*, 2008; Wibowo *et al.*, 2011). I then continue with experiments investigating regeneration after treatment with gentamicin and cisplatin, ototoxins that had not previously been used to study hair cell regeneration in zebrafish, though they have been used to explore the mechanisms of hair cell death (Ou *et al.*, 2007; Owens *et al.*, 2008; Owens *et al.*, 2009; Ou *et al.*, 2010). Chapter 4 contains further study of these four ototoxins to determine if the regeneration observed occurs by proliferation or direct transdifferentiation. I also describe my efforts to block proliferation and determine if a secondary non-proliferative mechanism can rescue regeneration. I examine the role of innervation and lateral line hair cell regeneration in Chapter 5, beginning with a comparison of aneurogenic fish that develop hair cells without innervation and denervated fish in which the lateral line nerve has been removed by axotomy and degeneration. I then examine the possible role of myelinating Schwann cells in lateral line hair cell regeneration to explain the differences observed between aneurogenic and denervated fish.

Finally, Chapter 6 presents the conclusions I derived from my experiments and how this research enhances our understanding of mechanosensory hair cell regeneration not only in the lateral line but also in other non-mammalian vertebrates and in the mammalian inner ear. I discuss the future direction of this research and its implications. Some of my results continue the progress reported in studies by other members of the Raible laboratory (Ma *et al.*, 2008; Owens *et al.*, 2009; Namdaran *et al.*, 2012), and my experiments on the role of innervation in hair cell

regeneration may prove useful in our efforts to derive a molecular mechanism responsible for initiating hair cell regeneration.

# CHAPTER 1: Hearing Loss and Mechanosensory Hair Cells

## 1.0 OVERVIEW

Hearing loss affects almost all elderly individuals to some degree and is an increasing public health concern for children and young adults. Approximately 16% of adults between the ages of 20 and 69 in the United States suffer from hearing loss in the frequency range of audible speech (Agrawal *et al.*, 2008), impairing their ability to interact with other individuals and their environment. Serious effects on quality of life may result, as a person may be unaware of dangerous situations, may be unable to enjoy music or other auditory stimuli, and may retreat from interpersonal relationships when conversation becomes difficult (Hallam *et al.*, 2006). Death of mechanosensory hair cells in the inner ear, which transduce sound waves into neuronal activity, is a frequent cause of poor hearing or deafness. Excessive noise, exposure to pharmaceuticals with ototoxic side effects, and progressive age-related hearing loss (presbycusis) are all leading causes of damage to auditory hair cells, which do not regenerate in adult mammals (Roberson & Rubel, 1994; Chardin *et al.*, 1995). However, many other vertebrates including birds, amphibians, and fish do regenerate mechanosensory hair cells (Roberson *et al.*, 1996; Baird *et al.*, 1996; Baird *et al.*, 2000; Harris *et al.*, 2003; Roberson *et al.*, 2004; Taylor & Forge, 2005; Hernández *et al.*, 2007; Ma *et al.*, 2008; Wibowo *et al.*, 2011). Efforts to characterize regeneration in these species and understand those factors regulating it may result in new therapeutic opportunities for humans.

## 1.1 INTRODUCTION TO MECHANOSENSORY HAIR CELLS

Hair cells are mechanoreceptors that convert physical motion into neuroelectrical impulses *via* small hair-like projections called stereocilia. These “hairs” form a hair bundle arranged in a sloping pattern from shortest to tallest and are joined by tip links (Hudspeth, 1989; Fettiplace & Hackney, 2006). Deflection of the hair bundle is thought to cause these tip links to open mechanically gated ion channels that permit entry of potassium ions from the surrounding environment. Potassium-mediated depolarization then opens calcium channels. Calcium-mediated signals induce neurotransmitter release onto afferent synapses that communicate information about the stimulus to the nervous system (Hudspeth, 1989). Mechanosensory hair cells can be found in the auditory and vestibular epithelia of all vertebrates as well as in the

lateral line of aquatic species. The mechanical displacement necessary to stimulate such cells comes from a variety of sources, including sound waves transduced by the basilar membrane of the auditory cochlea, head movements resulting in redistribution of fluid in the vestibular semicircular canals, or water currents and vibrations in an aquatic environment detected by the lateral line system.

**Organization of auditory hair cells in humans and other mammals**

Auditory hair cells within the mammalian cochlea are arranged in three rows of outer hair cells and one row of inner hair cells (Figure 1.1). Non-sensory support cells can be loosely classified as any non-hair cell in the sensory epithelium. They surround the hair cells, providing physical structure and various potential regulatory signals. Inner hair cells are the primary source of auditory detection, while the outer hair cells function as cochlear amplifiers, receiving efferent feedback from the central nervous system so as to alter the intensity and selectivity of stimuli detected by the inner hair cells (Hudspeth, 1989; Fettiplace & Hackney, 2006).

Hair cells and support cells are located above the basilar membrane and below the tectorial membrane within a specialized cochlear structure called the organ of Corti. Sound waves are transduced into physical movement of the basilar membrane that creates a shearing force with the tectorial membrane, deflecting the hair cell stereocilia (Fettiplace & Hackney, 2006). When the cochlea is unrolled, it becomes apparent that hair cells are arranged in a tonotopic map: those that detect high frequency vibrations are toward the basal end near the oval window, and those that detect low frequency vibrations are toward the apical end, also called the helicotrema (Fettiplace & Hackney, 2006). In the absence of regeneration, damage to hair cells causes a failure to perceive their respective frequencies. However, most auditory stimuli are a combination of many frequencies that combine to produce complex tones. Noticeable hearing loss may not occur until after accumulated damage, although higher frequencies are among the first affected (Agrawal *et al.*, 2008).

**Drug-mediated hearing loss in humans**

Many compounds with therapeutic value, such as the aminoglycoside antibiotics neomycin and gentamicin as well as the chemotherapeutic cisplatin, cause hair cell damage and hearing loss. Estimates of aminoglycoside-induced hearing loss extend up to 20% of the 2

million individuals annually treated with this class of antibiotics in the in the U.S. (Forge & Schacht, 2000), though these numbers are declining with the creation of alternative treatments that have fewer side effects. Although neomycin and gentamicin are used more commonly in research on hair cell loss and regeneration, other aminoglycosides including streptomycin, kanamycin, and amikacin also have use in therapeutic settings. Developing societies value the high efficacy and low cost of aminoglycosides for treating *M. tuberculosis* and various Gram-negative bacteria despite the potential drawbacks, which also include nephrotoxicity (Swan, 1997; Edson & Terrell, 1999). Similarly, some chemotherapeutics like cisplatin, carboplatin, and oxaliplatin act by forming DNA adducts and inducing apoptosis (Eastman, 1999; Alderden *et al.*, 2006), not just in cancerous cells but also in auditory and vestibular hair cells. Research to block these ototoxic side effects or to stimulate subsequent regeneration in humans may reduce the need for prescriptive tradeoffs.

## 1.2 REGENERATION IN NON-MAMMALIAN VERTEBRATES

Auditory and vestibular hair cells are not replaced in adult mammals, but other vertebrates have more robust regenerative capacities (Figure 1.2). Fish, amphibians, and birds are among the most popular models of non-mammalian sensory hair cell regeneration. Progenitor cells are derived from the surrounding support cells, which generally exhibit less stereotypic arrangement compared to those in the mammalian cochlea, and regeneration can occur by either direct transdifferentiation or proliferative regeneration (Brignull *et al.*, 2009). In direct transdifferentiation, one support cell becomes a new hair cell without an intermediate phase of cell division. Proliferative regeneration, however, involves division into two daughter cells and could be symmetric, producing two hair cells, or asymmetric, producing one hair cell and a replacement support cell.

There have been several reports of observations in the chick (Roberson *et al.*, 1996; Roberson *et al.*, 2004) and some amphibians (Baird *et al.*, 1996; Baird *et al.*, 2000; Taylor & Forge, 2005) that demonstrate direct transdifferentiation can occur in the inner ear. However, this initial period of direct transdifferentiation is followed by a period of mitotic regeneration that also makes a major contribution to hair cell replacement in the chick basilar papilla (Roberson *et al.*, 2004). Although significant hair cell regeneration still occurs when proliferation is blocked in the basilar papilla, the number of replacement hair cells and remaining support cells are reduced

when regeneration is limited to transdifferentiation (Shang *et al.*, 2010). Complete regeneration in the chick can require several weeks, so this early transdifferentiation appears to provide an immediate restoration of partial auditory capability while allowing proliferative regeneration to complete the process. Similarly, amphibians possess both proliferative and non-proliferative forms of regeneration (Baird *et al.*, 1996; Baird *et al.*, 2000; Taylor & Forge, 2005), but proliferation has a dominant role.

The zebrafish (*Danio rerio*) lateral line is a popular model for studying mechanosensory hair cell death and regeneration, in part because hair cells begin to regenerate less than 24 h after damage (Harris *et al.*, 2003; Ma *et al.*, 2008). Multiple studies have found that progenitor cells almost always give rise to pairs of new hair cells (López-Schier & Hudspeth, 2006; Ma *et al.*, 2008) and that treatment with bromodeoxyuridine (BrdU), a thymine analog that incorporates into DNA during S-phase, will label a majority of new hair cells during regeneration (Harris *et al.*, 2003; Hernández *et al.*, 2007; Ma *et al.*, 2008). At least one report found evidence of non-proliferative regeneration in younger zebrafish embryos (Hernández *et al.*, 2007), however it may be difficult to distinguish normal hair cell addition from regeneration at this stage during initial development of the lateral line. In more mature larvae, evidence of a distinct early phase of direct transdifferentiation could be obscured by the rapid rate of proliferative regeneration, and an early non-proliferative phase may not be necessary or may not have an adaptive advantage. Furthermore, some post-mitotic yet immature hair cells are also resistant to ototoxins (Murakami *et al.*, 2003; Santos *et al.*, 2006) and could be misinterpreted as originating through a non-proliferative mechanism when their differentiation is complete.

In addition to regeneration *en masse*, the zebrafish lateral line is thought to undergo continuous hair cell turnover (Williams & Holder, 2000), although the reason for this is unclear. Some hair cells may occasionally suffer localized damage, or perhaps all hair cells have a finite lifespan and must be replaced as the animal ages. At present, there are no data to indicate that hair cell regeneration during normal turnover is different from that occurring after ototoxic damage.

## 1.3 REGULATORS OF MECHANOSENSORY HAIR CELL REGENERATION

Numerous genes and proteins have been identified that affect hair cell proliferation or differentiation, and together they are beginning to suggest a mechanism for how regeneration is

regulated. Many of these regulatory components were previously identified during studies of the initial development of the sensory epithelium, which will be discussed in greater detail in Chapter 2. Examples include the Notch signaling pathway and downstream effectors such as *atonal homolog-1* (*atoh1*), a basic helix-loop-helix (bHLH) transcription factor necessary for hair cell differentiation (Bermingham *et al.*, 1999; Zheng *et al.*, 2000; Woods *et al.*, 2004; Matei *et al.*, 2005). Additional candidates have been identified from their ability to regulate cell proliferation *in vitro*, such as fibroblast growth factors (FGFs).

**Regulators of progenitor cell proliferation**

Interestingly, FGFs, which serve an important role in promoting cell proliferation during embryonic development of the inner ear and the lateral line (Nechiporuk & Raible, 2008; Ma & Raible, 2009; Sweet *et al.*, 2011), can become negative regulators of proliferation in the mature sensory epithelium after hair cell damage (Oesterle & Stone, 2008). FGF2 inhibits proliferation *in vitro* in cultures of the chick basilar papilla and vestibular epithelium (Oesterle *et al.*, 2000). FGF receptor (FGFR) 3 is highly expressed among support cells of the basilar papilla, but it is later downregulated after hair cell damage *in vivo* (Bermingham-McDonogh *et al.*, 2001). FGFR3 is not expressed during regeneration and is instead delayed until support cells exit the cell cycle after hair cells are replaced. Conversely, hair cells do not regenerate in the rat organ of Corti (Oesterle & Stone, 2008), but FGFR3 is upregulated in support cells after noise damage (Pirvola *et al.*, 1995). This contrast suggests that, after their role in proliferation during earlier development, FGFs become important for maintaining support cells in a quiescent state specifically in organisms that are otherwise capable of significant proliferation and regeneration.

A variety of important signaling proteins, including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), phosphatidyl inositol 3-kinase (PI3K), and mammalian target of rapamycin (mTOR) are required for cell proliferation. Inhibiting these proteins will decrease cell proliferation *in vitro* in the sensory epithelia of adult chickens (Witte *et al.*, 2001) and neonatal mice (Montcouquiol & Corwin, 2001). However, all these kinases are components of broad signaling pathways with roles in many unrelated biological processes. It is difficult to draw many conclusions about their individual roles specific to regulating cell proliferation and cell cycle re-entry in sensory epithelia.

**Notch signaling and cell fate specification**

Notch signaling requires direct cell-cell interactions between the Notch receptor and ligands such as Delta to induce proteolytic cleavage of the Notch intracellular domain (NICD) by γ-secretase. The NICD then migrates to the nucleus and modifies gene expression (Artavanis-Tsakonas, 1999). Notch signaling is present in all metazoans (multicellular animals) and is involved in several important biological processes, including those that rely on lateral inhibition, such as hair cell differentiation. In particular, Notch-regulated expression of *atoh1* serves an important role in specifying hair cell fate in the migrating sensory primordium of the zebrafish lateral line (Itoh & Chitnis, 2001; Nechiporuk & Raible, 2008) and has also been implicated in subsequent regeneration of lost hair cells. Expression of *notch3*, *deltaA*, and *atoh1* are increased during regeneration after aminoglycoside damage (Ma *et al.*, 2008). Notch ligands *deltaB* and *deltaC* have also been found to play a role in specifying hair cell fate during initial development of the lateral line (Smithers *et al.*, 2000; Itoh & Chitnis, 2001) but have not been studied in the context of regeneration.

Lateral line hair cell precursors express *atoh1* prior to differentiation (Itoh & Chitnis, 2001), but this is later downregulated after hair cell maturation is complete (Ma *et al.*, 2008). Overexpression of *atoh1* can induce ectopic hair cells derived from mature, presumably post-mitotic cells in inner ear cultures from adult rats (Zheng *et al.*, 2000; Shou *et al.*, 2003), indicating that this gene is necessary but also sufficient to specify a hair cell fate. Therefore, by inhibiting *atoh1* expression during development, Notch signaling is thought to be critical for maintaining the progenitor population and preventing excessive hair cell differentiation.

Regeneration after hair cell death is associated with a significant increase in *atoh1* expression within 24 h (Ma *et al.*, 2008). Expression of *notch3* is associated with a support cell fate, and like *atoh1*, expression peaks shortly after neomycin treatment (Ma *et al.*, 2008). Finally, expression of *deltaA* appears to regulate an increase in support cell proliferation (Ma *et al.*, 2008), perhaps giving rise to additional hair cell progenitors as they divide and differentiate into hair cell precursors. Expression of *deltaA* peaks at 12 to 16 h after treatment, overlapping with the earliest expression of *notch3* but prior to *atoh1*. Lateral inhibition between Notch and its ligands limits the number of regenerated hair cells, and reducing Notch signaling activity with the γ-secretase inhibitor DAPT during regeneration produces excess hair cells (Ma *et al.*, 2008).

Similar to its role in zebrafish, *delta1* is highly upregulated among proliferating cells in both the avian utricle and the basilar papilla after aminoglycoside damage. *Delta1* expression persists in the newly-generated hair cells but decreases in cells that do not ultimately acquire a hair cell fate (Stone *et al.*, 1999). Although not normally expressed in support cells, *atoh1* is upregulated in the nuclei of progenitor cells that produce new hair cells through mitosis as well as transdifferentiation, and it is later highly expressed and confined to hair cells after their differentiation (Cafaro *et al.*, 2007). By contrast, *notch1*, which is normally expressed only in support cells, increases in the post-mitotic cell pairs during regeneration (Stone *et al.*, 1999). Treating *in vitro* cultures of the chick basilar papilla with DAPT increases the number of regenerated hair cells at the expense of support cells (Daudet *et al.*, 2009) and also promotes hair cell regeneration in inner ear cultures form rodents (Zhao *et al.*, 2011; Lin *et al.*, 2011). The role of Notch signaling in hair cell regeneration appears similar to that in development, acting through lateral inhibition to limit the number of hair cells produced.

In studies of the zebrafish inner ear and lateral line, the transcription factor *sox2* is one of the few genes that appears to be important for hair cell regeneration (Hernández *et al.*, 2007) but which is dispensable during early development (Millimaki *et al.*, 2010). Interestingly, *sox2* is still required for development of the mammalian inner ear (Kiernan *et al.*, 2005; Puligilla *et al.*, 2010), where it acts upstream of *atoh1* (Kiernan *et al.*, 2005) and remains expressed in support cells even as it is downregulated in maturing hair cells (Dabdoub *et al.*, 2008). Although the function of *sox2* is less certain in the lateral line, it has been shown that co-expression of *sox2* and *atoh1*, but not of *atoh1* alone, can induce ectopic sensory development in the zebrafish inner ear (Sweet *et al.*, 2011), confirming that *sox2* serves a permissive function when specifying a hair cell fate.

## 1.4 SUMMARY

Humans and all other vertebrates possess mechanosensory hair cells that convey information about different sensory modalities. A variety of genetic and environmental causes exist for hair cell damage, but only non-mammalian vertebrates are capable of robust regeneration. In humans, permanent loss of hair cells results in poor vestibular and auditory perception with consequences for personal safety and quality of life. Research on hair cells in birds, amphibians, and fish has provided insight into the mechanisms of hair cell death and

regeneration, including their timing and regulation. However, there remain many unanswered questions.

A considerable amount of our current knowledge is derived from studies in the chick and other birds, but the inner ear sensory epithelia in these animals is relatively inaccessible, and certain genetic tools are unavailable to researchers. Therefore, zebrafish have recently become more prevalent in hair cell research, particularly because of the functional and morphological similarities of lateral line hair cells to those in the inner ear. The development and organization of the lateral line will be discussed in Chapter 2.

## 1.5 FIGURES AND LEGENDS

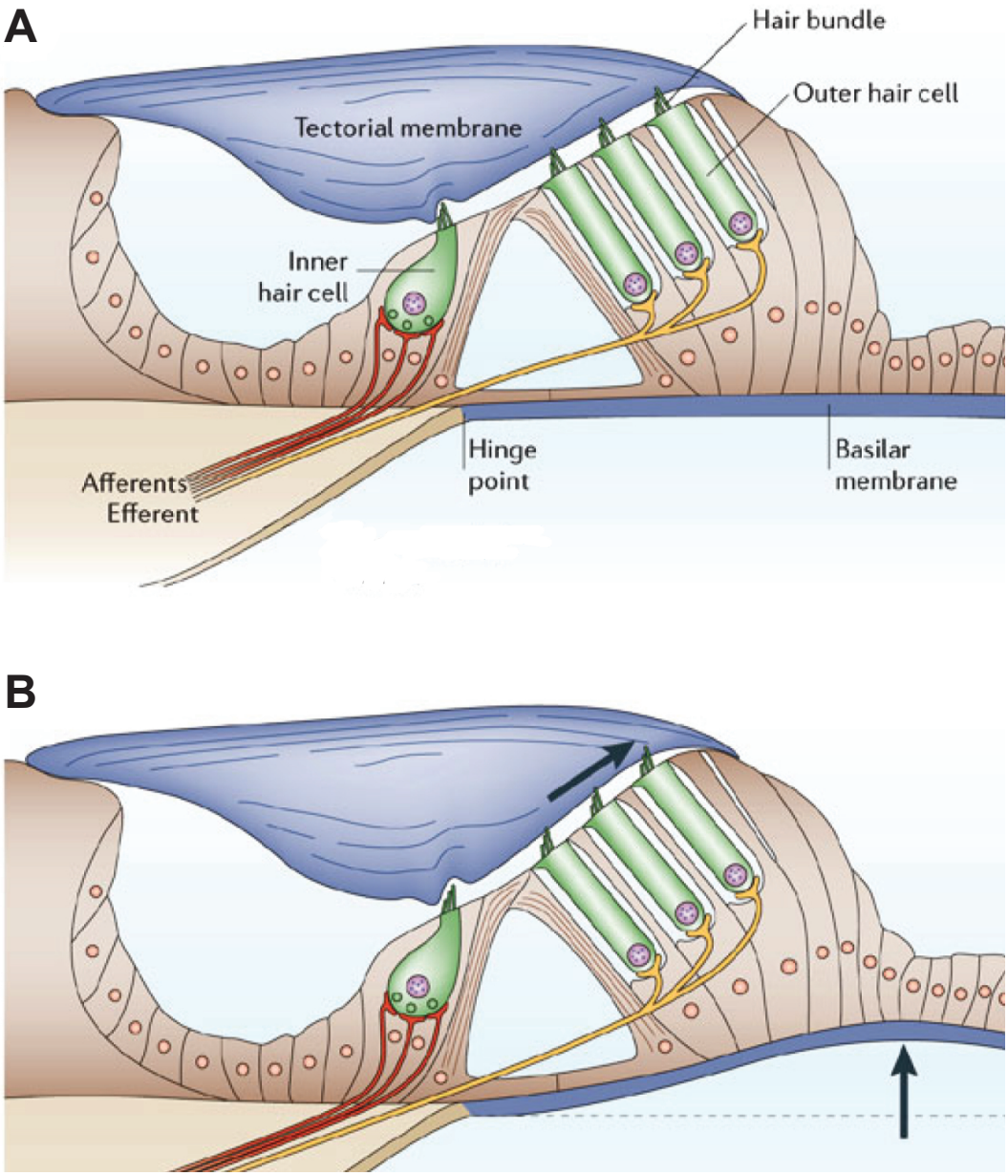


**Figure 1.1: Schematic diagram of the mammalian organ of Corti.**
**(A)** This cross-section through the mammalian cochlea shows the different structures and cell types found within the auditory sensory epithelium. One row of inner hair cells and three rows of outer hair cells (green) are innervated by afferent and efferent fibers. Hair cells are surrounded by non-sensory support cells (brown) and organized above the basilar membrane. **(B)** When this membrane is displace upward, hair bundles are deflected against the tectorial membrane by a shearing force (arrows). Sensory information is sent from the inner hair cells to the brain through afferent fibers of the VIII$^{th}$ cranial nerve. Outer hair cells function as cochlear amplifiers, adjusting frequency sensitivity in response to efferent signals that carry feedback from the central nervous system. This figure is adapted from Fettiplace and Hackney (2006).

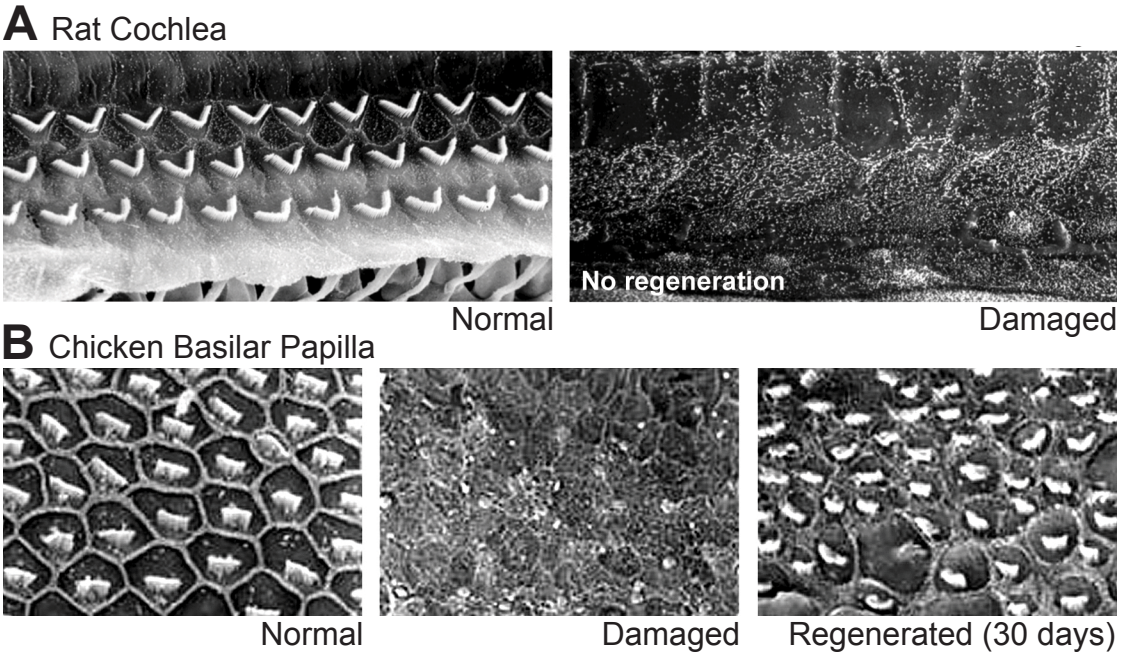


**Figure 1.2: Hair cell death and regeneration in rodent and chick auditory epithelia.**
**(A)** Scanning electron micrographs show normal and damaged hair cells in the rat cochlea. **(B)** Similar images of the chick basilar papilla show normal, damaged, and regenerated hair cells after 30 days. These images are courtesy of M. Lenoir and E. Ma.

# CHAPTER 2: Introduction to the Zebrafish Lateral Line

## 2.0 OVERVIEW

Zebrafish are well suited to developmental research owing to their high fecundity, short generation time, and optically transparent larvae. Most organ systems begin to develop within 36 hours post-fertilization (hpf), and larvae begin to feed independently at 4 to 5 days post-fertilization (dpf). They have been used for a variety of research purposes, including studies of retinal development, tissue regeneration, pigmentation, cell fate specification and migration, and simple learning tasks. Although several other vertebrates have been used to study mechanosensory hair cells as described earlier, the auditory and vestibular sensory epithelia in those organisms are located within the inner ear and are relatively difficult to access. In contrast, lateral line hair cells are found on the outside of the body, and even those within the zebrafish inner ear are visible during early development.

## 2.1 INTRODUCTION TO THE LATERAL LINE

The lateral line is a sensory structure found in fish, sharks, amphibians, and other aquatic vertebrates that use it to detect water movement, providing information such as the presence of other organisms and the direction of current that are used for behavior such as courtship (Blaxter, 1989), predator and prey detection (Hoekstra & Janssen, 1985; Montgomery *et al.*, 1997; Feitl *et al.*, 2010), and rheotaxis (Arnold, 1974; Montgomery *et al.*, 1997; Montgomery *et al.*, 2000; Suli *et al.*, 2012). Clusters of mechanosensory hair cells form neuromasts that are the basic functional unit of the lateral line (Figure 2.1). These may be found either within subdermal canals or on the surface of the organism (Webb & Shirey, 2003). The ciliary hairs and a single, longer kinocilium on each sensory hair cell are surrounded by a gelatinous cupula. Water motion forces the cupula to bend, and the hairs pull on each other to open or close tip link ion channels.

Hair cells rely on rate coding, *i.e.*, variations in a tonic baseline firing rate, to convey information about the direction of a stimulus (Hudspeth, 1989). Hairs are arranged in a sloping pattern in fish and other vertebrates such that movement toward the tallest hairs and kinocilium depolarizes the cell by opening tip link channels, and excitatory neurotransmitter release increases (Hudspeth, 1989). Conversely, movement toward the shortest hairs hyperpolarizes the cell. All hair cells within a neuromast are aligned along the same axis but show opposite polarity

so that they respond maximally to motion in a single direction. Although one neuron may innervate multiple neuromasts, it forms synapses only with hair cells that respond to stimuli in the same direction, *e.g.*, from anterior to posterior (Figure 2.1). Hair cells that respond maximally to force in the opposite direction are innervated separately. Additional neuromasts may be oriented along different axes to provide a more complex perceptual map of the environment. Development of the lateral line contributes significantly to this polarization, which is maintained throughout hair cell regeneration (Wibowo *et al.*, 2011).

## 2.2 ZEBRAFISH LATERAL LINE DEVELOPMENT

Each neuromast contains a central cluster of hair cells surrounded by various non-sensory support cells. In zebrafish, this sensory system is divided into the anterior lateral line (aLL), surrounding the head and eye, and the posterior lateral line (pLL), which runs along the trunk and tail (Raible & Kruse, 2000; Nechiporuk & Raible, 2008). Both subdomains have been studied extensively in the contexts of development and regeneration, and the placement of neuromasts is highly stereotypic. Many of the initial experiments in this dissertation will refer to the aLL since several neuromasts can be viewed at once. However, the linear organization of the pLL makes it well suited to studies of neuromast deposition, the maturation of the first sensory hair cells, and their innervation, which will become essential in Chapter 5.

Development of the pLL primordium and the accompanying pLL ganglion neurons are closely related. Within the pre-pLL sensory placode, neurons are marked by expression of *neuroD* and *neurogenin-1* (*ngn*) (Mizoguchi *et al.*, 2011). These neurons and the cells constituting the first pLL neuromast (L1) remain in place while the cells adjacent to the pLL ganglion give rise to the first primordium (Figure 2.2), a group of self-renewing stem cells that deposit additional neuromasts as they migrate along the horizontal myoseptum (Sapède *et al.*, 2002; Nechiporuk & Raible, 2008). This process occurs between 20 and 40 hours post-fertilization (hpf), producing between seven and nine neuromasts (L2 through L8/10). Neuromasts are deposited in rosettes of sensory epithelial progenitors (Figure 2.3) that later mature into a central cluster of functional hair cells surrounded by various support cells (López-Schier *et al.*, 2004; Ma & Raible, 2009). Subsequent primordia deposit additional neuromasts, although some neuromasts may form by division of interneuromast cells (Sapède *et al.*, 2002; Ledent, 2002).

Axon projections from the pLL ganglion neurons accompany the migrating primordium, a phenomenon regulated by glial cell line-derived neurotrophic factor (GDNF; Schuster *et al.*, 2010). Loss of GDNF produces defects in innervation because the nerve is unable to track the migrating primordium, and GDNF also serves to guide re-innervation during axon regeneration (Schuster *et al.*, 2010; Villegas *et al.*, 2012). Similarly, many of the genes expressed during primordial migration and early neuromast formation also serve a role in regeneration and were discussed in Chapter 1. Many of these factors have already been reviewed elsewhere (Ma & Raible, 2009).

Mature hair cells can be identified with styryl dyes such as DASPEI (Harris *et al.*, 2003) and FM 1-43 (Seiler & Nicolson, 1999), which transiently label the plasma membrane, and the cyanine monomers YO-PRO-1 and TO-PRO-3 (Santos *et al.*, 2006), which label the hair cell nuclei and persist longer. Several transgenic lines such as *Brn3c:mGFP* (Xiao *et al.*, 2005) and *SqET4:GFP* (Parinov *et al.*, 2004) express fluorescent markers in hair cells at different developmental periods; *SqET4:GFP* is among the earliest markers and is found in pre-mitotic progenitors as well as fully differentiated hair cells. Finally, hair cells can be visualized in fixed larvae with antibodies against a variety of markers including parvalbumin (Eybalin & Ripoll, 1990) and myosin-VI and -VIIa (Self *et al.*, 1998; Self *et al.*, 1999; Coffin *et al.*, 2007).

## 2.3 LATERAL LINE HAIR CELL DEATH AND REGENERATION

Although humans suffer loss of hair cells for a variety of reasons, including acoustic trauma or genetic disposition, pharmacological insults are most amenable to laboratory research with zebrafish. Many compounds with therapeutic value, such as the aminoglycoside antibiotics neomycin and gentamicin as well as the chemotherapeutic cisplatin, have been used extensively to study hair cell death and regeneration in the lateral line. Copper sulfate is also used due to the potency of dissolved copper as an environmental pollutant (Linbo *et al.*, 2006), although it does not normally pose a threat to humans.

Neomycin kills hair cells very rapidly, achieving near complete loss within 30 to 60 minutes followed by complete regeneration within 72 hours (Harris *et al.*, 2003; Ma *et al.*, 2008). Damage is limited to functionally mature hair cells, defined by their expression of mechanotransduction channels that also take up various fluorescent dyes. However, concentrations in excess of 400 μM can cause nephrotoxicity and death (Harris *et al.*, 2003).

Copper is effective at much lower concentrations than neomycin, but cell death and regeneration occur at a similar timescale (Linbo *et al.*, 2006; Hernández *et al.*, 2006; Hernández *et al.*, 2007). One disadvantage is that copper can potentially damage immature hair cells and support cells (Olivari *et al.*, 2008), confounding some attempts to make comparisons with neomycin. Concentrations of copper greater than 50 μM have been found to prevent regeneration in the pLL and severely hamper regeneration in the aLL (Hernández *et al.*, 2007).

Gentamicin has effects similar to neomycin but is different in that it can cause cell death by means of two distinct intracellular signaling pathways (Owens *et al.*, 2009), the specifics of which have yet to be clearly identified. The first pathway, an acute response, is similar to that of neomycin and causes rapid cell death following high concentrations of the ototoxin. The second, a chronic response, may require as much as 24 h in the presence of low concentrations. Cisplatin similarly kills hair cells at a variable rate as a product of concentration and exposure, but there is no evidence that different mechanisms are at work as in gentamicin. Although rapid cell death is attainable, lower concentrations kill most hair cells after 24 h (Ou *et al.*, 2007) while limiting investigator exposure.

Greater understanding of the development and function of lateral line hair cells has assisted efforts to prevent or treat hair cell loss. For example, tip link mechanotransduction channels provide an entry point for ototoxic compounds not found in most other cell types, which may explain why aminoglycosides selectively affect mature hair cells (Murakami *et al.*, 2003; Santos *et al.*, 2006). In contrast to aminoglycosides, copper and cisplatin both appear to enter through copper ion transport channels responsible for maintaining important trace levels of intracellular copper (More *et al.*, 2010), explaining their broader effects on other cell types. Additional experiments have probed the cell death pathways activated by these different ototoxins after entry, including the possibility that they may act on specific organelles or disrupt intracellular calcium stores. With greater understanding of the entry points and mechanisms that ototoxins use to induce hair cell death, it might be possible to temporarily block their action on hair cells without impairing therapeutic efficacy.

## 2.4 SUMMARY

The zebrafish lateral line develops according to a stereotypic pattern that facilitates study of developmental phenomena such as the regeneration of mechanosensory hair cells. In addition,

the location of hair cells on the body exterior, the rapid development of this sensory structure, the transparency of larval zebrafish, and the convenient use of multiple labeling approaches together make the zebrafish an ideal model organism for studying the lifecycle of mechanosensory hair cells. Although their small size makes possible detailed, *in vivo* studies of the lateral line at a cellular level, zebrafish also possess high fecundity and a short generation time that facilitate their use in high-throughput screens of hair cell death and regeneration. These features have contributed to the growing use of zebrafish in auditory neuroscience research.

Many previous studies have examined regeneration after treatment with the ototoxins described in this chapter, but few direct comparisons exist to highlight any similarities or differences between them. The rate and specificity of hair cell death can vary widely, even when they may be related compounds or have similar mechanisms of entry. For example, similar rates of hair cell death follow high concentrations of neomycin and gentamicin, but only gentamicin can cause hair cell loss through a slower secondary mechanism. Copper more easily damages these mature hair cells, but greater concentrations can have increasingly non-specific effects on other, non-sensory cell types. This dissertation will present evidence that the regenerative mechanism in the zebrafish lateral line consistently relies on proliferation despite these differences in hair cell death. While this may represent a divergence from our understanding of regeneration in other vertebrates such as the chick, which can also regenerate through transdifferentiation, it suggests the possibility of a single therapeutic approach to regenerating sensory hair cells in humans.

## 2.5 FIGURES AND LEGENDS

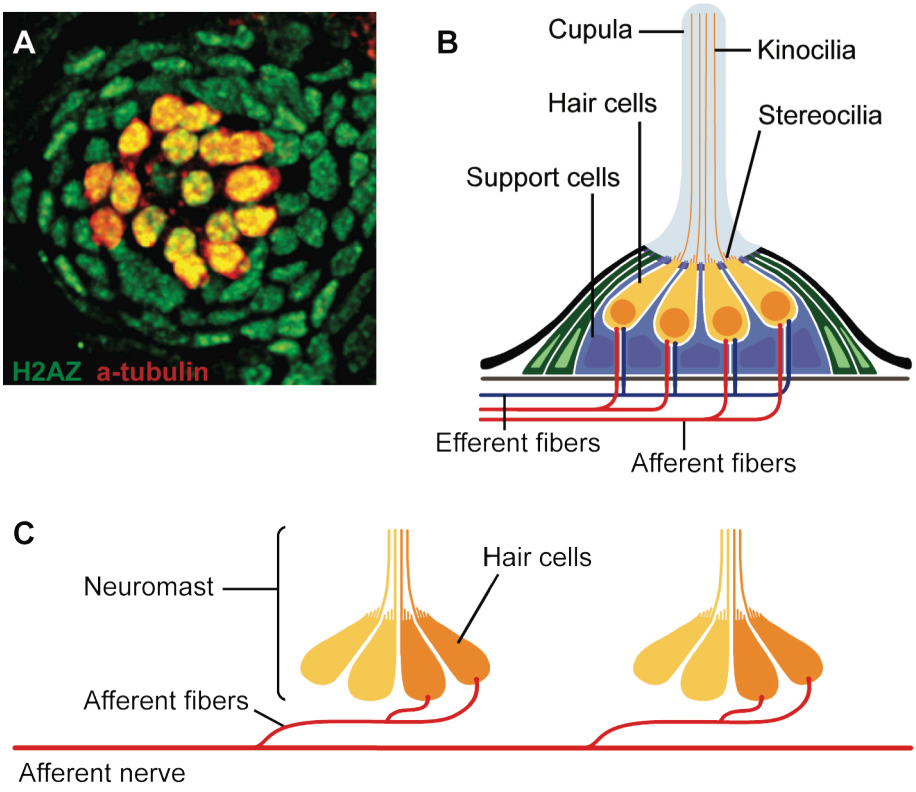


**Figure 2.1: Schematic of a zebrafish neuromast and the lateral line.**
**(A)** Top view of a neuromast in a transgenic *H2AZ-GFP;α-tubulin:tdTomato* fish. All cell nuclei express GFP (green) and hair cells express tdTomato (red). **(B)** Cross-sectional diagram of a lateral line neuromast representing the arrangement of different cell types and their innervation. **(C)** Diagram showing selective innervation of hair cells of the same polarity (orange) but in different neuromasts innervated by a single afferent nerve. Hair cells of the opposing polarity (yellow) are innervated separately. This figure is adapted from Ma and Raible (2009).

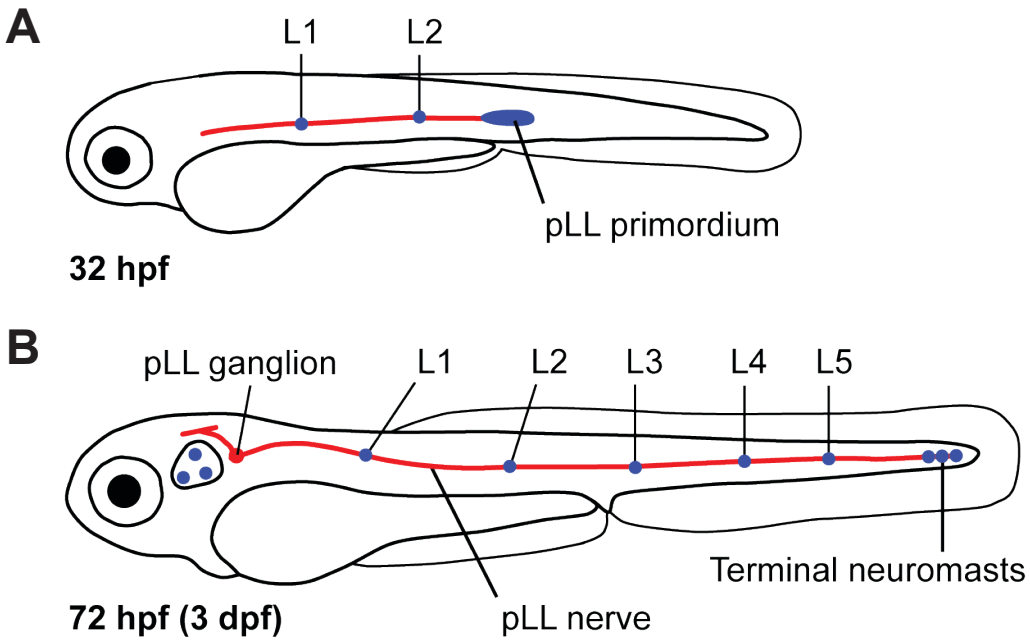


**Figure 2.2: Development of the posterior lateral line.**
**(A)** At 32 hpf, the first pLL primordium has migrated halfway down the length of the trunk and deposited two neuromasts, labeled L1 and L2. **(B)** At 72 hpf (3 dpf), the pLL consists of five neuromasts (L1-L5). Terminal neuromasts at the end of the trunk are apparent, and the pLL is now innervated. Some neuromasts being to appear in the aLL but are not labeled. This figure is adapted from Ma and Raible (2009).

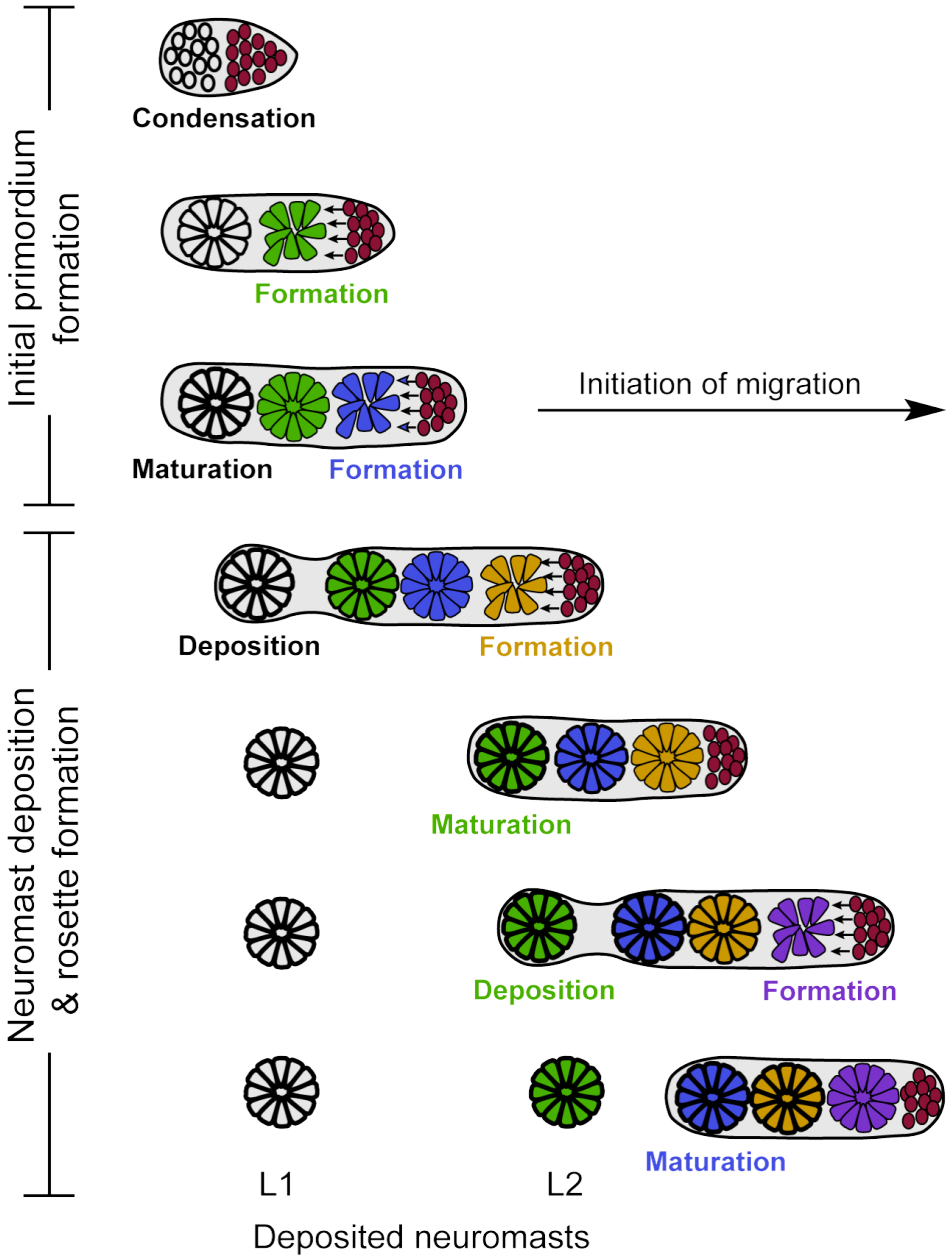


**Figure 2.3: Model of neuromast deposition in the posterior lateral line.**
The developing pLL primordium is organized into 2 or 3 individual rosettes (top) that form protoneuromasts, each labeled here with a different color. The posterior region of the primordium forms the leading zone, in which additional rosettes are formed and push previously formed rosettes toward the trailing end, where they are deposited. Hair cells in the protoneuromast then undergo final maturation. This figure is courtesy of E. Ma.

# CHAPTER 3: Non-specific Ototoxins Delay Hair Cell Regeneration

## 3.0 SUMMARY

Multiple ototoxins have been used to study mechanosensory hair cell death and regeneration. However, regeneration of lateral line hair cells has only been examined after treatment with neomycin (Harris *et al.*, 2003; Ma *et al.*, 2008; Behra *et al.*, 2009; Namdaran *et al.*, 2012) or copper (Linbo *et al.*, 2006; Hernández *et al.*, 2007; Hernández & Allende, 2008; Olivari *et al.*, 2008). In response to the growing use of these and other ototoxins to study lateral line hair cells, I treated fish with neomycin, gentamicin, copper, and cisplatin to determine if these compounds differentially affect the rate of subsequent hair cell regeneration. Treatment with neomycin and gentamicin as well as low concentrations of copper resulted in complete hair cell regeneration within 72 hours post-treatment (hpt). Incomplete regeneration was observed in a dose-dependent manner after treatment with greater concentrations of copper, consistent with reports that these conditions also damage the surrounding support cells (Olivari *et al.*, 2008). Cisplatin has been previously reported to block hair cell regeneration in the chicken basilar papilla (Slattery & Warchol, 2010), but I observed significant, albeit delayed, regeneration of lateral line hair cells. These findings indicate that, although the zebrafish lateral line maintains a robust regenerative capacity, certain ototoxins may compromise the rate of regeneration if they have nonspecific effects on the support cells that give rise to replacement hair cells.

## 3.1 INTRODUCTION

Several lines of evidence suggest that the type and extent of damage alter the course of hair cell regeneration. Differential exposure to copper affects the capacity for regeneration: treatment with lower concentrations of copper elicits regeneration, while treatment with high concentrations prevents most hair cell replacement (Hernández *et al*., 2006). Exposure at moderate to high concentrations also damages support cells (Olivari *et al*., 2008), preventing their differentiation into new hair cells. Cisplatin has been shown to have analogous effects on the regeneration of avian cochlear and vestibular sensory epithelia, killing hair cells and preventing both proliferative regeneration and interfering with direct transdifferentiation (Slattery & Warchol, 2010). Taken together these data suggested that different types or levels of damage could elicit different responses with potentially distinct underlying molecular regulation.

I compared the regenerative responses to copper, cisplatin, and the aminoglycosides neomycin and gentamicin, which other members of the Raible lab have previously suggested kill zebrafish lateral line hair cells by both distinct and overlapping mechanisms (Owens *et al*., 2008; Owens *et al*., 2009).

Neomycin (Harris *et al.*, 2003; Ma *et al.*, 2008; Owens *et al.*, 2008; Ou *et al.*, 2009) and copper (Hernández *et al.*, 2006; Hernández *et al.*, 2007; Olivari *et al.*, 2008; Villegas *et al.*, 2012) are commonly used to study hair regeneration in the lateral line because they kill hair cells quickly and thoroughly. Neomycin especially has very selective effects on mature, functional hair cells below the concentration at which it begins to cause nephrotoxicity and death (Harris *et al.*, 2003). However, many studies that seek to understand the mechanism of hair cell death have used either gentamicin (Steyger *et al.*, 2003; Ton & Parng, 2005; Chung *et al.*, 2006; Coffin *et al.*, 2009; Ou *et al.*, 2009; Wang & Steyger, 2009) or cisplatin (Ton & Parng, 2005; Ou *et al.*, 2007; Chiu *et al.*, 2008; Owens *et al.*, 2008; Giari *et al.*, 2012). Gentamicin conjugated to Texas Red is commonly used to monitor aminoglycoside uptake and transport within hair cells (Steyger *et al.*, 2003; Ou *et al.*, 2009), and other fluorescent tags are being developed in the Raible laboratory for studying aminoglycoside-induced cell death. Cisplatin and related platinum-based chemotherapeutics are used extensively even in developed nations, although there are efforts to develop compounds that block their effects on inner ear hair cells (Ton & Parng, 2005; Kim *et al.*, 2008; Owens *et al.*, 2008; More *et al.*, 2010). In contrast to these pharmaceuticals, copper poses almost no threat to humans, but it is a cheap and effective means of killing hair cells for regeneration studies (Hernández *et al.*, 2006) and is also an environmental pollutant in streams, lakes, and watersheds (Linbo *et al.*, 2006; Linbo *et al.*, 2009).

Studies of gentamicin- and cisplatin-mediated hair cell loss suggest they might have unique effects on subsequent regeneration. Gentamicin, like neomycin, is capable of inducing rapid hair cell death at high concentration, but exposure to much lower concentrations results in hair cell loss over a significantly longer time scale (Owens *et al.*, 2009). This distinction between acute and chronic treatments suggests that two alternate cell death pathways are present in mechanosensory hair cells. Activation of one or the other might also affect the intercellular signals that communicate the death of a hair cell to its neighboring support cells and perhaps influence the type of regenerative response. Cisplatin does not appear to have a similar dual mechanism, but its effects persist long after drug washout. A recent study of the chick basilar

papilla found that cisplatin continued to cause apoptosis in dividing support cells and prevented new hair cell regeneration (Slattery & Warchol, 2010). I was curious if cisplatin would similarly prevent regeneration in the zebrafish lateral line.

In this chapter, I summarize results of treating wild type zebrafish larvae with copper, neomycin, gentamicin, or cisplatin at different concentrations to compare and contrast their effects on the rate of lateral line hair cell regeneration. Both a semiquantitative scale that approximates hair cell number (Harris *et al.*, 2003) and exact hair cell counts were used to provide accurate comparisons under approximately equal treatment conditions. Treatments with the aminoglycoside antibiotics neomycin and gentamicin were followed by rapid and equal rates of hair cell regeneration regardless of the concentration used. However, copper and cisplatin treatments resulted in delayed hair cell regeneration, which I conclude is a result of their non-specific effects on nearby support cells and hair cell progenitors (Olivari *et al.*, 2008; Slattery & Warchol, 2010).

## 3.2 RESULTS

### Treatment with copper kills hair cells rapidly and can impair regeneration

The Raible lab previously demonstrated that media composition alters hair cell response to toxic compounds (Coffin *et al*., 2009). To ensure copper treatments effectively killed hair cells under conditions comparable to those used with aminoglycoside antibiotics, I performed a dose response assay using 5 dpf wild type *AB larvae treated with concentrations of dissolved copper(II) sulfate ranging from 0.3-30 μM and continuous exposure times ranging from 30 min to 10 h (Figure 3.1). These data show a consistent relationship between concentration, duration of exposure, and hair cell death that is similar to previous reports (Linbo *et al*., 2006; Hernández *et al*., 2006). While 1 μM copper killed nearly all hair cells within 4 hours, higher concentrations were effective within 30-60 minutes. The interplay between concentration and time of treatment suggests that copper may act in a cumulative fashion to damage hair cells.

I next compared hair cell regeneration after treatment with copper or neomycin on larvae at 5 dpf, when neuromast development is largely complete. As reported previously (Harris *et al*., 2003; Ma *et al*., 2008), full regeneration was observed after treatment with varying concentrations of neomycin (Figure 3.2A). In contrast, not all larvae treated with copper experienced full regeneration (Figure 3.2B). Larvae treated with 1 μM copper for 30 minutes

fully recovered, but larvae treated with 10 μM copper for 30 minutes exhibited incomplete regeneration, as did larvae treated with either concentration for 2 hours (data not shown). These results are consistent with previous studies describing the effects of copper on hair cell regeneration (Hernández *et al*., 2006) and damage to hair cell progenitors (Olivari *et al*., 2008) in younger, 3 dpf embryos.

**Gentamicin or cisplatin treatment do not block regeneration**

I next tested whether the rate of hair cell renewal varies after damage by other ototoxic compounds. Gentamicin can induce both rapid and delayed patterns of cell death, which appear to be regulated by intracellular pathways that may be distinct from those that follow neomycin treatment (Owens *et al*., 2009) and indicate there may also be differences in the rate of subsequent hair cell regeneration. Cisplatin is a platinum-derived chemotherapeutic that forms DNA adducts and induces apoptosis (Eastman, 1999). Previous work in cultures of the chick basilar papilla found that even after cisplatin was washed out, there was a decrease in proliferation, sustained apoptosis in support cells, and hair cells did not regenerate (Slattery & Warchol, 2010). Cisplatin has been used to study death of lateral line hair cells (Ton & Parng, 2005; Ou *et al*., 2007; Owens *et al*., 2008), but there were no studies examining subsequent regeneration in zebrafish.

To determine whether gentamicin treatment altered the rate of lateral line hair cell regeneration, I treated 5 dpf larvae with acute (200 μM, 30 minutes) or chronic (50 μM, 6 hours) gentamicin and measured regeneration until 96 hpt. Approximately half of the hair cells remained at 6 hours after the beginning of treatment and continued to decrease until 24 hpt (Figure 3.3A), consistent with previous results (Owens *et al*., 2009). Complete regeneration was observed in both groups 72 hour after this nadir.

I also determined the time course and extent of regeneration after cisplatin exposure. Wild type 5 dpf larvae were treated with chronic cisplatin (50 μM, 24 hours), after which regeneration was monitored until 96 hpt (Figure 3.3B). Although higher concentrations of cisplatin will kill hair cells more rapidly, chronic treatment was previously found to eliminate most hair cells (Ou *et al*., 2007) and limits the risk of investigator exposure. Initial loss of hair cells was comparable to neomycin- and gentamicin-treated larvae, but regeneration was significantly delayed and remained incomplete at 96 hpt ($p < 0.001$). These results suggest that

persistent apoptosis may also occur in fish as in chick, reducing the success of proliferating progenitor cells. However, regeneration is not fully compromised.

## 3.3 DISCUSSION

There have been few direct comparisons of regeneration following treatment with different ototoxins commonly used to study sensory hair cells in zebrafish. Neomycin and gentamicin are among the most commonly used ototoxins and are both aminoglycoside antibiotics. However, whereas neomycin has been used in zebrafish to study regeneration (Harris *et al.*, 2003; Ma *et al.*, 2008; Moon *et al.*, 2011; Namdaran *et al.*, 2012) and also to perform screens for hair cell protection and survival (Murakami *et al.*, 2003; Ton & Parng, 2005; Santos *et al.*, 2006; Ou *et al.*, 2009), gentamicin has been used more often to study the mechanism of aminoglycoside entry and hair cell death (Ton & Parng, 2005; Coffin *et al.*, 2009; Ou *et al.*, 2009; Wang & Steyger, 2009). This is in part due to the greater ease of monitoring the localization of gentamicin conjugated to Texas Red (Steyger *et al.*, 2003; Ou *et al.*, 2009), but it still raises the question whether neomycin and gentamicin induce hair cell damage in the same way. T. Linbo and others in the Raible laboratory are currently developing new conjugated markers to observe neomycin activity within hair cells. Meanwhile, the data presented here have demonstrated that regeneration after treatment with either ototoxin is very similar, increasing the likelihood that all aminoglycosides function the same way in the lateral line.

Copper has been used in several studies with zebrafish, but those reports that measure regeneration either examined a limited number of neuromasts (Linbo *et al.*, 2006), used a different measure of regeneration (Hernández *et al.*, 2006; Villegas *et al.*, 2012), or used relatively young embryos (Hernández *et al.*, 2006; Hernández *et al.*, 2007; Villegas *et al.*, 2012) that may not be comparable to 5 dpf larvae typically used by other investigators. The data I present here indicate that although copper is sometimes followed by hair cell regeneration similar to neomycin, there is a narrow range of effective and useful doses. Specifically, a very low (0.3 μM) concentration of copper does not cause effective hair cell loss, and much higher concentrations (3-10 μM) can impair subsequent hair cell regeneration. This latter observation is consistent with previous reports that severe copper exposure can permanently block hair cell regeneration (Hernández *et al.*, 2006) or damage non-sensory support cells (Olivari *et al.*, 2008). I also found that regeneration was less affected by higher concentrations of copper in zebrafish

embryos with a less developed lateral line, indicating that age should be considered when comparing the results of different treatments.

Finally, I examined hair cell regeneration after treatment with cisplatin, which has been previously studied in the chick (Slattery & Warchol, 2010) but not in zebrafish. There is evidence that cisplatin and copper may enter the cell or otherwise cause damage through similar mechanisms (Katano *et al.*, 2002; More *et al.*, 2010), which emphasizes the value of performing a comparative analysis of the effects of different ototoxins. Cisplatin was reported to completely block hair cell regeneration in cultured tissue from the avian basilar papilla (Slattery & Warchol, 2010), but in zebrafish the lateral line remained capable of considerable regeneration, albeit significantly delayed. This establishes the possibility of performing genetic or chemical screens in zebrafish treated with cisplatin and provides a baseline for measuring changes in regeneration. Because cisplatin induces hair cell damage through a unique mechanism that directly damages DNA, such screens might yield unique regulators of hair cell survival and regeneration.

I continue my comparison of these four ototoxins in Chapter 4, examining the mechanism of regeneration after different treatments. There has been some evidence of non-proliferative direct transdifferentiation in zebrafish treated with a low concentration of copper (Hernández *et al.*, 2007), but the overwhelming body of literature using neomycin indicates that most or all hair cell regeneration occurs through division of progenitor cells (Harris *et al.*, 2003; Ma *et al.*, 2008; Wibowo *et al.*, 2011; Namdaran *et al.*, 2012). Because both proliferative and non-proliferative mechanisms of regeneration have been observed in other model organisms, I examined more closely the possibility that certain treatment variables such as concentration or age might bias the type of hair cell regeneration that occurs in the lateral line.

## 3.4 FIGURES AND LEGENDS

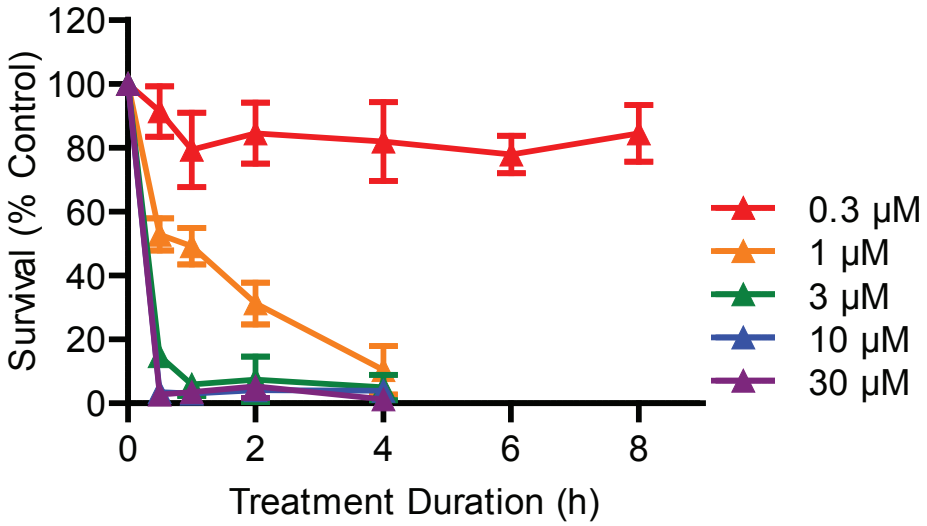


**Figure 3.1: Rapid hair cell loss following copper exposure.**
Wild type larvae were treated at 5 dpf with serial dilutions of copper(II) sulfate for a period of 30 min to 8 h. Hair cell death was rapid in most cases, although 0.3 µM copper had a minimal effect. *N* = 8 fish per group per time point. Error bars are +/- SD.

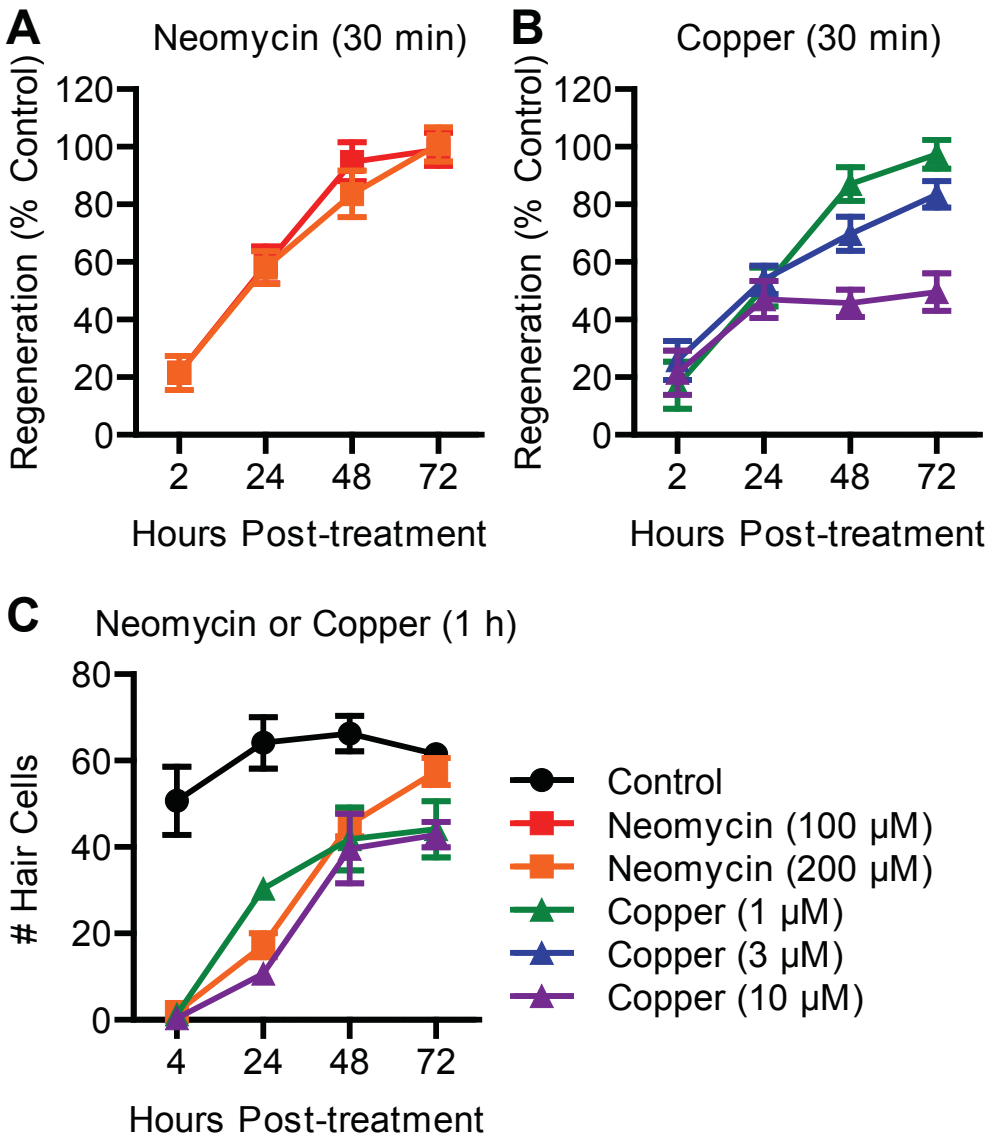


**Figure 3.2: Copper may impair hair cell regeneration.**
**(A, B)** Wild type larvae were treated at 5 dpf with serial dilutions of neomycin or copper for 30 min. Hair cell regeneration was scored using FM 1-43FX and normalized to controls. Regeneration was incomplete in groups treated with 3 or 10 µM copper, but other groups recovered fully. **(C)** Individual counts of hair cells labeled with antibodies against parvalbumin confirmed incomplete regeneration in larvae treated for 1 h with copper. *N* = 8 fish per group per time point. Error bars are +/- SD.

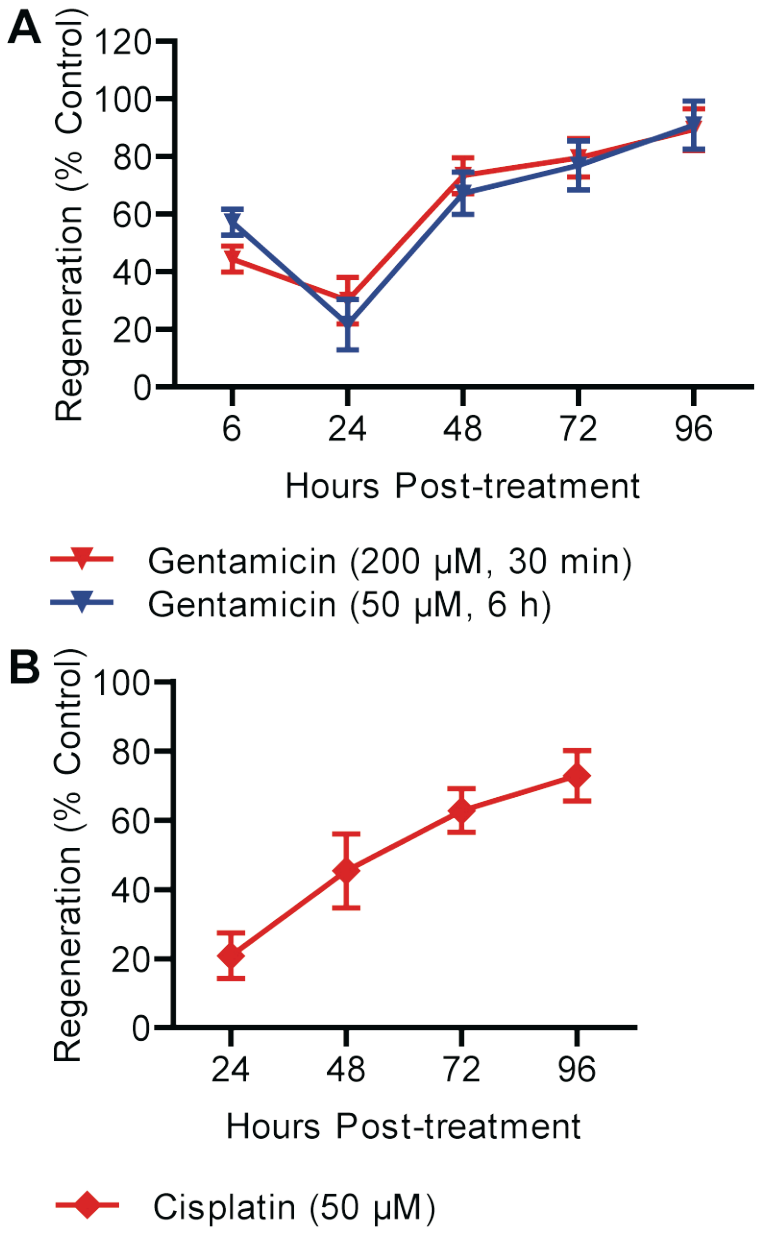


**Figure 3.3: Hair cell regeneration after treatment with gentamicin or cisplatin.**
**(A)** Wild type larvae were treated at 5 dpf with 200 µM gentamicin for 30 min (acute) or 50 µM gentamycin for 6 h (chronic) and allowed to recover for 96 h from the beginning of treatment. Gentamicin-induced death was delayed compared to neomycin. There was no dose-dependent effect on regeneration, which was complete by 96 hpt. **(B)** Wild type larvae were treated at 5 dpf with 50 µM cisplatin for 24 h and allowed to recover for 72 h (96 h from the beginning of treatment). Regeneration was considerable yet remained incomplete. *N* = 8 fish per group per time point. Error bars are +/- SD.

**Table 3.1: Parvalbumin hair cell numbers after continuous copper treatment.**
*N* = 8 per group. Control means were used for normalization.

| | | Continuous Copper Exposure | | | | | |
|---|---|---|---|---|---|---|---|
| **Treatment and Duration** | | **30 min** | **1 h** | **2 h** | **4 h** | **6 h** | **8 h** |
| **Control** | *Mean* | 70.75 | 76.75 | 68.75 | 69.88 | 70.00 | 62.00 |
| | *SD* | 5.85 | 6.25 | 8.61 | 10.22 | 5.48 | 5.95 |
| **0.3 µM Copper** | *Mean* | 64.63 | 60.88 | 58.13 | 57.25 | 54.50 | 52.38 |
| | *SD* | 5.58 | 8.95 | 6.56 | 8.60 | 4.11 | 5.53 |
| **1 µM Copper** | *Mean* | 37.38 | 37.75 | 21.50 | 7.25 | | |
| | *SD* | 3.54 | 4.43 | 4.47 | 5.26 | | |
| **3 µM Copper** | *Mean* | 10.50 | 4.50 | 5.13 | 3.50 | | |
| | *SD* | 2.07 | 2.67 | 4.91 | 2.78 | | |
| **10 µM Copper** | *Mean* | 2.50 | 2.38 | 2.88 | 2.75 | | |
| | *SD* | 1.60 | 0.92 | 1.64 | 2.05 | | |
| **30 µM Copper** | *Mean* | 2.00 | 2.75 | 3.63 | 0.88 | | |
| | *SD* | 1.51 | 1.83 | 2.45 | 1.36 | | |

**Table 3.2: FM 1-43FX hair cell numbers after neomycin or copper treatment at 5 dpf.**
*N* = 7 per group. Control means were used for normalization.

| Neomycin or Copper Treatment (30 min) | | | | | |
|---|---|---|---|---|---|
| **Time Post-treatment** | | **2 h** | **24 h** | **48 h** | **72 h** |
| **Control** | *Mean* | 16.57 | 16.71 | 16.57 | 16.43 |
| | *SD* | 0.79 | 1.11 | 0.79 | 0.98 |
| **100 µM Neomycin** | *Mean* | 3.57 | 9.86 | 15.71 | 16.29 |
| | *SD* | 0.53 | 1.07 | 1.11 | 0.95 |
| **200 µM Neomycin** | *Mean* | 3.57 | 9.71 | 13.86 | 16.57 |
| | *SD* | 0.98 | 0.95 | 1.35 | 0.98 |
| **1 µM Copper** | *Mean* | 2.86 | 8.57 | 14.43 | 16.00 |
| | *SD* | 1.35 | 1.13 | 0.98 | 0.92 |
| **3 µM Copper** | *Mean* | 4.29 | 9.00 | 11.57 | 13.71 |
| | *SD* | 1.11 | 0.82 | 0.98 | 0.56 |
| **10 µM Copper** | *Mean* | 3.57 | 7.86 | 7.57 | 8.14 |
| | *SD* | 1.27 | 1.07 | 0.79 | 1.07 |

**Table 3.3: Parvalbumin hair cell numbers after chronic or acute gentamicin treatment.** *N* = 8 per group. Control means were used for normalization.

| | | Gentamicin Treatment (1 or 6 h) | | | | |
|---|---|---|---|---|---|---|
| **Time Post-treatment** | | **6 h** | **24 h** | **48 h** | **72 h** | **96 h** |
| **Control** | *Mean* | 74.88 | 67.50 | 65.63 | 63.00 | 64.50 |
| | *SD* | 4.05 | 10.18 | 4.75 | 5.81 | 5.86 |
| **200 µM Gentamicin (Acute)** | *Mean* | 33.25 | 20.25 | 48.13 | 50.13 | 57.63 |
| | *SD* | 3.37 | 5.42 | 4.05 | 4.19 | 4.72 |
| **50 µM Gentamicin (Chronic)** | *Mean* | 42.88 | 14.63 | 44.13 | 48.50 | 58.63 |
| | *SD* | 3.36 | 5.93 | 4.85 | 5.35 | 5.40 |

**Table 3.4: Parvalbumin hair cell numbers after chronic cisplatin treatment.**
*N* = 8 per group. Control means were used for normalization.

| Cisplatin Treatment (1 h) | | | | | |
|---|---|---|---|---|---|
| **Time Post-treatment** | | **24 h** | **48 h** | **72 h** | **96 h** |
| **Control** | *Mean* | 77.00 | 77.75 | 75.88 | 73.50 |
| | *SD* | 5.68 | 5.06 | 6.10 | 5.32 |
| **50 µM Cisplatin** | *Mean* | 16.00 | 35.25 | 47.63 | 53.50 |
| | *SD* | 5.07 | 8.29 | 4.84 | 5.32 |

# CHAPTER 4: Proliferation is Required for Hair Cell Regeneration

## 4.0 SUMMARY

Chapter 3 examined the ability of lateral line hair cells to regenerate after treatment with different ototoxins, but it has also been suggested that the severity of exposure affects the mechanism of regeneration in the zebrafish lateral line system. Specifically, exposure to a low concentration of copper resulted in non-proliferative regeneration and treatment with a high concentration resulted in proliferative regeneration (Hernández *et al*., 2007). By contrast, most evidence suggests that proliferation is the dominant regenerative mechanism in the zebrafish lateral line following treatment with neomycin (López-Schier & Hudspeth, 2006; Ma *et al*., 2008; Wibowo *et al*., 2011). I measured incorporation of the thymine analog 5-bromo-2'-deoxyuridine (BrdU) to confirm that the majority of new hair cells in the lateral line derive from dividing precursors. Treatment with the mitotic inhibitor flubendazole, which disrupts microtubule organization (Spagnuolo *et al.*, 2010), blocked hair cell regeneration. Flubendazole-treated progenitor cells also displayed increased expression of phosphohistone-H3 (PHH3), which is elevated during M phase (Hendzel *et al.*, 1997) and evidence that flubendazole acts by arresting progenitor cell division rather than by disrupting differentiation. Additional progenitors did not accumulate, indicating that mitosis is a rate-limiting step. These results indicate proliferation is the dominant mechanism of mechanosensory hair cell regeneration in the lateral line and that direct transdifferentiation does not make a significant contribution even in the absence of proliferation.

## 4.1 INTRODUCTION

Two mechanisms of hair cell regeneration have been observed in non-mammalian vertebrates (Brignull *et al.*, 2009). Proliferative regeneration occurs when a hair cell progenitor divides into two hair cell precursors, which then differentiate into new hair cells. The Raible laboratory has previously presented evidence suggesting that proliferation is the dominant regenerative mechanism in zebrafish following treatment with neomycin (Ma *et al.*, 2008), consistent with the work of others (López-Schier & Hudspeth, 2006; Wibowo *et al.*, 2011). However, studies in the chick (Roberson *et al.*, 1996; Roberson *et al.*, 2004) and amphibians (Baird *et al.*, 1996; Baird *et al.*, 2000; Taylor & Forge, 2005) have also found evidence of direct

transdifferentiation occurring in the absence of proliferation, providing an alternative mechanism that researchers could exploit to replace lost hair cells in the human inner ear.

Whether direct transdifferentiation can occur in the zebrafish lateral line is uncertain, in part because of its rapid regeneration and also because it is difficult to exclude the potentially confounding influence of immature yet post-mitotic hair cell precursors. Hernandez *et al.* (2007) reported that treatment with copper yielded non-proliferative regeneration in a dose-dependent manner. A low concentration of copper was followed exclusively by non-proliferative regeneration in young, 3 dpf embryos, whereas treatment with a high concentration resulted in proliferative regeneration similar. These data suggested that different levels of damage could elicit different responses with potentially distinct underlying molecular mechanisms. I therefore sought to determine if this phenomenon was a specific response to copper or unique feature of regeneration in developmentally immature embryos.

In this chapter, I demonstrate that potential evidence of direct transdifferentiation in young larvae should be interpreted not as hair cell regeneration but rather as the maturation of post-mitotic hair cell precursors resistant to ototoxic damage. The mitotic inhibitor flubendazole was used to inhibit cell proliferation in an effort to reveal a latent capability for hair cell replacement by direct transdifferentiation. However, no such secondary mechanism was observed. Additional experiments using gentamicin and cisplatin also yielded evidence of proliferative regeneration. Although a small amount of transdifferentiation may still occur below the threshold of detection, this mechanism does not appear to contribute meaningfully to hair cell regeneration in the lateral line.

## 4.2 RESULTS

### Robust proliferative regeneration occurs after treatment with neomycin or copper

To examine whether treatment with neomycin or copper might bias regeneration toward proliferative or non-proliferative mechanisms, I used a pulse-chase paradigm to label new hair cells arising after damage with BrdU, a thymine analog that incorporates into DNA during S phase. Wild type *AB larvae were treated at 5 dpf with 200 μM neomycin, 1 μM copper, or 10 μM copper for 1 hour and then incubated in 5 mM BrdU for the first 23 hours of recovery (24 hours from the beginning of treatment). The BrdU was replaced with fresh embryo medium, and larvae recovered for an additional 48 hours at which time they were fixed and stained with

antibodies recognizing BrdU and parvalbumin to reveal mature hair cells derived from dividing precursors. Hair cells without BrdU staining presumably derived from non-dividing progenitor cells or from progenitors that proliferated after exposure to BrdU.

All experimental conditions resulted in significantly more proliferation compared to controls (Figure 4.1A). One-way ANOVA revealed a highly significant main effect of treatment ($p < 0.0001$). Approximately 40-50% of all hair cells in ototoxin-treated groups were BrdU-positive compared to ~10% in control larvae, evidence of significant proliferative regeneration during the first 23 h of recovery ($p < 0.001$; Figure 4.1B). Although there was a small but significant difference in the proportion of BrdU-positive hair cells between larvae treated with neomycin and 1 μM copper ($p < 0.05$), there was no such difference between 1 μM and 10 μM copper. Taken together, these data support the idea that hair cells are derived from dividing precursors after damage from multiple ototoxins.

**Little direct transdifferentiation occurs after treatment with neomycin or copper**

Given that proliferative regeneration occurred after treatment with neomycin and with both low and high concentrations of copper, I sought to determine if preventing proliferation would uncover evidence of direct transdifferentiation. I treated 5 dpf larvae with neomycin or copper for 1 h and then incubated them in 5 μM flubendazole throughout the recovery period. Flubendazole blocks tubulin polymerization, preventing assembly of a mitotic spindle required for chromosome segregation (Spagnuolo *et al*., 2010), and has been identified as a drug that blocks hair cell regeneration (Namdaran *et al*., 2012). Importantly, flubendazole shows little toxicity to hair cells themselves, removing a potential confounding effect of other mitotic inhibitors such as genistein and colchicine (Wibowo *et al*., 2011). Larvae were collected up to 48 hpt to establish a time course of regeneration with and without flubendazole exposure. All ototoxic treatments resulted in near-complete loss of hair cells in 5 dpf larvae, with substantial recovery by 48 hpt (Figure 4.2). Treatment with flubendazole alone had little effect on hair cell number but significantly impaired regeneration compared to control ($p < 0.001$). These results support the idea that hair cells are derived from dividing precursors.

Impairment of microtubule assembly with flubendazole might impair maturation of hair cells instead of blocking progenitor cell division. I therefore assayed the extent of regeneration during pharmacological inhibition of proliferation using transgenic *ET4:GFP* fish that express

GFP in mature hair cells as well as pre-mitotic progenitors (López-Schier & Hudspeth, 2006). In the absence of flubendazole, the increase in GFP-positive cells significantly preceded the increase in parvalbumin-positive cells (Figure 4.2A; $p < 0.001$). However, there was no significant difference between GFP- and parvalbumin-positive cells in larvae treated with flubendazole (Figure 4.2B), indicating pre-mitotic progenitors did not accumulate. Analysis of larvae with antibodies against phosphohistone H3 (PHH3), which is expressed during mitosis, revealed incubation in flubendazole produced a significant increase in the proportion of PHH3-positive nuclei in GFP-positive cells during regeneration after neomycin ($p < 0.05$; Figure 4.3) and copper ($p < 0.001$). This result strongly suggests that flubendazole arrests hair cell progenitors during mitosis by interfering with spindle microtubules, not at a later stage of differentiation.

A previous report demonstrated non-proliferative addition of hair cells after copper treatment in younger, 3 dpf animals during late embryogenesis (Hernández *et al*., 2007). I therefore directly compared regeneration after different treatment conditions using neomycin or copper at 3 dpf. We observed that hair cell regeneration in all experimental groups was rapid and complete when compared to controls (Figure 4.4A,B). However, I also observed that control embryos added substantial numbers of hair cells over the course of the experiment (Figure 4.4C). The overall increase in hair cell number in ototoxin-treated groups closely followed the addition of hair cells in controls. This finding suggests that damage caused during embryonic growth can be compensated for by developmental mechanisms and many of the hair cells that arose after damage would have been added irrespective of toxin exposure.

**Proliferation is required for regeneration after treatment with gentamicin or cisplatin**

Treatment with neomycin or copper, two unrelated ototoxins, produced similar results indicating that proliferation is the major mechanism of hair cell regeneration present in the zebrafish lateral line. In Chapter 3, I examined the rate of hair cell regeneration after treatment with gentamicin and cisplatin. I also indicated these ototoxins have unique properties that could potentially affect the mechanism of hair cell regeneration. Gentamicin can induce both rapid and delayed patterns of cell death that appear to be regulated by distinct intracellular pathways (Owens *et al.*, 2009). Previous work using cultures of the avian inner ear found that cisplatin inhibits proliferation and continues to induce apoptosis after treatment, preventing hair cell

regeneration (Slattery & Warchol, 2010). I hypothesized that these ototoxins might affect hair cell progenitors in zebrafish and the signaling mechanisms that direct proliferative regeneration.

Wild type larvae were treated at 5 dpf with acute (200 μM, 30 min) or chronic (50 μM, 6 h) gentamicin followed by incubation in 5 μM flubendazole. Flubendazole significantly blocked regeneration in both groups when measured at 48 hpt ($p < 0.001$; Figure 4.5A). I performed a similar experiment using 50 μM cisplatin for 24 h followed by flubendazole and observed that regeneration was similarly prevented ($p < 0.001$; Figure 4.5B). These results confirm the role of proliferative regeneration in lateral line hair cells and do not support the hypothesis that different ototoxins or variations in exposure could promote alternative mechanisms of regeneration.

## 4.3 DISCUSSION

My results support a model in which new lateral line hair cells are derived exclusively from dividing progenitors. By labeling proliferating cells with BrdU during regeneration, I found extensive incorporation of this marker under a variety of treatment conditions. This is consistent with other studies that used time-lapse microscopy and BrdU to observe hair cell regeneration. Previous reports have found that nearly all BrdU-positive hair cells regenerate in pairs (Ma *et al.*, 2008), suggesting that regeneration involves symmetric division of a progenitor into two daughter hair cells. Some progenitor cells may divide to form immature hair cell precursors prior to ototoxic damage, but incubation in BrdU both before and after neomycin treatment results in near complete incorporation of the marker (Wibowo *et al.*, 2011). Finally, live imaging studies of ET4 larvae have followed the movement and division of ET4-positive cells from their earliest expression as pre-mitotic progenitors through division until their final differentiation as mature hair cells (Wibowo *et al.*, 2011).

Proliferation is not only sufficient but also necessary for hair cell regeneration. The anthelmintic drug flubendazole successfully blocked the production of new hair cells in regenerating neuromasts, consistent with reports using this and other mitotic inhibitors (Wibowo *et al.*, 2011; Namdaran *et al.*, 2012). I also found that flubendazole does not block differentiation of post-mitotic progenitors into new hair cells, but it does prevent the accumulation of additional progenitors. This suggests that the mechanism responsible for production of hair cell progenitors is sensitive to the rate of hair cell regeneration. A recent report found that progenitors form in regions of low Notch expression located in polar compartments on opposite ends of the

neuromast (Wibowo *et al.*, 2011). There may be other signals produced by post-mitotic hair cell precursors that influence this rate-limiting step.

Several other organ systems have been used to model auditory hair cell regeneration, and many of these make use of proliferative as well as non-proliferative regeneration, *i.e.*, direct transdifferentiation. Although proliferative regeneration has been observed in birds (Ryals & Rubel, 1988) and appears to be the dominant mechanism in the salamander (Jones & Corwin, 1996), there is abundant evidence that early phases of direct transdifferentiation also occur in birds (Baird *et al.*, 1996; Roberson *et al.*, 1996; Roberson *et al.*, 2004), frogs (Baird *et al.*, 1996; Baird *et al.*, 2000), and newts (Taylor & Forge, 2005). These multiple mechanisms of regeneration mark a key difference between the zebrafish and other vertebrates, complicating efforts to translate some experimental results.

A few possible explanations exist for the absence of extensive direct transdifferentiation during regeneration of lateral line hair cells. Rapid proliferation may be so successful that an alternate mechanism is unnecessary. Regeneration in the zebrafish occurs within days compared to one or two months in the chick (Bermingham-McDonogh & Rubel, 2003; Brignull *et al.*, 2009). The early phase of direct transdifferentiation in the chick basilar papilla might serve to restore minimal auditory perception as rapidly as possible, relying on subsequent proliferation for full regeneration. However, proliferation is not necessarily required. Aphidicolin was used to block proliferative regeneration in cultures of chick basilar papilla treated with gentamicin. Division of support cells was completely inhibited, as measured by the absence of BrdU incorporation. However, significant numbers of new hair cells were still generated, labeled with myosin-VI and FM 1-43FX (Shang *et al.*, 2010). However, direct transdifferentiation alone produced fewer hair cells than a combination of proliferative and non-proliferative mechanisms, and the number of support cells significantly decreased in the absence of proliferation (Shang *et al.*, 2010). While proliferative regeneration is not required for regeneration of the basilar papilla, it appears to be an important contributor alongside transdifferentiation. It is also possible that hair cells in the zebrafish ear would respond differently than those the lateral line. Evidence of non-proliferative regeneration has been reported in the utricular macula after laser ablation at 2 dpf (Millimaki *et al.*, 2010), suggesting a distinct response from the drug-induced hair cell loss described in the present study.

Although direct transdifferentiation seems simpler, it is not necessarily clear if proliferation or transdifferentiation provides a better opportunity to replace the large numbers of sensory hair cells required to restore auditory function in humans. Support cells undergo terminal mitosis in the mammalian cochlea while those in the vestibular system continue to proliferate in limited amounts (Warchol *et al.*, 1993; Rubel *et al.*, 1995). With or without proliferation, differentiation of progenitors into sensory hair cells requires expression of the transcription factor *atoh1*, which specifies hair cell fate during both development and regeneration (Bermingham *et al.*, 1999; Itoh & Chitnis, 2001) and is regulated through lateral inhibition established by Notch signaling (Itoh & Chitnis, 2001).

Notch signaling is required for specification of prosensory domains during early development of the inner ear (Daudet & Lewis, 2005; Daudet *et al.*, 2007; Hartman *et al.*, 2010) but can serve as a hindrance during hair cell regeneration. For example, *atoh1* is expressed in some support cells in the mouse utricle after damage, but these rarely become new hair cells. Inhibition of Notch signaling increases both the amount of *atoh1* expression and the number of cells that successfully transdifferentiate (Lin *et al.*, 2011). In zebrafish, which produce hair cells by proliferation, Notch is important for regulating the production of new hair cell progenitors (Wibowo *et al.*, 2011) prior to their division and differentiation into sensory cells. Inhibition of Notch signaling during either development or regeneration results in supernumerary hair cells (Ma *et al.*, 2008; Wibowo *et al.*, 2011).

In some ways, regeneration of the lateral line recapitulates early development. The role of proliferation, like Notch-regulated expression of *atoh1*, is conserved during both phenomena. The migrating primordium that establishes the posterior lateral line is unique to development, and it deposits several protoneuromasts that already contain a small number of immature hair cells (Ghysen & Dambly-Chaudière, 2007). Additional hair cells are then added through the same mechanism of progenitor division and differentiation that occurs during regeneration. Therefore, one of the challenges to regenerating human auditory hair cells appears to be re-initiating these early developmental steps without disrupting the fully formed and complex structure of the inner ear.

## 4.4 FIGURES AND LEGENDS

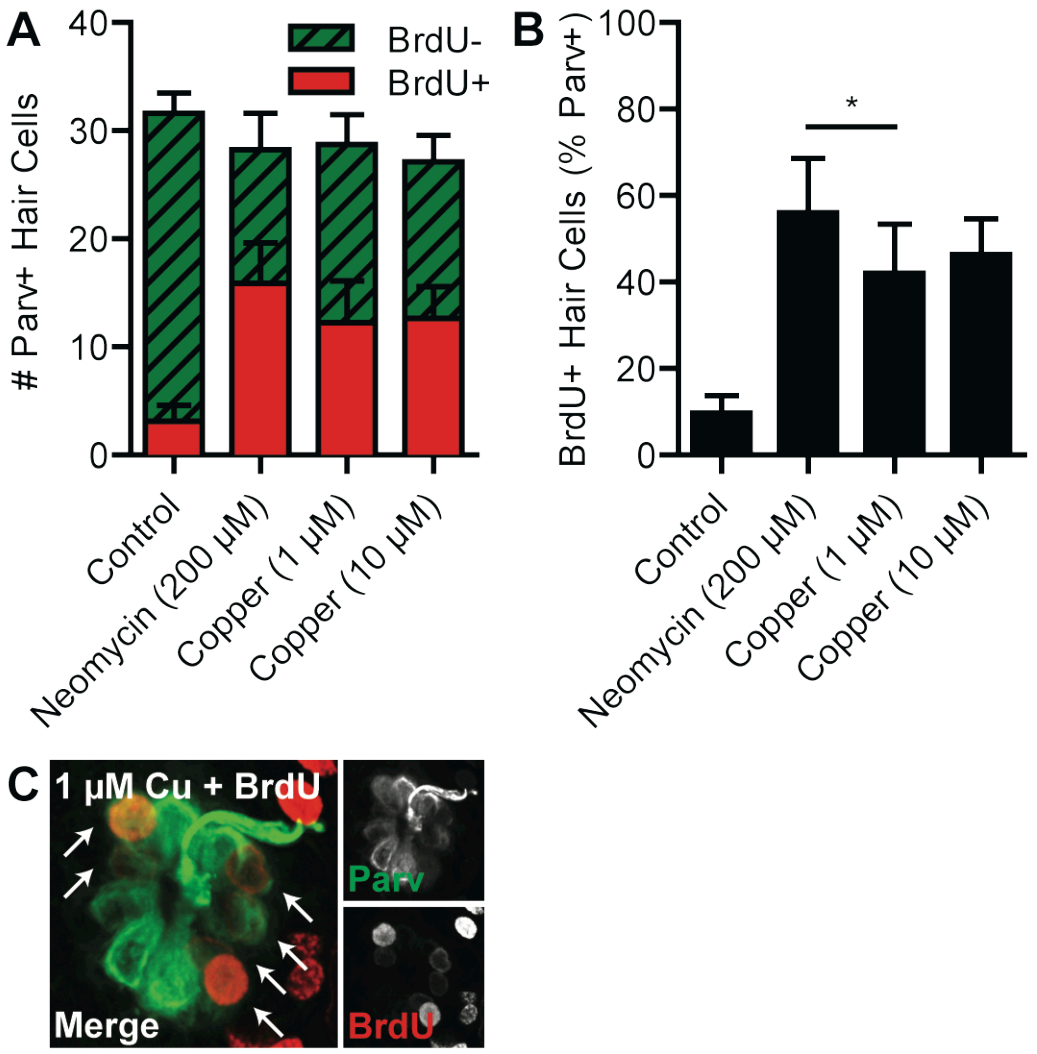


**Figure 4.1: Hair cells are derived from dividing precursors.**
**(A)** Wild type larvae were treated at 5 dpf with neomycin or copper for 1 h followed by recovery in 5 mM BrdU for the first 23 hr followed by fresh embryo medium. Greater numbers of BrdU-positive hair cells were observed in all ototoxin-treated groups at 72 hpt. **(B)** One-way ANOVA followed by a Tukey post-hoc analysis of the proportion of BrdU-positive hair cells revealed significant increases in all ototoxin-treated groups (***, $p < 0.001$). No significant difference was observed between 1µM and 10 µM copper that would suggest a dose-dependent effect on proliferative regeneration, although a small difference was present between neomycin and 1 µM copper (*, $p < 0.05$). $N = 8$ fish per group per time point. Error bars are + SD. **(C)** Example neuromast at 72 hpt from a fish treated with 1 µM copper. Arrows mark BrdU-positive hair cells.

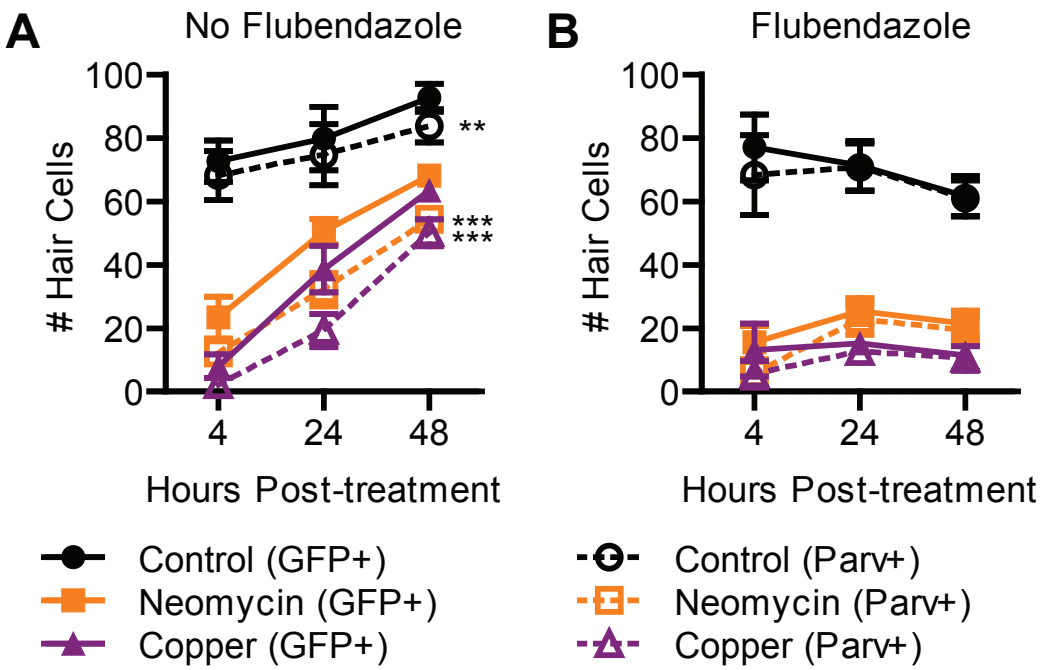


**Figure 4.2: Inhibiting mitosis blocks hair cell regeneration.**
*ET4:GFP* larvae were treated at 5 dpf with neomycin or copper for 1 h and incubated in flubendazole for the duration of recovery. **(A)** Those not treated with flubendazole continued to add mature hair cells. Significantly more cells expressed GFP than parvalbumin, indicating the presence of hair cell precursors. Two-way ANOVA followed by Bonferroni post-hoc analysis found a significant difference in all treatment groups between parvalbumin- and GFP-positive hair cell counts at 48 hpt (**, $p < 0.01$; ***, $p < 0.001$). **(B)** Those treated with flubendazole exhibited little or no increase in mature hair cells. There were at most 1 or 2 parvalbumin-negative precursors present per neuromast, indicating that undifferentiated precursors did not accumulate. $N = 8$ fish per group per time point. Error bars are +/- SD.

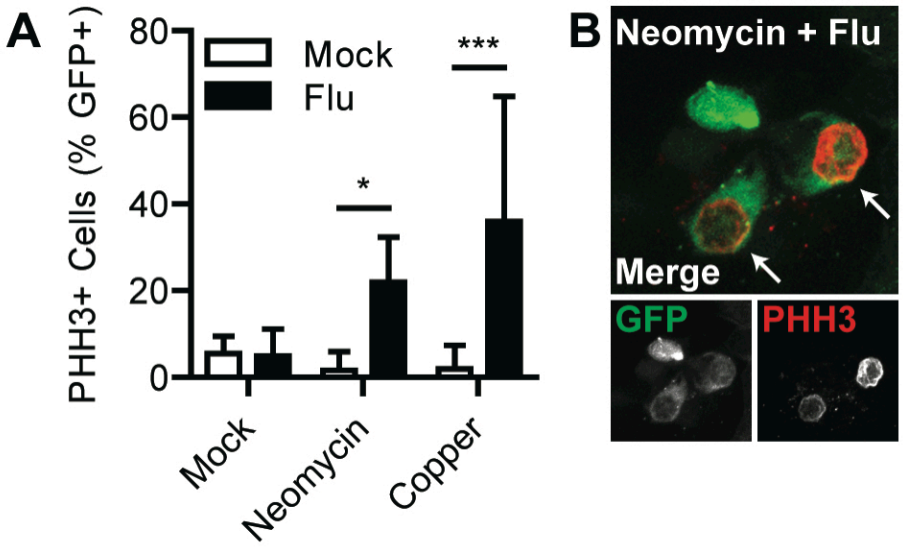


**Figure 4.3: Flubendazole impairs division of hair cell progenitors.**
*ET4:GFP* larvae were treated at 5 dpf with neomycin or copper for 1 h and incubated in flubendazole for 24 h. Immunohistochemistry was performed for GFP and PHH3, which is upregulated during mitosis. **(A)** Two-way ANOVA followed by Bonferroni post-hoc analysis found significant increases in the proportion of PHH3-positive cells in neomycin- and copper-treated larvae, demonstrating that cell division was arrested in GFP-positive hair cell precursors (*, $p < 0.05$; ***, $p < 0.001$). $N = 8$ fish per group. Error bars are + SD. **(B)** Example neuromast at 24 hpt with two PHH3-positive progenitors after treatment with neomycin and flubendazole. Arrows mark PHH3-positive nuclei.

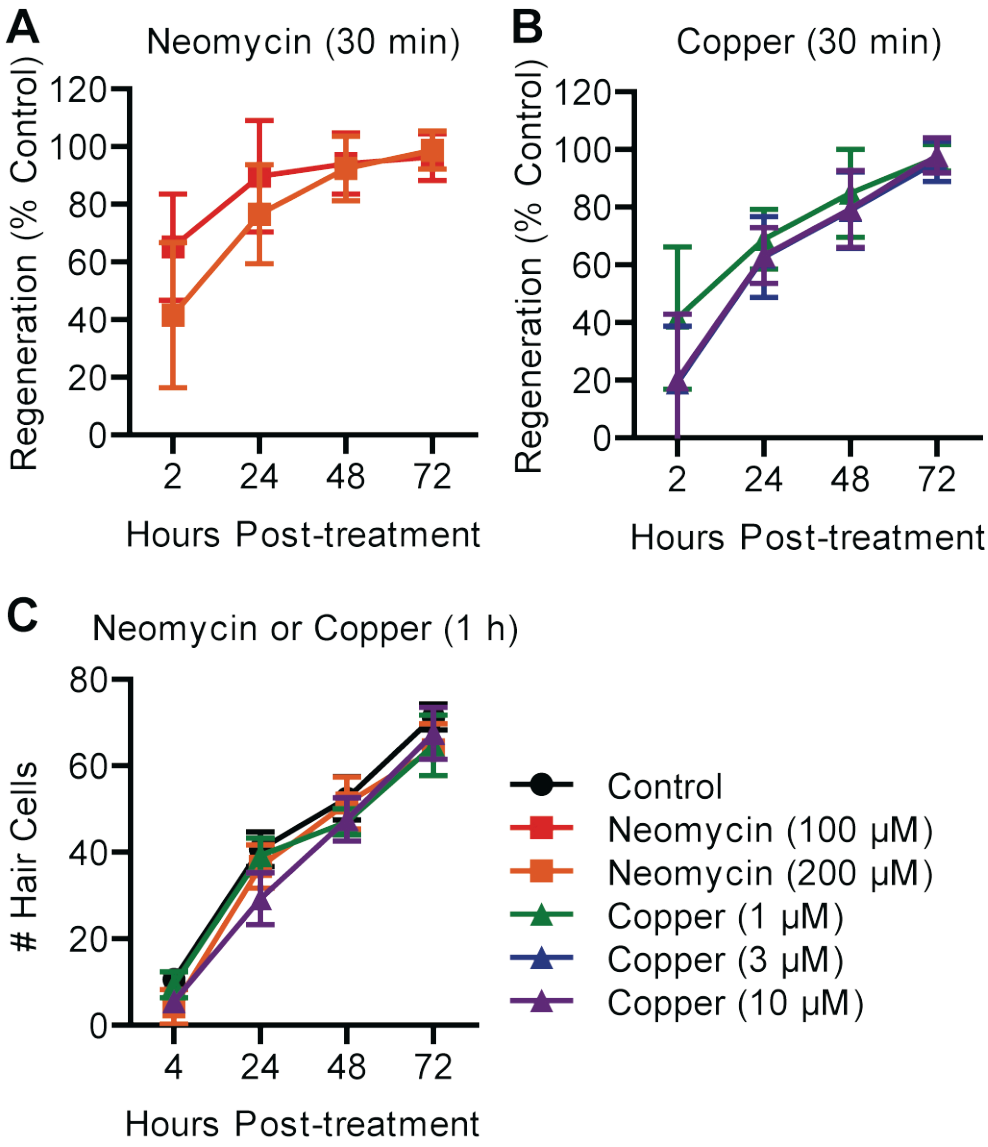


**Figure 4.4: Regeneration is obscured by growth of the immature lateral line.**
**(A, B)** Wild type larvae were treated at 3 dpf with serial dilutions of neomycin or copper for 30 min. Hair cell regeneration was scored using FM 1-43FX and normalized to controls. Complete regeneration was observed in all groups. **(C)** Individual counts of hair cells labeled with antibodies against parvalbumin confirmed complete regeneration after 1 h treatment with copper or neomycin. However, all ototoxin-treated groups closely matched control larvae, which had few hair cells at the time of treatment. Instead of regeneration, most hair cell addition appeared related to early development of the lateral line. *N* = 8 fish per group per time point. Error bars are +/- SD.

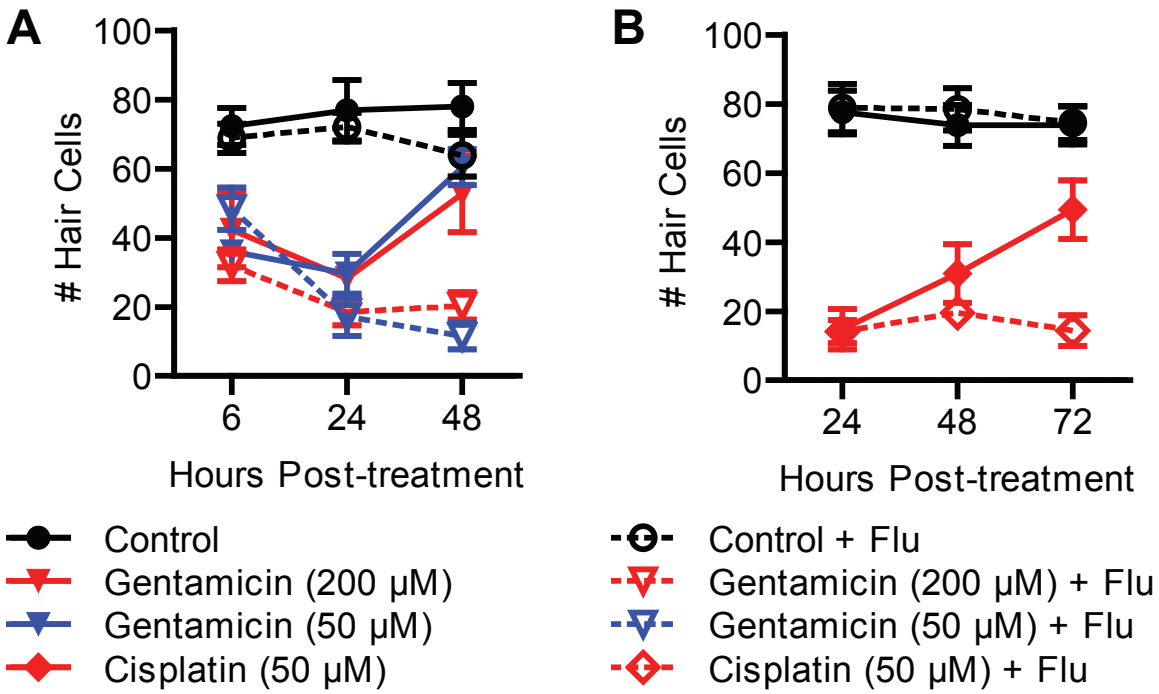


**Figure 4.5: Proliferation is required for regeneration after gentamicin and cisplatin.** **(A)** Wild type larvae were treated at 5 dpf with 200 µM gentamicin for 30 min (acute) or 50 µM gentamycin for 6 h (chronic) and were incubated in flubendazole for 48 h after treatment. Minimal regeneration was observed in after either acute or chronic treatment, indicating no difference in the mechanism of regeneration. **(B)** Wild type larvae were treated at 5 dpf with 50 µM cisplatin for 24 h and were incubated in flubendazole for 48 h after treatment. Again, minimal regeneration was observed, suggesting that although the lateral line can regenerate after cisplatin treatment, this requires dividing progenitors. *N* = 8 fish per group per time point. Error bars are +/- SD.

**Table 4.1: FM 1-43FX hair cell numbers after neomycin or copper treatment at 3 dpf**
*N* = 14 per group. Control means were used for normalization.

| Neomycin or Copper Treatment (30 min) | | | | | |
|---|---|---|---|---|---|
| **Time Post-treatment** | | **2 h** | **24 h** | **48 h** | **72 h** |
| **Control** | *Mean* | 6.36 | 14.00 | 17.00 | 17.71 |
| | *SD* | 2.79 | 1.47 | 1.11 | 1.07 |
| **100 µM Neomycin** | *Mean* | 4.14 | 12.57 | 16.00 | 17.07 |
| | *SD* | 1.17 | 2.71 | 1.80 | 1.44 |
| **200 µM Neomycin** | *Mean* | 2.65 | 10.71 | 15.71 | 17.50 |
| | *SD* | 1.60 | 2.40 | 1.90 | 1.16 |
| **1 µM Copper** | *Mean* | 3.00 | 9.64 | 14.43 | 17.21 |
| | *SD* | 1.57 | 1.45 | 2.59 | 0.80 |
| **3 µM Copper** | *Mean* | 1.21 | 8.79 | 13.43 | 17.00 |
| | *SD* | 1.25 | 1.97 | 2.24 | 1.24 |
| **10 µM Copper** | *Mean* | 1.29 | 8.86 | 13.50 | 17.36 |
| | *SD* | 1.44 | 1.35 | 2.28 | 1.19 |

**Table 4.2: GFP+ and PHH3+ hair cell numbers after ototoxic damage.**
*N* = 8 per group. Control means with and without flubendazole treatment were used for normalization.

| | | ET4:GFP+ Cells | | PHH3+ Cells | |
|---|---|---|---|---|---|
| **Treatment (1 h)** | **Recovery** | *Mean* | *SD* | *Mean* | *SD* |
| **Control** | | 43.63 | 7.07 | 2.38 | 2.00 |
| **200 µM Neomycin** | **Control** | 14.38 | 1.51 | 0.25 | 0.71 |
| **10 µM Copper** | | 13.63 | 2.67 | 0.25 | 0.71 |
| **Control** | | 40.25 | 5.65 | 1.88 | 2.36 |
| **200 µM Neomycin** | **5 mM Flubendazole** | 8.13 | 2.17 | 1.75 | 0.89 |
| **10 µM Copper** | | 5.25 | 1.16 | 1.75 | 1.16 |

# CHAPTER 5: Hair Cell Regeneration Is Impaired by Loss of Prior Innervation

## 5.0 SUMMARY

Extensive research on nerve-dependent limb regeneration in amphibians (Brockes & Kumar, 2005; Carlson, 2007) suggested that innervation might also influence the regenerative capacity of lateral line hair cells. Transgenic fish containing an insertion of GFP within the *neurogenin-1* gene (*ET33-mi20* fish, referred to as $ngn^{33}$ fish; Kondrychyn *et al.*, 2009) were used to produce an aneurogenic naïve state in which lateral line hair cells develop normally without innervation. Other transgenic fish expressing GFP under control of the *neuroD* promoter (*NeuroD:GFP* fish; Obholzer *et al*., 2008) were used to produce a denervated state in which the lateral line nerve was labeled so the pLL could be axotomized by laser ablation. Aneurogenic and denervated larvae were treated with neomycin to kill lateral line hair cells at 5 dpf, and hair cell regeneration was subsequently observed over 72 to 96 hpt. Although aneurogenic fish exhibited normal regeneration indistinguishable from wild type siblings, denervated fish exhibited delayed regeneration. This delay was limited to those neuromasts that had lost innervation, but the pLL nerve also regenerated and re-innervated neuromasts rapidly. Neuromasts that remained denervated displayed significantly fewer hair cells when compared to neuromasts that were never denervated or that were recently re-innervated. These results suggest that lateral line hair cell regeneration is nerve-dependent but also that this dependence is an acquired trait. Additional experiments also examined the role of myelinating Schwann cells in promoting hair cell regeneration. The small molecule inhibitor PD 158780 was used to disrupt ErbB signaling necessary for differentiation of Schwann cells during embryogenesis, producing a naïve agliogenic state. PD 158780 was also used to silence the function of existing Schwann cells in fish with normal development. However, neither manipulation disrupted the rate of hair cell regeneration. These experiments suggest that innervation by the lateral line nerve, but not the association with nearby Schwann cells, is important for hair cell regeneration.

## 5.1 INTRODUCTION

Numerous studies using the salamander, newt, axolotl, and other amphibians have examined the role of innervation in tissue regeneration since the first report on this phenomenon almost two centuries ago (Todd, 1823). Amphibians have a remarkable capacity for tissue

regeneration, but transecting the spinal nerves at the base of the limb and distal to the amputation site prevents tissue regeneration, indicating that regeneration of the lost limb is nerve-dependent (Singer, 1952). Even more interesting is that when the nerve is previously ablated during early development prior to innervating the limb, tissue growth can occur normally without innervation. This aneurogenic limb will regenerate when amputated, unlike the denervated limb. These results demonstrate that limb regeneration is nerve-dependent but also that this is an acquired characteristic itself dependent on innervation.

Marcus Singer and his colleagues made significant advances in our understanding of limb regeneration during the middle of the twentieth century. Nerve-dependent regeneration may be a misnomer as the functions normally associated with the nervous system, such as the distinction between efferent and afferent innervation and the ability of neurons to conduct electrical impulses and release neurotransmitters, appear unimportant. Selective ablation of the motor or sensory nerves did not inhibit limb regeneration (Sidman & Singer, 1951), indicating that it was innervation itself and not the type of innervation that was required for regeneration. Furthermore, blocking nerve activity in an amputated limb by multiple injections of botulinum toxin did not inhibit regeneration, indicating that nerve conduction and neurotransmitter release were not required (Drachman & Singer, 1971).

Regeneration of an amputated amphibian limb is a form of epimorphic regeneration. The wound site forms a blastema of proliferative cells that express pattern formation genes (*e.g.*, members of the Hox family) just as in the original development of the limb (Kragl *et al.*, 2009). Missing bone, vasculature, musculature, and so forth are produced to create a fully functioning limb relatively indistinguishable from what was lost. Recent studies in the newt have suggested that anterior gradient protein (AG) functions as a trophic factor to promote limb regeneration and is secreted by myelinating Schwann cells (Kumar *et al.*, 2007), explaining how tissue regeneration can be nerve-dependent without requiring nerve function. Other proteins, such as FGF (Mullen *et al.*, 1996), neuregulin (Brockes & Kintner, 1986; Wang *et al.*, 2000), transferrin (Mescher *et al.*, 1997), and substance P (Globus *et al.*, 1991), have also been implicated in limb regeneration, and FGFs were previously discussed in Chapter 1 in the context of development and regeneration of mechanosensory hair cells.

Previous chapters of this dissertation described a common mechanism for hair cell regeneration in the zebrafish lateral line, specifically the role of dividing progenitors at the

exclusion of transdifferentiation. Those experiments did not, however, demonstrate what signals induce regeneration after hair cells are lost. Given the proximity of the lateral line nerve and its associated glia as well as the supporting evidence from earlier studies in amphibians, I explored the possibility that innervation may serve a role in hair cell regeneration. In this chapter, I describe experiments using aneurogenic transgenic fish that develop lateral line hair cells without innervation and also denervated transgenic fish in which the lateral line nerve has been labeled with GFP to facilitate laser axotomy. I treated aneurogenic and denervated fish with neomycin to kill lateral line hair cells and determine if hair cell regeneration is nerve-dependent in a manner similar to the studies on limb regeneration in amphibians. I also performed similar experiments on fish treated with the small molecule inhibitor PD 158780, which interferes with the binding of neuregulin to the ErbB receptor, to disrupt the development of myelinating Schwann cells or to inhibit the function of existing Schwann cells. Analogous to experiments testing the role of innervation, this experimental design allowed me to test two treatment conditions in which the organism was either naïve to pre-existing Schwann cells or in which Schwann cells had developed normally but were functionally silent.

Aneurogenic fish regenerated hair cells normally and were indistinguishable from wild type siblings, but denervated fish exhibited delayed hair cell regeneration. Hair cell regeneration appeared linked to regeneration of the pLL nerve, and those neuromasts that became re-innervated also regenerated lost hair cells more rapidly than those that still lacked innervation. In contrast, Schwann cells did not appear to have a direct role in hair cell regeneration. Preventing Schwann cell differentiation during early embryogenesis or inhibiting Schwann cell function during regeneration did not impair hair cell regeneration. There may, however, be indirect effects of combining PD 158780 treatment with pLL axotomy. Preliminary evidence suggested in some cases that absence of Schwann cells might impair clearance of the axotomized nerve or impair nerve regeneration given the reduction in axon guidance cues. However, regeneration of hair cells still appeared linked to the presence or absence of the pLL nerve.

## 5.2 RESULTS

### Hair cell regeneration is not disrupted in aneurogenic fish

To determine if innervation by the pLL nerve is required for lateral line hair cell regeneration, I used *ngn*$^{33}$ transgenic zebrafish in which a gene encoding GFP has been inserted

in the existing coding region for *neurogenin-1*. Neurogenin-1 is a basic helix-loop-helix (bHLH) transcription factor that is required for neuronal differentiation (Ma *et al.*, 1996). Homozygous mutants will express GFP in neuronal precursors that become unable to differentiate, and the absence of the pLL nerve removes a necessary anchor for migrating neural crest cells that would differentiate into myelinating Schwann cells (Gilmour *et al.*, 2002). Because glia normally inhibit neuromast formation by interneuromast cells, ectopic neuromasts form between the stereotypic, named neuromasts deposited by the migrating primordia (López-Schier & Hudspeth, 2005). The number of neuromasts present in mutant larvae was approximately 2- to 3-fold greater than in wild type siblings. Both heterozygous and homozygous mutants will express GFP, but only homozygous mutants will fail to generate the pLL nerve and can be identified by pre-screening larvae stained with FM 1-43FX to label supernumerary neuromasts. Wild type siblings were used as a negative control.

Mutant and sibling *ngn*$^{33}$ larvae were treated with neomycin at 5 dpf to kill mature hair cells and then collected and fixed at 2, 24, 48, and 72 hpt. Larvae were labeled with antibodies against parvalbumin before performing hair cell counts of neuromasts L1 through L5, which are deposited by the first pLL primordium in wild type siblings. In mutants with supernumerary neuromasts, hair cell counts were performed on five neuromasts in approximately similar locations along the pLL. Neuromasts in the PLL have approximately 8-12 hair cells each, and similar, robust regeneration was observed in both mutant and sibling larvae over 72 h (Figure 5.1A). One-way ANOVA identified no significant effect of genotype on mean hair cell counts per neuromast at any time point, and the presence of supernumerary neuromasts did not appear to affect the number of hair cells per neuromast.

I confirmed that regeneration was unaffected in aneurogenic larvae by performing hair cell counts in seven neuromasts of the aLL. The number of hair cells per neuromast is more variable in the aLL, and supernumerary neuromasts do not form. As with the pLL, I treated 5 dpf larvae with neomycin and measured the average number of hair cells per neuromast. One-way ANOVA identified no significant effect of genotype on total hair cell count per fish between mutant and sibling larvae at any time point, and regeneration was robust through 48 hpt when compared to untreated siblings and mutants (Figure 5.1B).

**Hair cell regeneration is delayed in denervated fish**

I next sought to determine whether the removal of pre-existing innervation would affect hair cell regeneration. Studies in amphibians have indicated that innervation sensitizes the surrounding tissue, establishing nerve-dependent regeneration that requires either afferent or efferent innervation (Sidman & Singer, 1951; Kumar *et al.*, 2011). I used transgenic *NeuroD:GFP* larvae that express GFP within the axon processes of the pLL, clearly visualizing the pLL ganglion, the projecting nerve, and the basket-like web of terminal processes that innervate hair cells in each neuromast (Obholzer *et al*., 2008). The nerve was axotomized by laser ablation between neuromasts L2 and L3 of the first pLL primordium, establishing a class of "upstream" neuromasts (L1 and L2) anterior or proximal to the ablation site and a class of "downstream" neuromasts (L3-L5) toward the posterior, distal to the ablation site. The posterior portion of the pLL nerve, no longer connected to the cell bodies, exhibited progressive degeneration toward the most posterior neuromasts. The nerve and terminal baskets were heavily fragmented within 4 h post-ablation. Hair cells were then killed with neomycin at 6 h post-ablation, after most nerve degeneration was complete, and larvae were collected and fixed for hair cell counts.

Abundant hair cell regeneration occurred in both groups. However, while regeneration was largely complete in upstream neuromasts by 72 hpt, downstream neuromasts exhibited significantly fewer hair cells per neuromast at the 48 and 72 h time points (Figure 5.2). Hair cell regeneration recovered in downstream neuromasts by 96 hpt, approaching the number observed in upstream neuromasts. This suggests that hair cell regeneration can occur in the denervated lateral line, unlike in the amphibian limb, but that regeneration remains significantly delayed.

**Regeneration of lateral line hair cells recovers after re-innervation**

Hair cell regeneration was significantly reduced at the 48 and 72 h time points when comparing upstream and downstream neuromasts. However, the pLL nerve also regenerated rapidly, and almost all neuromasts were re-innervated by 72 hpt (78 h post-ablation; Figure 5.3A). The timing of re-innervation coincided with the recovery of hair cell number in downstream neuromasts between 72 and 96 hpt, suggesting that as the pLL nerve regenerated along the tail, it facilitated hair cell regeneration in nearby neuromasts. To examine the interaction between re-innervation and hair cell regeneration, I distinguished between those

neuromasts that lost innervation and were re-innervated ("re-innervated") and those that lost innervation but were not re-innervated ("denervated"). These were compared to neuromasts on the opposite side of the fish, where the pLL nerve remained intact ("uncut"). Because of the rapid rate of nerve regeneration, hair cell regeneration was only examined at 48 hpt. Axotomy was performed immediately posterior to the L1 neuromast, and regeneration was observed in neuromasts L2-5 of the first primordium and in the first three neuromasts deposited by the second primordium.

Considerable hair cell regeneration occurred in uncut neuromasts that remained innervated on the opposite side of the fish, but approximately half as many hair cells were observed in denervated neuromasts where the nerve was earlier axotomized (Figure 5.3B). Re-innervated neuromasts exhibited an intermediate phenotype that suggested the rate of regeneration was initially very low, as in denervated neuromasts, but recovered after re-innervation. Still, significantly fewer hair cells were observed in both denervated and re-innervated neuromasts when compared to uncut control neuromasts. These results confirm that lateral line innervation facilitates hair cell regeneration and that restoring the connection between the pLL nerve and its associated neuromasts permits recovery from an initial regeneration deficit.

**Hair cell regeneration is unaffected by the absence of Schwann cells**

I next examined the possibility that associated Schwann cells, which provide myelination and axon guidance cues to the pLL nerve, might also be important in facilitating regeneration of lateral line hair cells. Furthermore, Schwann cells might secrete various trophic factors that promote regeneration, similar to evidence that implicates their importance in regeneration of the amphibian limb (Kumar *et al.*, 2007). To examine the effects of Schwann cells on regeneration, I used the small molecule inhibitor PD 158780, which blocks the binding of neuregulin to the ErbB receptor (Fry *et al.*, 1997). This interaction is essential for differentiation of migrating neural crest cells into Schwann cells (Lyons *et al.*, 2005) and is also required for the proper function of existing Schwann cells. Two readily apparent phenotypes result in the absence of pLL myelination. First, additional neuromasts are formed as in transgenic *ngn*$^{33}$ mutants as described earlier. Second, Schwann cells are required for axon guidance and fasciculation (Gilmour *et al.*, 2002; Villegas *et al.*, 2012). In their absence, the migrating pLL nerve travels a

circuitous path, and individual axons may detour before returning to the main nerve (Figure 5.4A).

To prevent the differentiation of Schwann cells in the lateral line, zebrafish were treated with PD 158780 from 18 to 72 hpf during early embryogenesis. To confirm the success of this treatment, *NeuroD:GFP* and *Sox10:Eos* adults were crossed to produce doubly transgenic larvae. The *Sox10:Eos* transgene expresses Eos in Schwann cells and other cell types derived from the neural crest (Prendergast *et al.*, 2012). Exposure to ultraviolet light source will photoconvert Eos from green to red. No red Eos-positive cells were observed along the pLL nerve in *NeuroD:GFP;Sox10:Eos* fish treated with PD 158780 during embryogenesis, in contrast to several Schwann cells identified in untreated fish, confirming that these conditions blocked Schwann cell differentiation (Figure 5.4A). Additional neuromasts were also present as expected in the absence of Schwann cells, similar to $ngn^{33}$ mutant fish.

*NeuroD:GFP* fish were treated with PD 158780 during embryogenesis and then with neomycin at 5 dpf to observe if the absence of Scwhann cells in these "agliogenic" fish disrupted hair cell regeneration. Similar to $ngn^{33}$ larvae, which lack not only the pLL nerve but also Schwann cells, no defect in hair cell regeneration was observed (Figure 5.4B) Significant hair cell addition occurred between 2 and 72 hpt in those fish with and without exposure to PD 158780, and these hair cell counts closely resembled those in $ngn^{33}$ mutants and wild type siblings.

When PD 158780-treated fish were axotomized to examine the effects of combining both treatments, several larvae failed to regenerate the majority of the pLL nerve after axotomy and neomycin treatment, confirming a recent report that Schwann cells are required for nerve regeneration (Villegas *et al.*, 2012). Initial development of the pLL nerve and its ability to innervate pLL neuromasts remained intact despite some disorganization, perhaps because the nerve grows with the extension of the tail. However, there appeared to be insufficient guidance cues to facilitate subsequent regeneration. Typically two or three neuromasts were re-innervated immediately posterior to the ablation site. The remaining neuromasts were not re-innervated by the pLL nerve, which did not regenerate. In some fish, denervated neuromasts retained the basket of terminal neurites that synapse with the hair cells, suggesting that axon degeneration was incomplete. This is consistent with reports that Schwann cells are required to facilitate removal of cell debris by recruiting macrophages (Griffin *et al.*, 1992; Banner & Patterson, 1994;

Shamash *et al.*, 2002). When terminal processes were present, they appeared to facilitate hair cell regeneration even in the absence of a functioning nerve, but these findings remain preliminary.

**Inhibition of Schwann cell function does not disrupt hair cell regeneration**

Hair cell regeneration was unaffected when Schwann cells were not present. However, if Schwann cells do differentiate, they might sensitize neuromasts and be required for later hair cell regeneration. This distinction is analogous to the role of innervation in establishing subsequent nerve-dependent limb regeneration in amphibians. To examine the possibility that continued Schwann cell function is required for hair cell regeneration, *NeuroD:GFP* transgenic larvae were treated at 5 dpf with neomycin and then incubated in PD 158780 from 0 to 72 hpt. The number of regenerated hair cells in treated and untreated fish was very similar (Figure 5.5A), indicating that PD 158780 did not affect hair cell regeneration. In addition, a small number of ectopic neuromasts were observed and served as evidence that PD 158780 successfully inactivated some elements of Schwann cell function, which is responsible for suppressing the ability of interneuromast cells to proliferate and differentiate into additional neuromasts.

*NeuroD:GFP* larvae were also treated with PD 158780 after axotomy and neomycin treatment to determine if the combination of silencing Schwann cell function after pLL denervation has a compound effect on hair cell regeneration. These larvae displayed no obvious defects in nerve regeneration, and the numbers of regenerated hair cells in re-innervated and denervated neuromasts at 72 h post-neomycin were similar to those observed in axotomized larvae not treated with PD 158780 (Figure 5.5B). In addition, a small number of ecotopic neuromasts were observed and confirmed the effectiveness of the inhibitor at silencing Schwann cell function.

## 5.3 DISCUSSION

Innervation is required for hair cells to function properly and convey their information about the environment to the central nervous system. However, most research on regeneration in the lateral line has focused on hair cells and not the associated nerve. A recent report by Villegas *et al.* (2012) characterized regeneration of the pLL nerve and confirmed many of the findings in this dissertation and those reported elsewhere. Schwann cells are essential for axon guidance

during initial development and subsequent regeneration. If Schwann cells are absent from the nerve, it becomes defasciculated and additional ectopic neuromasts form.

It is particularly interesting that despite the obvious need for innervation in lateral line hair cell function, it is not necessarily required for the formation of these cells. Using *ngn*$^{33}$ mutants and subsequent experiments that inhibited differentiation of Schwann cells, I found that neurons and glia are both dispensable for hair cell development and regeneration in naïve states in which the neurons or glia are prevented from forming. However, these manipulations do have long-term consequences on animal survival. Larval *ngn*$^{33}$ homozygous mutants do not mature into adults, and PD 158780 sometimes has lethal side effects during embryogenesis.

When the pLL nerve was axotomized by laser ablation and subsequently degenerated, hair cell regeneration was significantly impaired. This conflicts with another report that found little or no effect of denervation on lateral line hair cells (Villegas *et al.*, 2012). However, in the present study, the only significant difference between neuromasts that retained or lost innervation was observed between 48 and 72 h after neomycin treatment. The pLL nerve also regenerated quickly, and by 72 hpt most or all of these neuromasts were re-innervated. Subsequent experiments demonstrated that re-innervated neuromasts regenerated hair cells at a faster rate than those that remained denervated. A defect in hair cell regeneration could be overlooked depending on how neuromasts are classified and when hair cell counts are recorded.

The delay in hair cell regeneration linked to regeneration of the pLL nerve indicates that innervation facilitates or may be required for complete regeneration of lateral line hair cells. In contrast, the apparent lack of effect on hair cell regeneration when Schwann cells were absent or inactivated indicates that these do not serve an important role in regulating proliferation and differentiation of nearby support cells. These findings contrast with recent studies in the newt that have suggested anterior gradient protein (AG) functions as a trophic factor to promote amphibian limb regeneration and is secreted by myelinating Schwann cells (Kumar *et al.*, 2007). Furthermore, expression of AG protein is downregulated in the developing limb after innervation (Kumar *et al.*, 2011). These observations suggest a molecular basis for nerve-dependent limb regeneration in amphibians and explain why regeneration is unaffected in aneurogenic limbs. Although a similar mechanism may exist in the zebrafish lateral line, it appears to be regulated by the nerve itself.

The lateral line nerve most likely interacts with the support cells of each neuromast to promote their proliferation and differentiation into new hair cells. It is possible that the terminal synapses of the pLL nerve secret a trophic factor that stabilizes newly formed hair cells, but I observed no evidence that hair cells formed only to immediately die in the absence of innervation. If the pLL nerve does communicate with support cells, failure to regenerate the nerve might permanently inhibit hair cell regeneration. This was difficult to test because of the rapid rate of nerve regeneration. Although PD 158780-treated larvae appeared to have some defects in nerve regeneration, there are superior approaches to examining this question. Laser ablation cannot penetrate deeply enough to damage the pLL ganglion, but other options include microsurgical removal or a chemical lesion with hydrogen peroxide.

As an alternative or in addition to releasing trophic factors that promote regeneration, the pLL nerve might release an inhibitory factor after axotomy that negatively impacts hair cell survival and replacement. Villegas *et al.* (2012) found no definite evidence that hair cell number was affected by denervation alone, and my own experiments were similarly inconclusive. However, it seems unlikely that the damaged nerve would be able to inhibit hair cell regeneration over the long term and that regeneration of the nerve somehow relieves this inhibition. Some hair cell addition was observed during the first 48 h after neomycin treatment even in denervated neuromasts. While an inhibitory factor might be released immediately after nerve degeneration, it would likely dissipate within hours rather than days.

This project draws inspiration from seminal studies of amphibian limb regeneration, but there is limited opportunity for direct comparison. Nerve regeneration in the zebrafish is extremely rapid but does not occur in the denervated amphibian limb. In addition, the zebrafish tail was not amputated, and only the sensory hair cells were damaged. However, these experiments are consistent in demonstrating that innervation serves an important role in the regeneration of surrounding tissue. The evidence reported here that denervation significantly delays lateral line hair cell regeneration might ultimately lead to a new understanding of how hair cell regeneration is regulated by surrounding tissue.

## 5.4 FIGURES AND LEGENDS

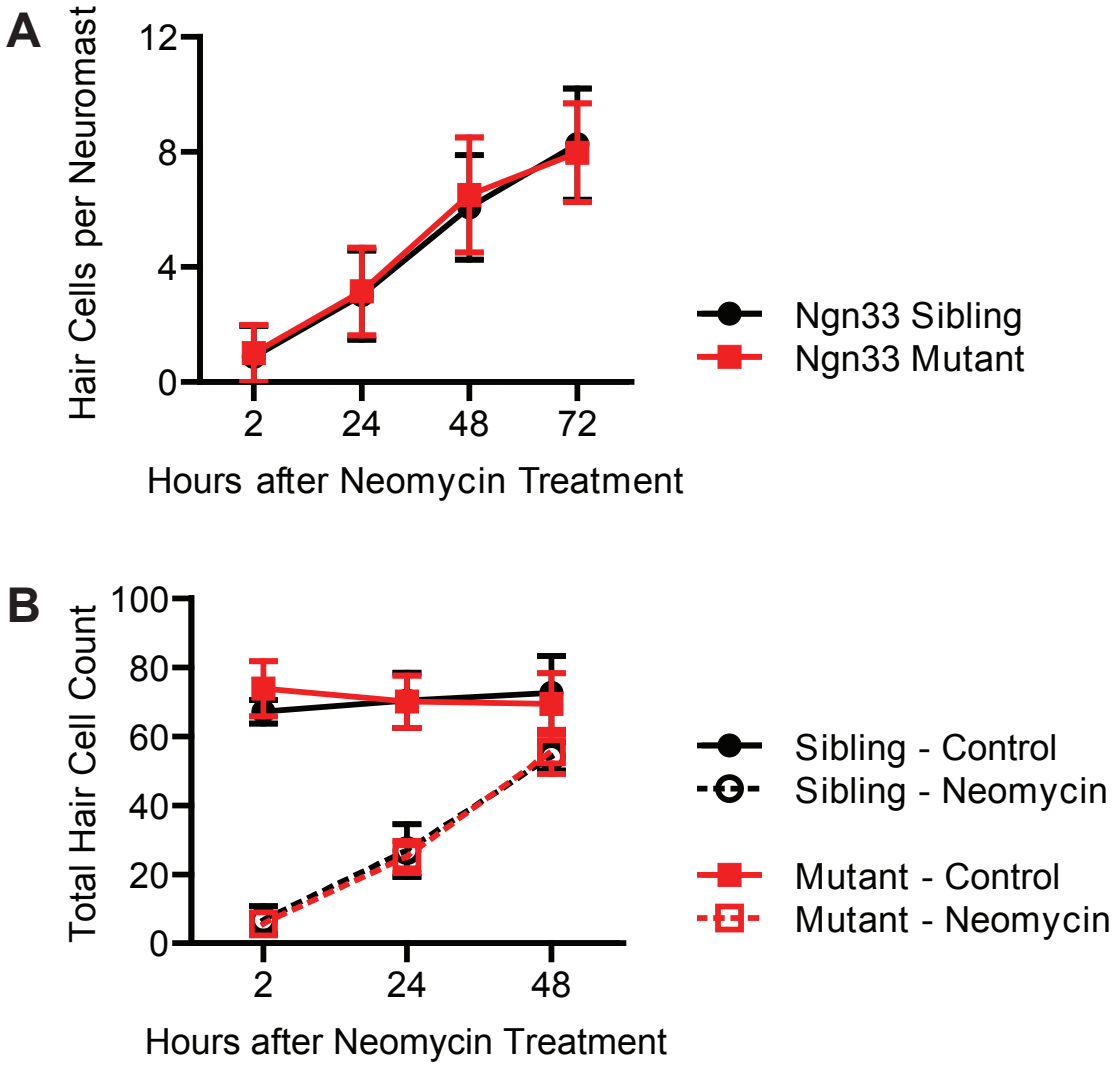


**Figure 5.1: Lateral line hair cell regeneration is unaffected in aneurogenic larvae**
Homozygous *ngn*$^{33}$ larvae fail to form the pLL nerve during development. Mutants lacking innervation continue to develop hair cells normally. **(A)** After 5 dpf larvae were treated with neomycin, regeneration was unaffected in mutant larvae, which had equal numbers of hair cells per neuromast when compared to siblings at all time points. **(B)** To confirm that regeneration was unaffected, regeneration was measured in 7 neuromasts in the aLL and compared to EM-treated controls. Again, hair cell numbers were comparable between mutant and sibling larvae in both control and neomycin-treated groups. *N* = 8 fish per group. Error bars are +/- SD.

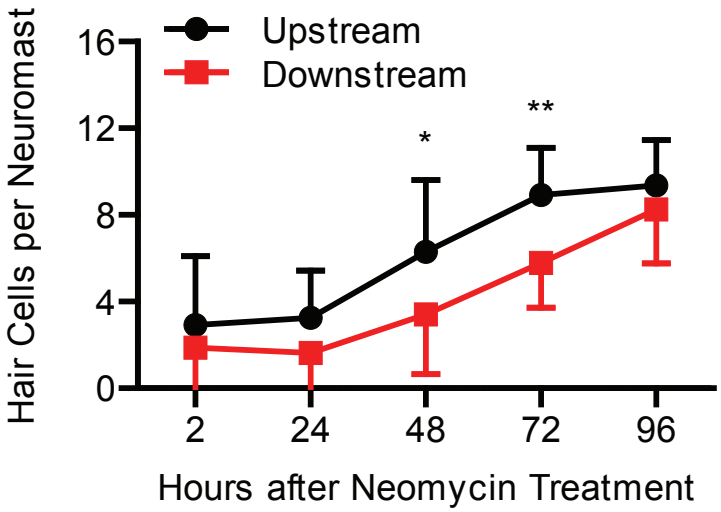


**Figure 5.2: Hair cell regeneration is delayed in denervated neuromasts.**
Transgenic *NeuroD:GFP* larvae were axotomized by using a laser to ablate the pLL nerve between neuromasts L2 and L3 in the first primordium. Fish were then treated with neomycin to kill hair cells 4 h later, after degeneration of the unconnected nerve fragment. Hair cells regenerated within 72 hours in "upstream" neuromasts that retained innervation, but hair cell regeneration was significantly impaired among "downstream" neuromasts where innervation was lost (*, $p < 0.05$; **, $p < 0.01$). $N = 8$ fish per group. Error bars are +/- SD.

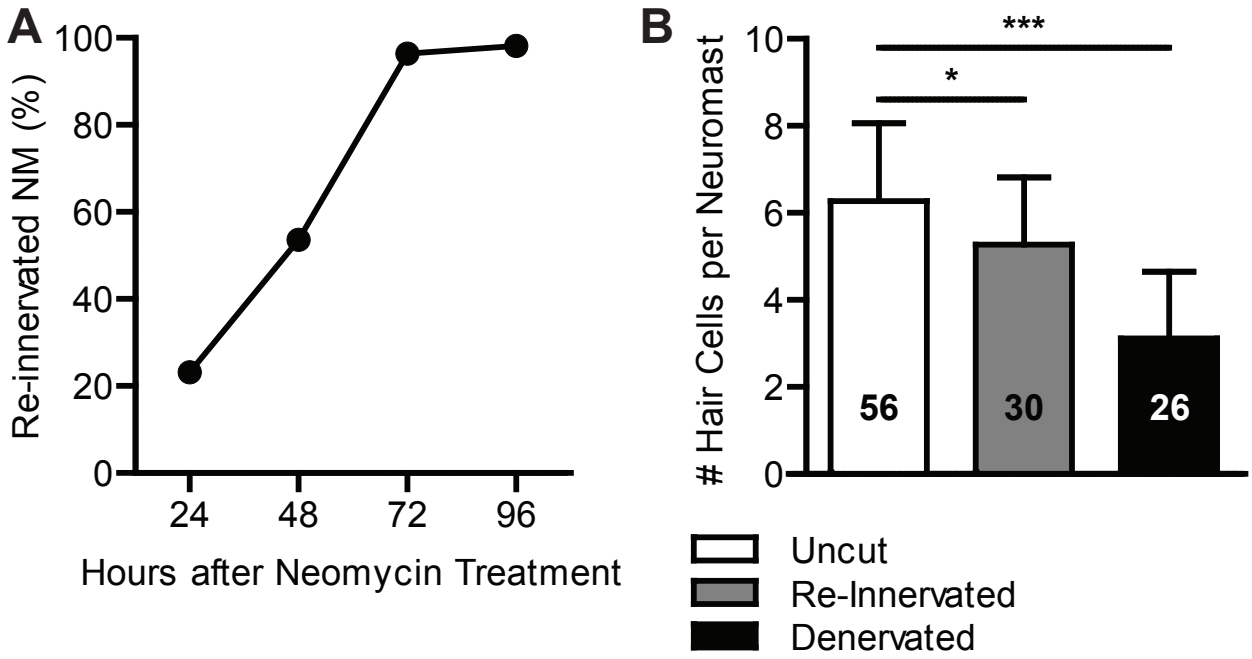


**Figure 5.3: Hair cell regeneration coincides with re-innervation**
*NeuroD:GFP* larvae were axotomized at 5 dpf posterior to the L1 neuromast on one side, followed 6 h later by treatment with neomycin. Hair cell regeneration was measured on both sides of the fish. **(A)** Neuromasts on the cut side were re-innervated rapidly, and nearly all neuromasts were successfully re-innervated by 72 h after neomycin treatment (78 h post-ablation). **(B)** When measured at 48 hpt, hair cell regeneration was greatest in neuromasts that retained innervation on the uncut side of the fish. Regeneration was significantly impaired on the cut side of the fish, both in denervated neuromasts and in re-innervated neuromasts, suggesting that hair cell regeneration is linked to nerve regeneration. There was a small but significant difference hair cell number between neuromasts that were uncut and those that were cut but re-innervated (*, $p < 0.05$). Regeneration was significantly reduced in neuromasts that remained denervated (***, $p < 0.001$). $N$ = number of neuromasts as indicated. Error bars are + SD.

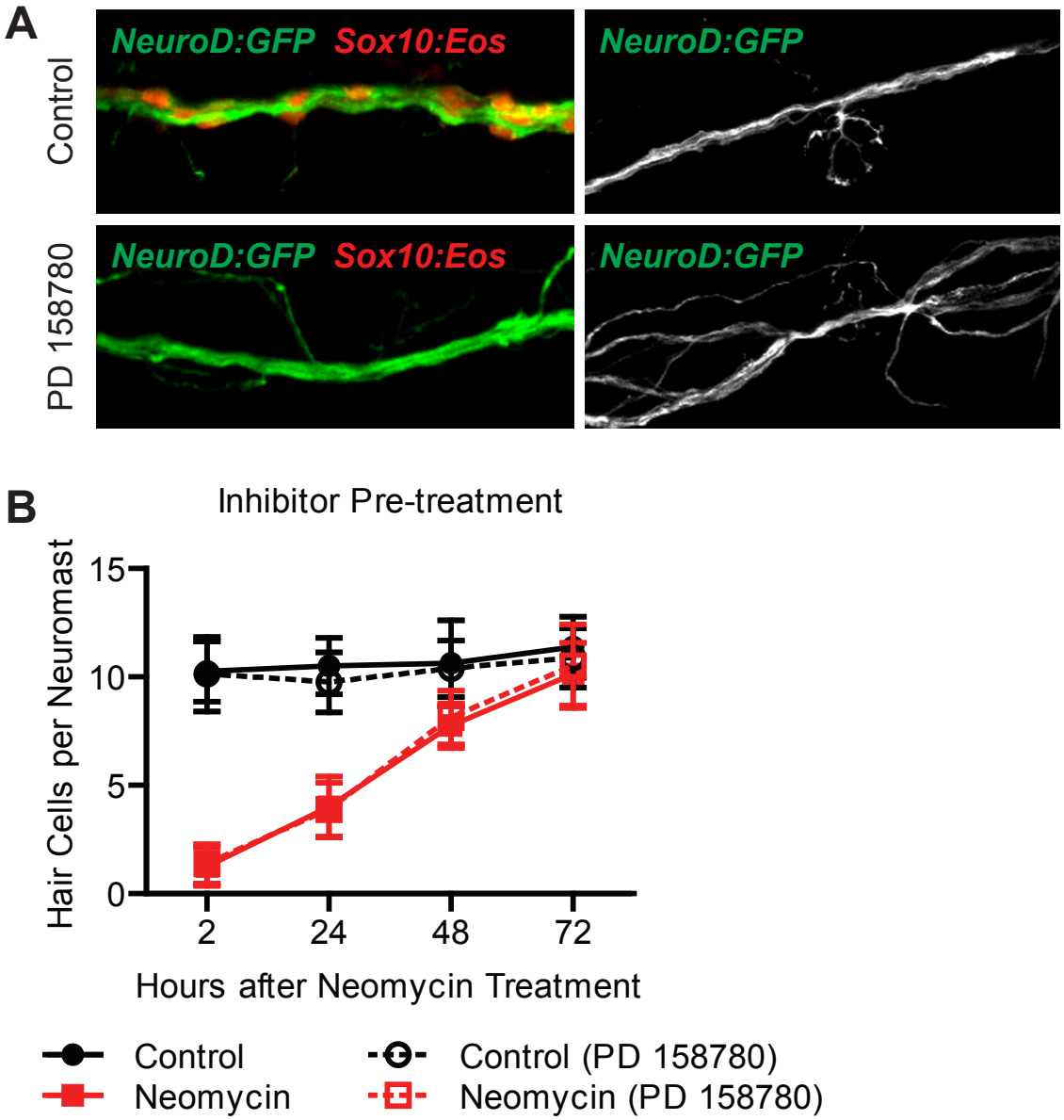


**Figure 5.4: Inhibiting ErbB disrupts pLL nerve formation but not hair cell regeneration.** Larvae were treated with the ErbB inhibitor PD 158780 to prevent formation of Schwann cells. **(A)** Transgenic *NeuroD:GFP;Sox10:Eos* larvae were used to demonstrate the effectiveness of PD 158780 treatment during embryogenesis. No Schwann cells were observed at 5 dpf, and the pLL nerve exhibited defasciculation. **(B)** When PD 158780-exposed larvae were treated with neomycin to kill hair cells, regeneration was unaffected compared to untreated controls. $N = 8$ fish per group. Error bars are +/- SD.

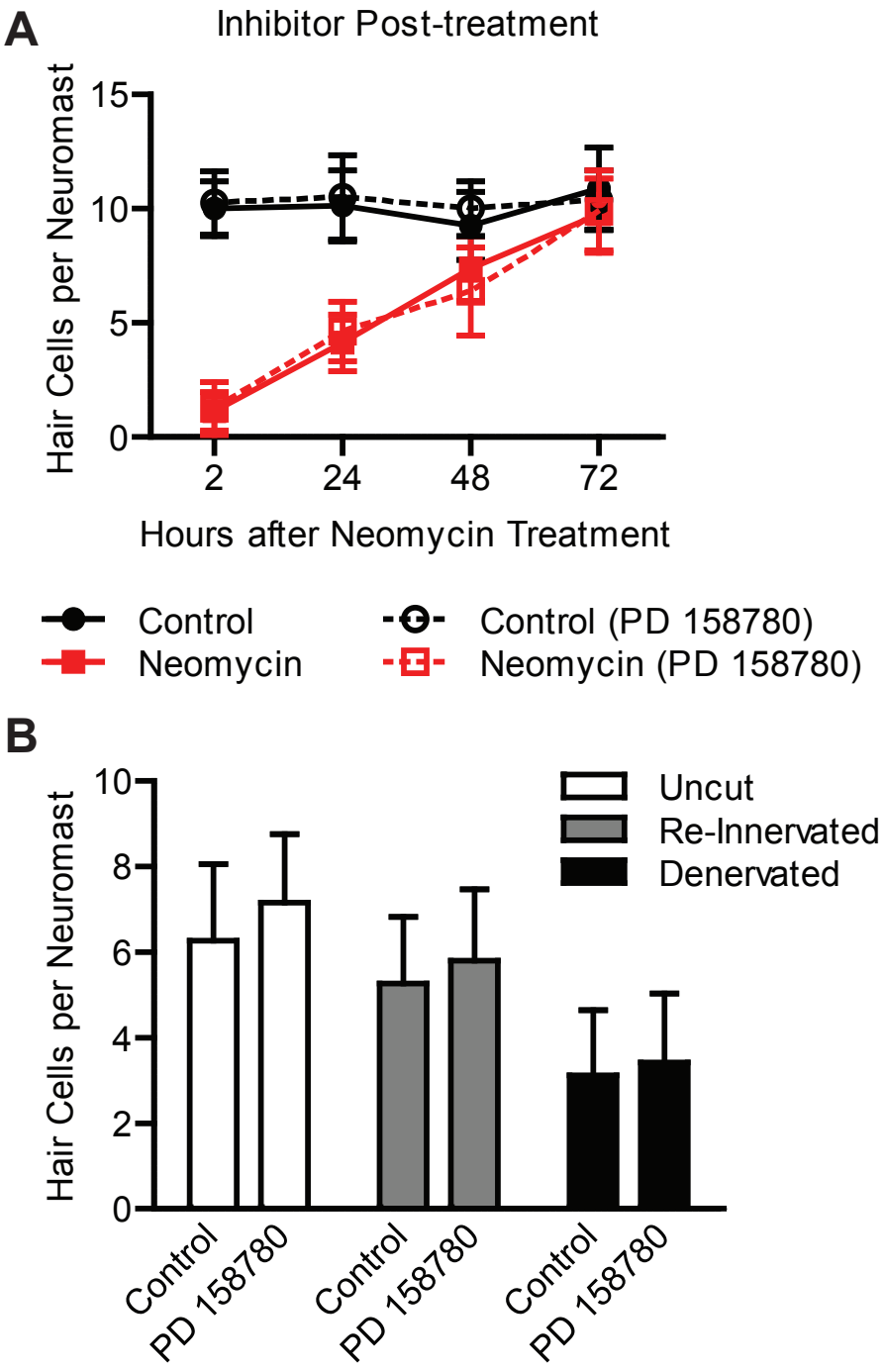


**Figure 5.5: Silencing Schwann cell function does not affect hair cell regeneration.**
**(A)** When *NeuroD:GFP* larvae were treated with PD 158780 during recovery after neomycin, hair cell regeneration was unaffected compared to untreated controls. **(B)** In addition, the combination of pLL axotomy and post-neomycin treatment with PD 158780 did not affect hair cell regeneration at 48 hpt. These experiments suggest that Schwann cell function is not directly required for hair cell regeneration and does have a compound effect with axotomy. $N = 8$ fish per group. Error bars are +/- SD.

# CHAPTER 6: Conclusions and Future Directions

## 6.0 OVERVIEW

In this dissertation, I have described my use of the zebrafish lateral line as a model system to study the regeneration of mechanosensory hair cells after damage by a variety of ototoxins. This has included examination of the mechanism of regeneration, whether it occurs by the division of hair cell progenitors or by their direct transdifferentiation, as well as whether regeneration of hair cells is influenced by the nearby lateral line nerve and associated Schwann cells. In this final chapter, I summarize the data and analysis presented in previous chapters and relate them to the current model of mechanosensory hair cell regeneration. I also discuss future studies that may expand on the results to provide greater understanding of how hair cell regeneration occurs in fish and other vertebrates.

## 6.1 SUMMARY AND CONCLUSIONS

There was no conclusive evidence that progenitors undergo direct transdifferentiation into new hair cells in the zebrafish lateral line. Instead, new hair cells exhibited BrdU-positive nuclei, a marker of recent cell division, and regeneration was largely blocked by treatment with flubendazole, an inhibitor of microtubule assembly and cell division. These findings are consistent with the observations of several previous reports that have used markers of recent cell proliferation (Harris *et al.*, 2003; Ma *et al.*, 2008; Namdaran *et al.*, 2012) and inhibition of cell proliferation (Wibowo *et al.*, 2011; Namdaran *et al.*, 2012). I have further examined the potential for direct transdifferentiation in non-conventional treatment paradigms, such as regeneration in larvae with underdeveloped lateral lines, treatment with different ototoxins, and variations in the duration or concentration of these ototoxins. These multiple conditions resulted in similar conclusions that proliferative regeneration is necessary for hair cell replacement.

Although a small increase in hair cell number was observed during regeneration in the presence of flubendazole, this provides weak evidence of transdifferentiation. Post-mitotic hair cell precursors, which exist in limited numbers, could resist ototoxic damage and continue to mature. This explanation could also be applied to an earlier report suggesting possible non-mitotic hair cell replacement in 3 dpf zebrafish embryos (Hernández *et al.*, 2007), which have a larger number of immature hair cells. Other investigators have found that when larvae are pre-

treated with BrdU before and after ototoxic damage, almost all replacement hair cells exhibit this proliferation marker (Wibowo *et al.*, 2011). This is because BrdU will incorporate into the small number of progenitors that are already dividing either to increase hair cell number or for regular hair cell turnover.

Although a limited amount of transdifferentiation may in fact occur, I suspect this is a rare occurrence and not a common phenomenon relied upon for regeneration of the lateral line. I found that when proliferation was prevented with the mitotic inhibitor flubendazole, cell division was arrested in progenitor cells, and additional progenitors did not accumulate. Others have reported that progenitor cells form individually after division of the previous progenitor, and that these progenitors form in regions of low Notch activity (Wibowo *et al.*, 2011). Though an errant progenitor might undergo transdifferentiation, the limited rate of new progenitor formation would appear to limit the potential usefulness of this approach for hair cell regeneration on a larger scale. These findings indicate a system particularly sensitive to the rate of new hair cell production, which may be related to one or more signals that regulate the number of regenerated hair cells.

Excess hair cells can result when the sensory epithelia in zebrafish (Ma *et al.*, 2008) and birds (Daudet *et al.*, 2009; Lin *et al.*, 2011; Zhao *et al.*, 2011) are treated with DAPT to inhibit Notch signaling. DAPT increases the number of hair cells produced through proliferative as well as non-proliferative mechanisms (Daudet *et al.*, 2009), so it remains unclear if certain regulatory genes or proteins can be used to bias this ratio of regeneration in organisms capable of both mechanisms. Still, evidence from birds suggests that a mix of proliferative and non-proliferative regeneration affords significant flexibility to the regenerating sensory epithelium. When proliferative regeneration is inhibited in the basilar papilla, significant regeneration still occurs through transdifferentiation (Shang *et al.*, 2010). The inability of lateral line hair cells to regenerate in the presence of flubendazole indicates that blocking proliferation is not sufficient in itself to promote non-mitotic regeneration. There may be additional regulatory genes activated during cell division that contribute to hair cell differentiation in the zebrafish lateral line.

Although treatment with different ototoxins did not favor alternate mechanisms of hair cell regeneration, they did have obvious effects on the rates of hair cell death and regeneration. Lateral line hair cell damage has been studied previously using all of the ototoxins described in this dissertation, including neomycin, gentamicin, copper, and cisplatin. However, there are no

studies that compared subsequent hair cell regeneration using roughly similar treatment conditions. Regeneration after all treatments with aminoglycoside antibiotics, including either acute or chronic gentamicin, required approximately 72 h after hair cell death was complete. By contrast, copper treatments had a variable effect on regeneration influenced by the duration and concentration of exposure. Although all conditions used in these experiments restored at least 50% of lost hair cells, higher concentrations have also been reported to impair or completely block regeneration (Hernández *et al.*, 2006; Linbo *et al.*, 2006).

This dissertation also demonstrated that hair cells do regenerate in zebrafish after treatment with cisplatin, which was found to block regeneration in the chicken basilar papilla (Slattery & Warchol, 2010). However, the regeneration observed in zebrafish was significantly delayed. Delayed hair cell regeneration after treatment with copper or cisplatin indicates that damage to surrounding non-sensory cell types may be a serious impediment to hair cell regeneration, with implications for other vertebrates, including mammals. Evidence that the lateral line remains capable of regenerating even under these extenuating circumstances suggests that either zebrafish possess a robust repair mechanism to correct cisplatin-induced damage or that the amount of cisplatin used in these experiments was insufficient to block proliferation in all support cells. Higher concentrations of cisplatin might block hair cell regeneration similar to the reported effects of high concentrations of copper (Hernández *et al.*, 2006). Despite this, if a novel repair mechanism does exist in the lateral line, it may provide a new area of investigation for developing regeneration therapies in humans.

Finally, this dissertation project is among the first studies of the role served by innervation in regeneration of lateral line hair cells. A previous report observed that the lateral line nerve is not required for hair cell regeneration in aneurogenic fish (López-Schier & Hudspeth, 2005), and a very recent study of pLL nerve regeneration briefly examined its effects on hair cells (Villegas *et al.*, 2012). This dissertation directly compared regeneration in aneurogenic *ngn*$^{33}$ mutants as well as transgenic *NeuroD:GFP* larvae in which the pLL nerve was damaged. Hair cell regeneration was significantly delayed only in the denervated preparation, indicating that this is a nerve-dependent phenomenon sensitized by prior innervation. Villegas *et al.*. (2012) did not observe a similar effect on hair cell regeneration, but it is possible this was overlooked by their experimental design. Evidence reported here found a significant delay was only apparent between 24 and 48 h after neomycin treatment. The effect

was more pronounced when examining only neuromasts that had not yet been re-innervated by the regenerating pLL nerve.

Furthermore, I observed that Schwann cells are not required for hair cell regeneration. This contrasts with studies of nerve-dependent limb regeneration in amphibians, which is mediated by anterior gradient (AG) protein derived from Schwann and gland cells (Kumar *et al.*, 2007). There was some evidence that nerve degeneration was compromised in the absence of Schwann cells, which recruit macrophages to facilitate debris removal (Perry & Brown, 1992), although this mechanism has recently been disputed in motor axons (Rosenberg *et al.*, 2012). Failure to eliminate the prior nerve could prevent its subsequent regeneration, which should be explored in future experiments. In some fish, basket-like nerve terminals continued to innervate neuromasts even though they were not connected to the pLL nerve, and hair cell regeneration in these fish appeared comparable to that in wild type *AB and mutant *ngn*$^{33}$ larvae. It is possible that AG or some other trophic factor is expressed by the nerve itself or even by support cells as a consequence of cell-cell interaction between neuromasts and the innervating neurons.

## 6.2 FUTURE DIRECTIONS

### Support cell fate decisions and cell identities

A significant portion of this dissertation has referred to the importance of non-sensory support cells in hair cell regeneration. Support cells give rise to new hair cells through proliferative regeneration in the lateral line and also through non-proliferative transdifferentiation in other non-mammalian vertebrates. Certain ototoxins significantly impair or delay hair cell regeneration due to their non-specific effects on support cells in zebrafish. Innervation serves an as yet undetermined role in promoting hair cell regeneration, perhaps through intercellular communication with support cells. However, support cells in the lateral line are vaguely defined and do not segregate into clearly defined subpopulations, partly due to a lack of cell-specific markers.

One of the few genes or proteins that differentiate support cells is the transcription factor Prox1, which is highly expressed in the developing chicken basilar papilla but becomes limited to a small number of support cells once this sensory epithelium matures and becomes largely quiescent (Stone *et al.*, 2004). Prox1 is upregulated in approximately 50% of support cells after hair cell damage, suggesting it is involved in specifying hair cell fate. Because not all support

cells express Prox1, there are likely other factors that predetermine which will contribute toward hair cell regeneration (Brignull *et al.*, 2009). Previous research by Ma *et al.*. (2008) in the Raible laboratory indicated the presence of two distinct subpopulations of support cells in the lateral line neuromasts. Internal support cells (iSCs) divide and differentiate to form new hair cells, and peripheral support cells (pSCs) renew the population of iSCs as well as replace themselves.

The distinction between iSCs and pSCs is based on cell location and morphology, and there is not a clear boundary between the two subpopulations. It is not known if all iSCs are capable of becoming hair cell progenitors or how cells migrate within and between the internal and peripheral regions. Recent evidence suggests that support cells take on a progenitor cell fate in polar compartments at opposing ends of each neuromast, and that all progenitor cells migrate to this region from other parts of the neuromast. Migration from other, undefined regions suggests that there may not be a niche of stem-like cells within the neuromast, or that these stem-like cells are widely distributed. Support cells might wander randomly, and those that happen to transit the polar compartments are selected to become new progenitors.

In the absence of specific markers for individual support cell subpopulations, I attempted to investigate these cell fate decisions indirectly with a targeted labeling approach. I created a stable transgenic line, *β-actin:NLS-Eos*, in which nuclear expression of the photoconvertible protein Eos is driven by the ubiquitous *β-actin* promoter. Any cell of interest, including support cells in different regions of the neuromast, can be labeled by photoconversion with an ultraviolet laser, turning green Eos into red, which is then stable for several days or weeks and clearly visible. However, this project proved technically challenging. It was difficult in many cases to clearly label individual support cells, which would be necessary to accurately track cell division and the migration of daughter cells.

Instead, I. Cruz in the Raible laboratory has used these fish to measure support cell turnover by photoconverting all support cells with solar ultraviolet radiation. All cells begin with approximately equal amounts of red Eos. Those cells that proliferate more frequently would replace this red Eos with new green Eos, and I. Cruz has identified localized polar regions that appear to represent slow cycling cells, one indicator of a stem cell niche (unpublished data). It may also be possible to return to the original objective of this experiment, to label and track individual cell nuclei, with in larvae injected with this construct at the single-cell stage. Stochastic expression of *β-actin:NLS-Eos* is likely to avoid accidental photoconversion of

adjacent cells, although this presents new challenges. Alternatively, two-photon microscopy may be used to provide more targeted photoactivation of individual cell nuclei.

**Characterizing nerve-dependent hair cell regeneration**

Hair cells possess afferent and efferent connections as described in Chapter 2, and these must be restored during hair cell regeneration. Experiments by A. Suli in the Raible laboratory have used rheotaxis (*i.e.*, orientation with the flow of water) to confirm that re-innervated hair cells are functional (Suli *et al.*, 2012). The angle of orientation can be determined by using computer-aided recognition of zebrafish larvae and comparing these images to the direction of water current in a flume. Larvae treated with neomycin to kill lateral line hair cells will align themselves in the direction of the current in ambient light but orient randomly under infrared illumination, indicating that hair cells are important for rheotaxis but also that the visual system can compensate in the absence of lateral line function. Rheotaxis gradually improves under infrared illumination during the course of hair cell regeneration, demonstrating that replaced hair cells are functional and contribute to behavior.

In Chapter 5, I demonstrated that the rate of hair cell regeneration appears to be nerve-dependent. Because the pLL nerve regrows so rapidly, it remains unclear if hair cells are replaced at a slower rate or not at all in neuromasts that remain denervated after axotomy. Current evidence indicates that hair cell regeneration occurs in denervated neuromasts but that innervation facilitates hair cell regeneration. There was a small increase in the number of hair cells in denervated neuromasts between 24 and 48 h after neomycin treatment, and preliminary evidence indicates that hair cell regeneration was unimpaired when nerve degeneration was incomplete in larvae pre-treated with PD 158780. Because Schwann cells were not present in PD 158780-treated fish, I suspect this putative trophic factor is released by the nerve itself or by surrounding support cells that respond to the nerve through intercellular signals. This disagrees with my initial hypothesis that lateral line regeneration may be very similar to regeneration of the amphibian limb. While both are nerve-dependent phenomena, the molecular mechanism in amphibians appears to rely on secretion of a trophic factor by the surrounding glia.

There has been significant evidence in amphibians suggesting that this glial-derived trophic factor is anterior gradient (AG) protein (Kumar *et al.*, 2007; Kumar *et al.*, 2010; Kumar *et al.*, 2011). AG protein expression is downregulated following innervation but increases again

after limb amputation (Kumar *et al.*, 2011). Although the expression of AG in zebrafish was not examined, at least two homologs have been identified for the AG receptor (Shih *et al.*, 2007). Whether non-glial cell types, such as support cells, might express AG in zebrafish is unknown, but expression of this or another trophic factor might be linked to intercellular signals between the nerve terminals and support cells. Such a mechanism could also help explain how hair cell regeneration is induced after damage. Loss of afferent signals from hair cells could result in feedback through efferent terminals to the surrounding support cells and stimulating the proliferation and differentiation of hair cell progenitors. Although it would be valuable to test the role of neural activity in hair cell regeneration, most transgenic and pharmacological approaches lack sufficient specificity and might also halt vital functions necessary for fish survival.

This dissertation relied on Wallerian nerve degeneration to examine the effects of denervation of hair cell regeneration. Wallerian degeneration describes the anterior-to-posterior gradient of nerve degeneration (Waller, 1849) observed after axotomy of the zebrafish lateral line and many other models of axon damage. Recent evidence from the laboratory of M. Granato indicates that motor axon degeneration in zebrafish is a prerequisite for successful regrowth (Rosenberg *et al.*, 2012). By expressing $Wld^S$, a dominant mutant allele originally discovered in mice (Lunn *et al.*, 1989), the axons of transgenic neurons will remain intact for multiple days in contrast to wild type axons that degenerate within hours (Rosenberg *et al.*, 2012). By expressing the $Wld^S$ allele in a subset of pLL neurons, it may be possible to compare hair cell regeneration between neuromasts that lose and restore innervation normally and those in which Wallerian degeneration and nerve regeneration is disrupted.

**Regeneration in adult and larval zebrafish**

The first lateral line hair cells mature at 3 dpf, but most neuromasts continue to add hair cells rapidly until 5 dpf. After this time, fewer new hair cells are formed, either through cell turnover, damage and regeneration, or through secondary growth as the lateral line expands to accommodate metamorphosis into adulthood (Ledent, 2002; Sapède *et al.*, 2002; Webb & Shirey, 2003). Most experiments on death and regeneration of lateral line hair cells have been performed in larvae up to 10 dpf (Williams & Holder, 2000; Harris *et al.*, 2003; Murakami *et al.*, 2003; López-Schier & Hudspeth, 2006; Santos *et al.*, 2006; Ou *et al.*, 2007; Ma *et al.*, 2008; Owens *et al.*, 2009; Ou *et al.*, 2010; Coffin *et al.*, 2010), and relatively little is understood about

whether or how hair cells respond differently to insults in older fish. Given that juvenile and adult zebrafish swim faster over greater distances and encounter more variable environments, they may be more likely to suffer physical damage, and their capability to regenerate lost hair cells is likely maintained.

Larval neuromasts are arranged around the head and along the tail, and their relatively low number and stereotypic placement make it easy to identify them. In contrast, adult neuromasts may form through budding of existing neuromasts into dorsoventral stiches, proliferation of interneuromast cells into new neuromasts, and deposition of protoneruomasts by additional migrating primordia. For example, the second pLL primordium closely follows the path of the first primordium, but neuromasts later migrate ventrally, changing not only their position relative to the horizontal myoseptum but also their polarity (López-Schier *et al.*, 2004). Whereas the primary neuromasts respond maximally along the anterior-posterior axis, secondary neuromasts respond to stimuli along the dorsal-ventral axis (Wibowo *et al.*, 2011). Finally, although adults retain the superficial neuromasts found in larvae, they also possess additional neuromasts within bony canals (Gompel *et al.*, 2001; Webb & Shirey, 2003). Unlike superficial neuromasts, which are small and round, canal neuromasts continue to growth, adding more hair cells and elongating during canal formation (Webb & Shirey, 2003). Differential aminoglycoside susceptibility between hair cells in superficial and canal neuromasts have been observed in the oscar (*Astronotus ocellatus*), in which a low dose of gentamicin was able to damage canal but not superficial neuromasts (Song *et al.*, 1995), but it is not known if a similar phenomenon occurs in zebrafish.

Experiments performed by I. Cruz in the Raible laboratory indicate that superficial neuromasts in the adult tail respond to neomycin in a similar manner as larval neuromasts. Hair cell death and regeneration in response to neomycin treatment occurs at the same concentrations and over the same time course of 72 h (unpublished data). In addition, adult fish are able to withstand multiple rounds of neomycin treatment, and the efficiency of hair cell regeneration does not diminish. Extrapolating from the number of support cells in a given neuromast, the pool of available progenitors should be exhausted after 8 to 10 cycles of neomycin-induced regeneration, but instead the numbers of support and hair cells in each neuromast remain relatively constant, indicating that support cells are capable of indefinite self-renewal (unpublished data).

**Screens for regulators of hair cell death and regeneration**

The zebrafish is an ideal model for high-throughput screens due to its small size, high fecundity, and stereotypic development. Forward genetic screens have used mutagenesis to successfully identify genes that are important in development of the inner ear and the lateral line (Granato *et al.*, 1996; Malicki *et al.*, 1996; Whitfield *et al.*, 1996; Nicolson *et al.*, 1998; Kappler *et al.*, 2004; Obholzer *et al.*, 2008; Brignull *et al.*, 2009). Small molecule screens for regulators of hair cell regeneration (Ton & Parng, 2005; Chiu *et al.*, 2008; Owens *et al.*, 2008; Ou *et al.*, 2009) or protection against neomycin-induced hair cell death (Owens *et al.*, 2008) have also been successful. T. Stawicki in the Raible laboratory recently began a screen for mutations that protect against cisplatin-induced hair cell loss and has already made some progress in characterizing one mutant family (unpublished data). The variety of dyes available for *in vivo* labeling of hair cells makes hair cell counts of large numbers relatively simple, and a new clutch of larval fish can be collected, treated, and screened every week. The Raible laboratory has developed a semiquantitative scoring method to approximate hair cell survival and regeneration on a scale from 0 to 20, and results using this scale are highly correlated with actual hair cell counts (Harris *et al.*, 2003). More detail on this method is available in Appendix A.

H. Brignull, with assistance from L. Clancey, L. Hericks, and myself, has identified over 10 mutant families from two mutagenesis screens with defects in hair cell regeneration. Mutants frequently display incomplete regeneration, in which a partial number of hair cells are replaced, or delayed regeneration, in which all hair cells are replaced after additional time (unpublished data). These phenotypes can be difficult to discriminate from normal regeneration in wild type siblings because the semiquantitative scoring method may only detect a difference in regeneration during a small temporal window, usually around 48 hpt. Sometimes mutants will also exhibit other phenotypes, including defects in membrane channel function that prevent vital dyes from labeling hair cells (labeling fixed larvae with antibodies against parvalbumin is possible but not generally feasible for high-throughput screens). Currently, *phoenix* is the only characterized mutation known to impair hair cell regeneration in zebrafish (Behra *et al.*, 2009). Genes that disrupt regeneration in zebrafish may have corresponding orthologs in mammals that could provide insight into why humans are unable to replace auditory and vestibular hair cells.

High-throughput screens for small molecules that affect hair cell death and regeneration have also been successful in the Raible laboratory (Chiu *et al.*, 2008; Owens *et al.*, 2008). Two

compounds, PROTO-1 and PROTO-2, were isolated from a library of several thousand candidates and found to protect against neomycin-induced hair cell loss (Owens *et al.*, 2008). Other screens using a library of FDA-approved pharmaceuticals have identified five compounds that protect against neomycin (Ou *et al.*, 2009) as well as others with previously unidentified ototoxic side effects (Chiu *et al.*, 2008). This research in this dissertation comparing hair cell death and regeneration in response to other ototoxins, including gentamicin, cisplatin, and copper, may facilitate small molecule screens. By comparing the results of screens using various ototoxins, it may be possible to identify common molecular features of protective compounds. There may also be unique chemical groups that provide protection against specific ototoxins, and these could allow physicians to provide tailored protection in different therapeutic contexts.

## 6.3 CONCLUDING REMARKS

Damage to mechanosensory hair cells is a significant risk for humans, one that grows as society continues to create and encourage new technologies that threaten them, such as jet engines, in-ear headphones, and ototoxic pharmaceuticals. Increased life expectancy in developed and developing nations will result in greater likelihood of age-related hearing loss. However, knowledge of hair cell function, death, and regeneration is also growing. The chick has long been a popular model for studying sensory hair cells, but the zebrafish is also earning well-deserved status as a valuable and flexible research tool, particularly as our understanding of the zebrafish genome becomes more complete. Zebrafish are considerably more amenable to forward and reverse genetic experiments, allowing not only screens for regulators of development and regeneration but also the opportunity to modify, insert, or replace specific genes to better understand their functions. Significant progress has been made in our understanding of the zebrafish lateral line within the last two decades.

In this dissertation, I characterized hair cell regeneration after treatment with a variety of different ototoxins, confirmed that the vast majority—if not all—hair cell regeneration in the lateral line occurs *via* proliferation, and determined that innervation is important for hair cell regeneration. These data will be useful to other researchers who are investigating such topics as the origin of hair cell progenitors, means of protection against hair cell death induced by aminoglycosides or cisplatin, and screens for new candidate genes that may protect against hair cell death or hinder regeneration. The Raible laboratory also works closely with investigators

who study mechanosensory hair cells the chick and other model organisms. These individuals may be interested to learn that direct transdifferentiation does not appear to contribute to regeneration of the lateral line as it does in the basilar papilla. Finally, my investigation of nerve-dependent hair cell regeneration may promote future projects to examine the mechanism underlying this phenomenon, perhaps providing an opportunity to identify protein or chemical signals that induce proliferation and differentiation. These and other experiments will expand our understanding of lateral line hair cell regeneration in zebrafish and how these processes and signaling pathways can translate to other organisms, benefitting humans with novel therapies to facilitate repair auditory and vestibular damage.

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# APPENDIX

## A.0 MATERIALS AND METHODS

### A.1 Zebrafish Strains and Maintenance

Zebrafish (*Danio rerio*) embryos were obtained from pairings of *AB wild type or various transgenic fish including *Et(krt4:EGFP)sqet4* ("*ET4:GFP* fish;" ZDB-GENO-070702-7 (Parinov *et al*., 2004; Go *et al*., 2010); gift of V. Korzh), *Et(krt4:EGFP)sqet33mi20* ("$ngn^{33}$ fish;" ZDB-GENO-110623-2 (Kondrychyn *et al*., 2009); gift of V. Korzh), *Tg(NeuroD:EGFP)* (ZDB-GENO-080701-3 (Obholzer *et al*., 2008)), and *Tg(sox10:Eos)* (ZDB-GENO-110721-5; (Prendergast *et al*., 2012)).

Adult zebrafish were maintained at 28.5 °C as described in Westerfield (1993). Embryos were produced by paired matings and raised in embryo media (EM; 40 μM $Na_2HPO_4$, 150 μM $KH_2PO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 500 μM KCl, 15 mM NaCl, pH 7.2). Embryos and larvae were maintained at a density of 50-60 per 100 $mm^2$ Petri dish. Larvae were fed live rotifers daily beginning at 4 days post-fertilization (dpf) with the exception of days when they were treated with an ototoxin. Experiments were conducted beginning at 3 or 5 dpf and were completed by 9 dpf. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

### A.2 Treatment with Ototoxic Compounds

Embryos at 3 dpf or 5 dpf were incubated for between 30 min and 10 hr in EM (control treatment), neomycin (100-200 μM; Sigma), copper(II) sulfate (1-30 μM; Fluka), gentamicin (50-200 μM; Sigma), or cisplatin (50 μM; Sigma). All solutions were prepared by serial dilution when appropriate. Larvae were rinsed twice in fresh EM after treatment then returned to fresh EM or another solution as described.

### A.3 Regeneration Assays in the aLL

Regeneration was measured in two ways. Initial experiments used a semi-quantitative scoring technique described previously (Harris *et al*., 2003) using the vital dye FM 1-43FX, a fixable form of FM 1-43, which selectively labels the cell membranes of functionally mature hair

cells. Larvae were incubated in 300 nM FM 1-43FX for 10 min at various hours post-treatment (hpt, measured from the beginning of treatment) at room temperature (RT), anesthetized in MESAB, and viewed with an epifluorescent dissecting microscope. Ten neuromasts of the aLL ($IO_1$, $IO_2$, $IO_3$, $IO_4$, $M_2$, $OP_1$, $MI_1$, $MI_2$, $O_1$, $SO_1$, and $SO_2$) were scored using a semiquantitative scale where 0 = no signal, 1 = partial signal, and 2 = full signal for a total possible score of 20. Larvae were screened only once. In other experiments, individual hair cells from 7 neuromasts ($IO_4$, $M_2$, $OP_1$, $MI_1$, $MI_2$, and $O_1$) labeled with antibodies against parvalbumin as described below and counted using a Zeiss Axioplan 2 epifluorescent microscope. Eight larvae (56-80 neuromasts) were assessed in each treatment group.

### A.4 Regeneration Assays in the pLL

Regeneration was observed in the pLL neuromasts for experiments studying the role of innervation. Whole mount immunohistochemistry was preformed as described in Appendix A.9, using a goat $\alpha$-mouse Alexa Fluor 568 antibody to label parvalbumin-positive hair cells and a goat $\alpha$-rabbit Alexa Fluor 488 antibody to label the GFP-positive pLL nerve. In most cases, five neuromasts were counted, including L1-L5 of the first pLL primordium.

For the experiments involving axotomy, the laser ablation was performed immediately posterior to the L1 neuromast, which was not included in those hair cell counts. The first three neuromasts of the second primordium were also counted to accommodate the speed of nerve regeneration, resulting in a total of 7 neuromasts per larva on both the cut and uncut sides and 8 larvae per treatment group (56 neuromasts).

When using the ErbB inhibitor described in Appendix A.7, additional neuromasts were present in the pLL. The average number of hair cells per neuromasts was not affected, but specific neuromasts could no longer be identified. Instead, the appropriate number of neuromasts was examined in their approximate locations on an untreated fish.

### A.5 Proliferation Assay

To detect evidence of recent cell proliferation, ET4 larvae were incubated in fresh EM containing 5 mM 5-bromo-2'-deoxyuridine (BrdU), a thymine analog, for 23 h immediately after treatment with an ototoxin for 1 h. Larvae were collected and labeled with antibodies against parvalbumin and BrdU as described below. BrdU was replaced with fresh EM at 24 hpt. Hair cell

counts to measure incorporation of BrdU assessed 3 neuromasts ($O_1$, $O_2$, and $MI_1$ in the aLL) per larva and 8 larvae per treatment group (24 neuromasts).

### A.6 Inhibition of Proliferation

To determine whether hair cell regeneration requires proliferation, larvae were incubated in fresh EM containing 5 μM flubendazole, which blocks microtubule assembly (Spagnuolo *et al*., 2010), until 48 hpt. Larvae were collected and labeled with antibodies against parvalbumin, GFP, or phosphohistone H3 (PHH3) as described in Appendix A.9. Hair cell counts assessed 3 or 7 neuromasts per larva and 8 larvae per treatment group (24-56 neuromasts).

### A.7 Inhibition of Glial Differentiation

Larvae were collected after fertilization and incubated at 28.5 °C for approximately 6 h, then incubated at RT overnight to slow development. The next morning, embryos were removed from their chorions at the 18- to 20-somite stage, when the tail begins to separate from the yolk (approximately 18 hpf). Dechorionated embryos were incubated in 1 μM PD158780 in EM containing 1% DMSO and kept in the dark to prevent photodegradation of the inhibitor. The solution was changed daily, and embryos were returned to normal EM at 72 hpf. Larvae were raised at a density of 30 per 100 $mm^2$ Petri dish to reduce the risk of disease if larvae died during treatment. The effectiveness of the inhibitor was confirmed by labeling hair cells with FM 1-43FX as described in Appendix A.4 and looking for supernumerary neuromasts in the pLL.

### A.8 Axotomy by Laser Ablation

Larvae were anesthetized with MESAB and restrained on a circular glass dish of under a small piece of netting Visualization and ablation of the pLL nerve were performed using a Marianas imaging system and accompanying SlideBook software (Intelligent Imaging Innovations). Hardware included a Zeiss Observer.Z1 spinning disk confocal microscope with 488 nm imaging laser and 532 nm photoablation laser. A 40X oil immersion objective (Zeiss) and FITC filter were used to visualize the pLL nerve and its terminal processes in transgenic fish using transillumination. The ablation laser was previously calibrated using a test slide to target a small rectangular region in the center of the visible field. Ablation was performed using two series of three 1-ms pulses at 85% full power. The ablation site was evaluated by

transillumination, and in some cases the ablation was repeated to achieve successful axotomy. Fish were then returned to fresh EM to recover for 6 h before neomycin treatment.

### A.9 Whole Mount Immunohistochemistry

Larvae were fixed in 4% paraformaldehyde and then rinsed in PBST (phosphate-buffered saline with 0.1% Tween-20) and dd$H_2O$. To label mature hair cells, larvae were blocked in 5% goat serum and incubated in mouse $\alpha$-parvalbumin primary antibody (1:500; Millipore) followed by goat $\alpha$-mouse Alexa Fluor 488 or 568 secondary antibody (1:500; Invitrogen). To label GFP in ET4 larvae, mouse (1:1,000; UC Davis/NIH NeuroMab Facility, clone N86/38) or rabbit (1:1,000; Invitrogen) $\alpha$-GFP primary antibody and goat $\alpha$-mouse or $\alpha$-rabbit Alexa Fluor 488 secondary antibody (1:500) were used. To label PHH3, rabbit $\alpha$-PHH3 primary antibody (1:500; Millipore) and goat $\alpha$-rabbit Alexa Fluor 568 secondary antibody (1:500) were used. Hair cells were then counted according to the regeneration assays described in Appendices A.3 and A.4.

For the proliferation assay measuring incorporation of BrdU, larvae were subsequently rinsed in PBSDT (PBST with 1% DMSO) and 1 M HCl before being blocked in 10% goat serum again. Larvae were incubated in rat $\alpha$-BrdU primary antibody (1:100; Abcam) followed by goat $\alpha$-rat Alexa Fluor 568 secondary antibody (1:400). Larvae were viewed on a Zeiss LSM 5 confocal microscope using a 40X oil immersion objective. PASCAL image acquisition software was used to collect *z*-stacks of 3 neuromasts per larva in 1 μm sections, which were later analyzed using ImageJ.

### A.10 Figure Preparation and Statistics

Figures were prepared using GraphPad Prism 5 and Adobe Illustrator CS3. Images were edited using Adobe Photoshop CS3. In all figures, error bars represent standard deviation (SD). Differences between groups and time points were analyzed using a one-ANOVA followed by a Tukey post-hoc analysis or two-way ANOVA followed by a Bonferroni multiple comparisons test.

## VITA

Scott Mackenzie was born in Mountain View, California, and raised in nearby Cupertino for the next eighteen years. After a strict parochial education, he excelled in mathematics and science at Cupertino High School and devoted his senior year to advanced coursework at De Anza Community College. He entered the University of California at Irvine with a full merit scholarship and graduated in 2007 with a Bachelor of Science in Neurobiology and a Bachelor of Arts in Psychology. In 2012, he earned a Doctor of Philosophy in Neurobiology and Behavior from the University of Washington under the mentorship of David Raible.