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Cisplatin ototoxicity and hair cell regeneration in the zebrafish lateral line

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CISPLATIN OTOTOXICITY AND HAIR CELL REGENERATION IN THE ZEBRAFISH LATERAL LINE

by

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Abstract: ***Hair cell death and regeneration on the zebrafish posterior lateral line was investigated after cisplatin administration. Hair cell regeneration was first observed by 24 hours of recovery and was further analyzed after specific recovery intervals. Disruption of the notch signaling pathways by the y-secretase inhibitor DAPT resulted in an increase in hair cells at three days of recovery.***

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INTRODUCTION

Cisplatin Ototoxicity

Cisplatin (cis-diamine-dichloroplatinum) is an effective chemotherapeutic agent used to treat a variety of soft tissue neoplasms, such as bladder, cervical, ovarian, testicular, and non-small cell lung cancers. It causes irreversible damage to the DNA of solid tumor cells, initiating cell death. Cisplatin chemotherapy often causes deleterious side effects such as nephrotoxicity and neurotoxicity. Ototoxicity is also a frequent side effect, resulting in tinnitus and permanent sensorineural hearing loss in a large percentage of treated individuals (Rybak, Whitworth, Mukherjea, & Ramkumar 2007). Evidence suggests that cisplatin ototoxicity targets the organ of Corti, cochlear lateral wall, and spiral ganglion cells through the generation of reactive oxygen species and depletion of antioxidant enzymes, ultimately eliciting apoptosis (Clerici, Hensley, DiMartino, & Butterfield 1996; Sergi, Fetoni, Ferraresi, Troiani, Azzena, Paludetti, & Maurizi 2004; Wang, Faulconbridge, Fetoni, Guitton, Pujol, & Puel 2003; Rybak *et al.* 2007).

Hair Cell Regeneration Following Ototoxicity

Ototoxic damage to hair cells in the mammalian ear is permanent; however, non-mammalian vertebrates (i.e., fish, amphibians, and birds) can regenerate hair cells via a process that is mediated by mitosis and direct transdifferentiation of inner ear supporting cells (Roberson, Alosi, & Cotanche 2004; Matsui & Ryals 2006). Cruz, Lambert, and Rubel (1987) were the first to evaluate hair cell regeneration after Gentamicin treatment in the chick cochlea, providing evidence of partial regeneration after approximately four weeks of recovery. Numerous subsequent studies have extensively demonstrated the regenerative processes in the avian inner

ear following treatment with other aminoglycosides. After cisplatin treatment, however, there is no evidence of a regenerative response in the avian ear (Slattery & Warchol 2010).

The Notch Signal Pathway

The Notch signaling pathway plays a significant role in regulating and inhibiting hair cell and supporting cell differentiation. For a cell to differentiate into a hair cell, the transcription factor *Atoh1* (also known as *Math1*) is required. *Atoh1* regulates the development of sensory receptors through induction and inhibition (Bermingham *et al.* 1999; Woods, Montcouquiol, & Kelley 2005). When *Atoh1* thresholds are reached in a particular cell, the transcription factor Delta is expressed, signaling to Notch receptors on adjacent cells. This signal then activates the enzyme □-secretase, leading to intracellular cleavage and release of the Notch intracellular domain into the nucleus. All surrounding cells are instructed to express the transcriptional repressor *Hes5* and differentiate as supporting cells. When Notch-mediated signaling is blocked through the use of a □-secretase inhibitor, an excess number of

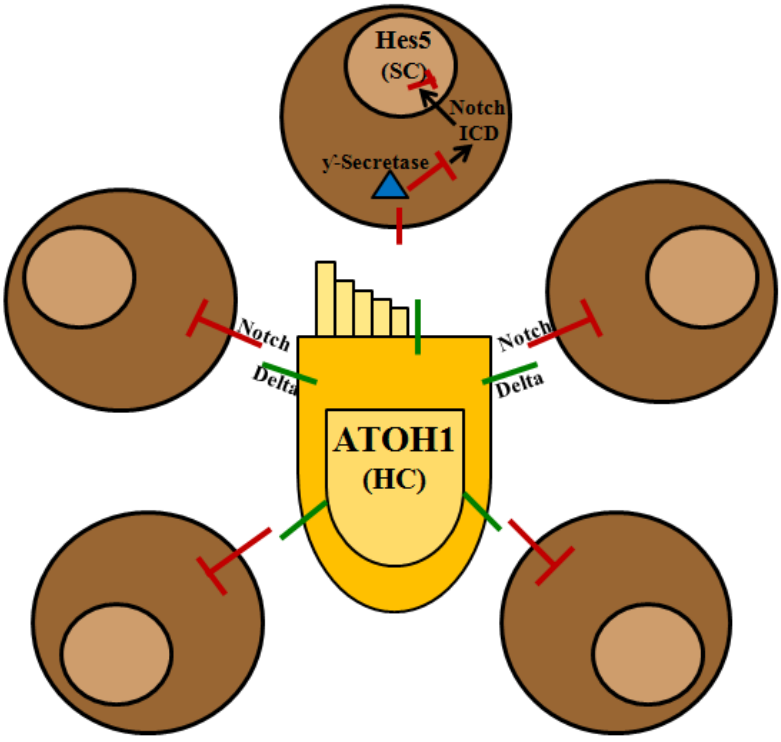

Figure 1. Delta/Notch signaling pathway involved in *Atoh1* hair cell differentiation and supporting cell *Hes5* expression.

hair cells is induced in the regenerating adult mouse utricle (Lin, Nguyen, Hume, Oesterle, & Stone 2011). After cisplatin exposure in the chick utricle, however, inhibition of the Notch pathways fails to enhance supporting cell transdifferentiation into new hair cells (Slattery & Warchol 2010).

The Use of a Zebrafish Model

The zebrafish lateral line is a sensory system comprised of multiple neuromasts. Each neuromast contains a sensory epithelium consisting of mechanosensory hair cells, surrounding supporting cells, and corresponding neurons with axons extending to the hindbrain (Ghysen & Dambly-Chaudiere 2004). The anterior lateral line consists of neuromasts on the head, whereas the posterior lateral line consists of neuromasts on the body, caudal fin, and tail. The system is sensitive to water perturbations and is responsible for school swimming and prey and predator detection (Dambly-Chaudiere, Sapède, Soubiran, Decorde, Gompel, & Ghysen 2003).

Due to the transparency and size of the zebrafish larvae, they allow for *in vivo* experiments and microscopic visualization without dissection. Furthermore, zebrafish

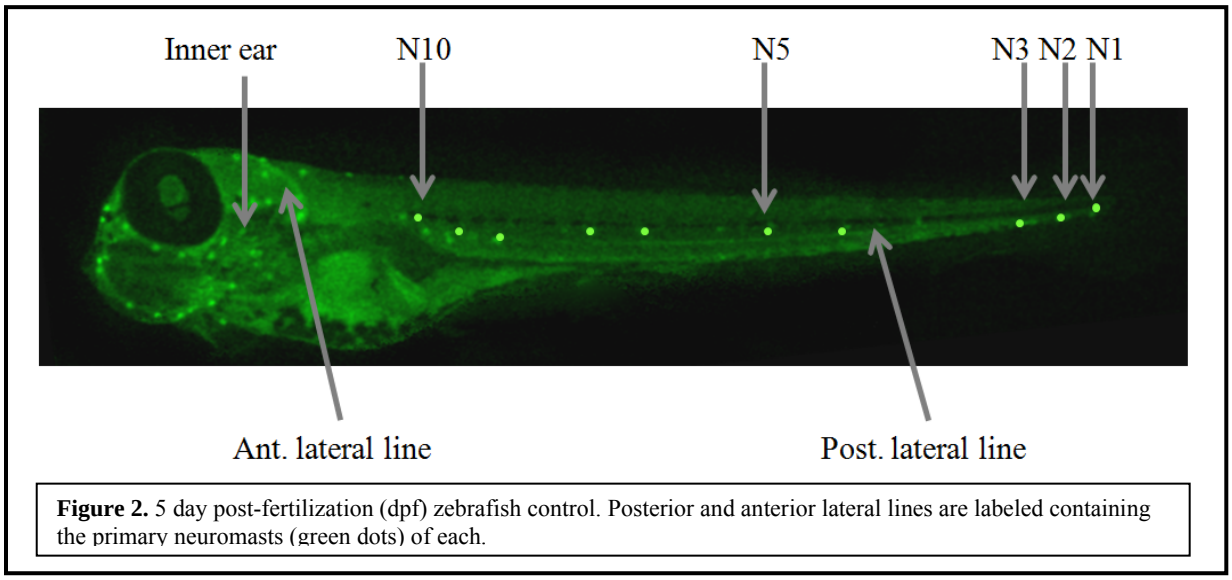

Figure 2. 5 day post-fertilization (dpf) zebrafish control. Posterior and anterior lateral lines are labeled containing the primary neuromasts (green dots) of each.

regenerative processes are significantly faster than their avian counterparts, allowing for greater sample sizes and data collection in a shorter amount of time. Recent studies have introduced the zebrafish as a model for ototoxic drug screening (Chiu, Cunningham, Raible, Rubel, & Ou 2008; Owens, Santos, Roberts, Linbo, Coffin, Knisely, Simon, Rubel, & Raible 2009; Ou, Santos, Raible, Simon, & Rubel, 2010) and for studying the mechanisms of cisplatin and aminoglycoside ototoxicity in greater detail (Ou, Raible, & Rubel 2007; Owens *et al.* 2009).

Current Study

Hair cell regeneration in the zebrafish lateral line has also been demonstrated following aminoglycoside-induced death (Harris, Cheng, Cunningham, MacDonald, Raible, & Rubel 2003). Furthermore, an excess number of hair cells is induced in the regenerating zebrafish lateral line through the use of a □-secretase inhibitor after aminoglycoside injury (Ma, Rubel, & Raible, 2007). The effects of cisplatin ototoxicity and regeneration in the zebrafish lateral line, however, are yet to be characterized. The current study analyzed hair cell death and regeneration on the zebrafish lateral line after cisplatin administration. Hair cell recovery through disruption of the Notch signaling pathway was also investigated.

MATERIALS AND METHODS

Animals

Zebrafish (*Danio rerio*) larvae, 5 days post-fertilization (dpf), of the AB wildtype strain were obtained from the Washington University in St. Louis zebrafish facility. According to Washington University Institutional Animal Research Committee guidelines, these zebrafish larvae are not yet considered vertebrae animals and thus do not need approval for animal experimentation.

Ototoxic Injury

Crystalline *cis*-platinum (II) diammine dichloride (cisplatin) was diluted in zebrafish "egg water" and stored at -20º C. The zebrafish were treated with 1000 μM of cisplatin for 4 hours. After treatment, the fish were thoroughly rinsed 3 times over 30 minutes.

DAPT Treatments

Notch signaling pathways were inhibited by the □-secretase inhibitor, DAPT (N-[N-(3,5-difluoro-phenacetyl)- L -alanyl]-S-phenylglycine t-butyl ester). DAPT was used at 50 μM with 0.5% dimethyl sulfoxide (DMSO).

Immunohistochemistry

For *in vivo* assessment and hair cell counts, all specimens were euthanized by treatment for 5 minutes in 10 μg/ml tricane (MS-222) and were fixed overnight in 4% paraformaldehyde at 4º C. Specimens were thoroughly rinsed in phosphate buffered saline (PBS) 3 times over 20 minutes. Nonspecific epitopes were blocked for 2 hours in a PBS solution with 5% normal horse

serum (NHS) and 1% DMSO. Hair cells were identified by the HCS-1 (Hair Cell Soma-1) primary mouse monoclonal antibody (1:500) (Goodyear *et al.* 2010). The primary antibody solution was made in PBS and contained 2% NHS and 1% Triton X-100. Specimens were incubated at room temperature for 24 hours.

The following day, specimens were rinsed 5 times over 20 minutes and incubated at room temperature for 2 hours in secondary antibody solutions. The secondary antibody used was Alexa488 anti-mouse IgG (1:200) for hair cell identification. The fish were rinsed 5 times over 30 minutes and mounted on microscope slides in glycerol/PBS (9:1) with their tail surfaces facing upwards.

Data Quantification and Imaging

The total number of surviving hair cells for each neuromast (N1, N2,...N10; see Fig.2) on the lateral line was counted using a Carl Zeiss LSM 700 Confocal Microscope with a 40x objective lens. Hair cells on the posterior lateral line were counted in the posterior to anterior direction and marked as present if they possessed a complete, distinctive circular flask-shape of a hair cell body. A total of 10 neuromasts were reliably visualized on each fish, and data from each of these 10 neuromasts were used for all subsequent experiments. All hair cell counts were compared to the hair cell numbers of the control fish from the same experiment. All data were organized in a Microsoft Excel spreadsheet and statistical analyses were performed.

Statistics

Averages and standard deviations were calculated and displayed graphically. Statistical significances were determined by using a 2-tailed Student's *t*-test. All data were computed and managed in Microsoft Excel.

RESULTS

Cisplatin Ototoxicity on the Zebrafish Lateral Line

To evaluate the extent of the ototoxic lesion caused by cisplatin on the zebrafish lateral line, 5 dpf zebrafish larvae were treated with 1000 μM of cisplatin for 4 hours. Following ototoxic injury, microscopic inspection of immuno-labeled specimens revealed a significant decline in neuromast hair cells across the entirety of the posterior lateral line, indicating a nearly complete lesion (Fig. 3). Although a limited amount of debris from dead or dying hair cells was observed, the few intact surviving hair cells were clearly labeled. In order to analyze the extent of the ototoxic damage, the total numbers of surviving hair cells in each neuromast was quantified. A hair cell was counted only if it possessed an intact membrane and maintained its distinctive morphology (Fig. 4). The resulting data indicated that cisplatin treatment caused a significant decrease in neuromast hair cell counts throughout the posterior lateral line (Fig. 3). A student's *t*-test confirmed that this decrease in hair cells was statistically significant ($p<0.0005$) when compared to the untreated control group.

In order to determine whether there was a difference in cisplatin toxicity from the anterior to the posterior end of the lateral line, hair cell numbers in the three most posterior neuromasts (N1, N2, N3) were compared to those in the three most anterior neuromasts (N8, N9, N10). At the time of the initial injury (i.e., immediately after cisplatin treatment), the three most posterior neuromasts had an average value of 2.2±1.7 hair cells, while the three most anterior neuromasts contained 1.6±1.5 hair cells. A Student's *t*-test revealed a significant difference ($p=0.003$) between the posterior and anterior groups. Furthermore, the three most posterior neuromasts in

the control (undamaged) fish at the time of the initial injury had an average value of 10.2±2.7 hair cells, while the three most anterior neuromasts yielded an average value of 9.6±4.2 hair cells. There was no significant difference (p=0.27) between the anterior and posterior neuromasts of the control fish, indicating that the anterior-most neuromasts were more susceptible to the cisplatin than their posterior neuromast counterparts (Fig. 5).

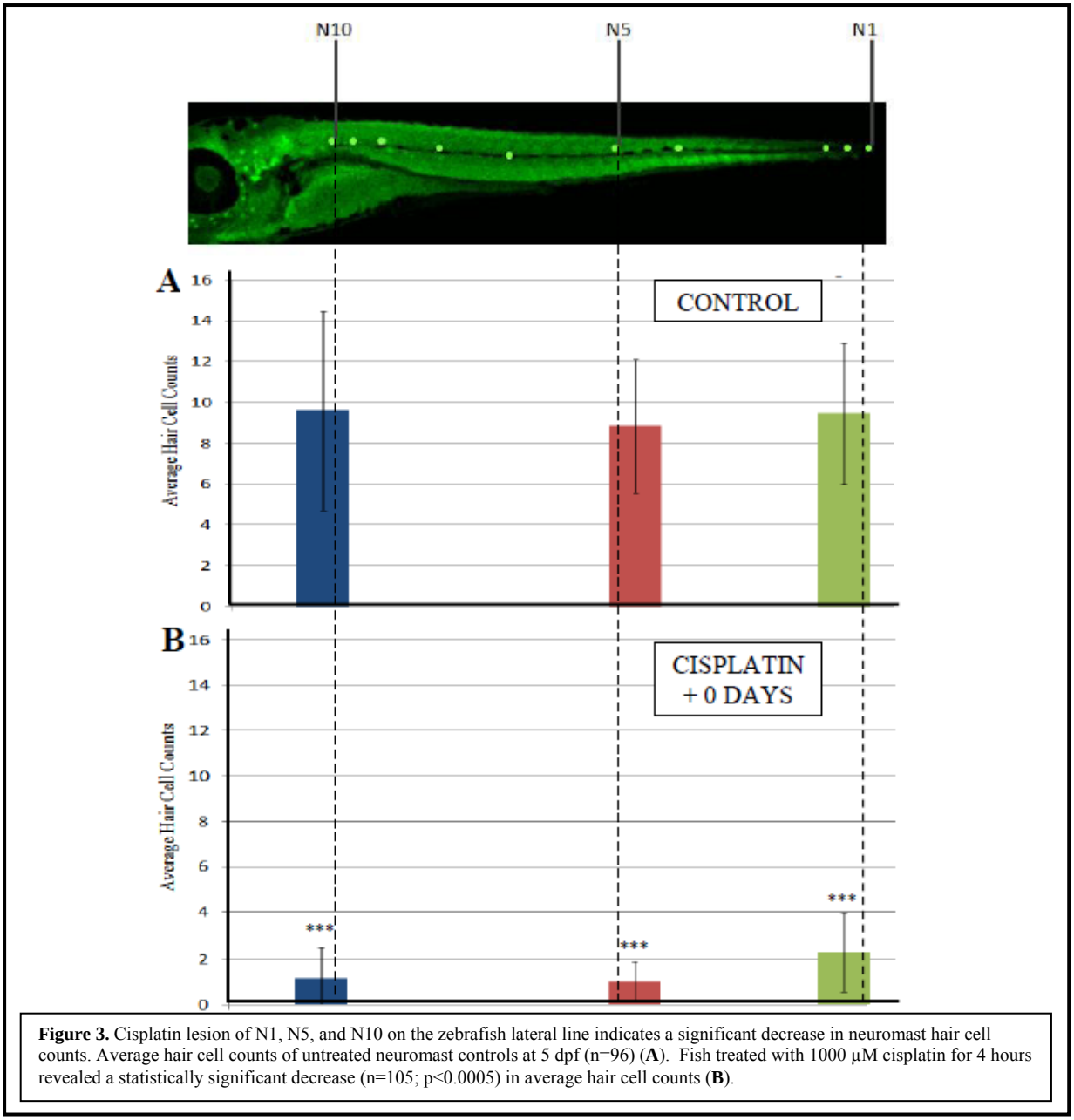

Figure 3. Cisplatin lesion of N1, N5, and N10 on the zebrafish lateral line indicates a significant decrease in neuromast hair cell counts. Average hair cell counts of untreated neuromast controls at 5 dpf (n=96) (**A**). Fish treated with 1000 µM cisplatin for 4 hours revealed a statistically significant decrease (n=105; p<0.0005) in average hair cell counts (**B**).

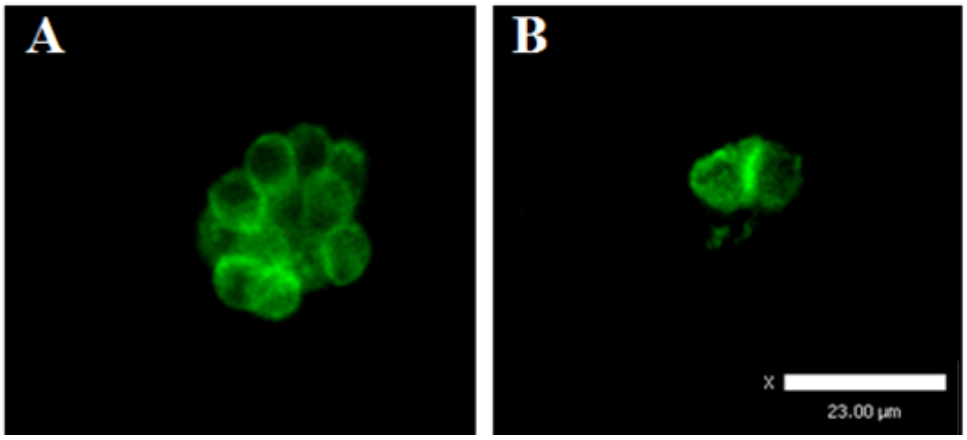

Figure 4. Zebrafish neuromast hair cells labeled with HCS-1. Untreated neuromast control at 5 dpf (**A**). Fish were treated with 1000 µM cisplatin and sustained for 24 hours (**B**).

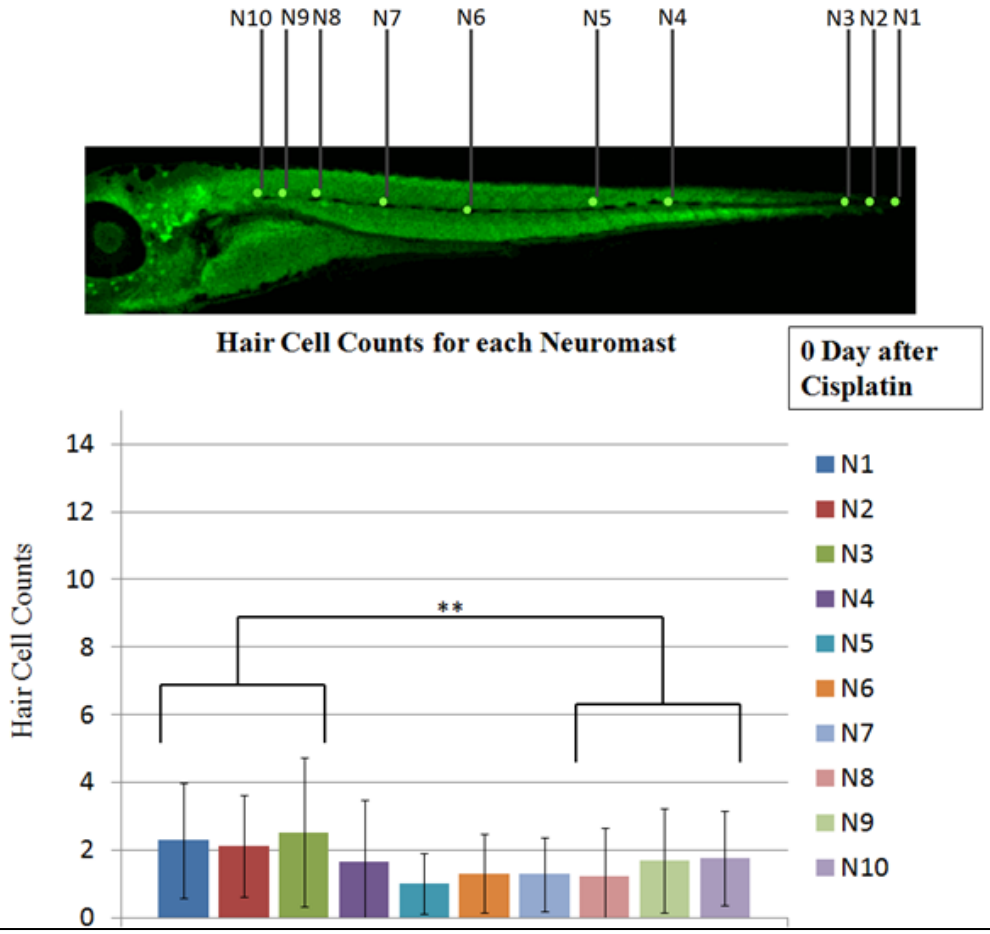

Figure 5. Average surviving hair cell counts for each neuromast (N1-N10) on the same day as the ototoxic injury. A significant difference (np=.003) was found between the three most anterior and posterior neuromasts (n=105 neuromasts/group), indicating a greater susceptibility to cisplatin in the anterior-most neuromasts.

Hair Cell Regeneration Following Cisplatin Injury

Zebrafish neuromast hair cells have been shown to regenerate following aminoglycoside ototoxicity (Song *et al.* 1995; Harris *et al.* 2003; Ma *et al.* 2007). Although no experiments have examined lateral line regeneration after cisplatin ototoxicity, data from *in-vitro* organotypic

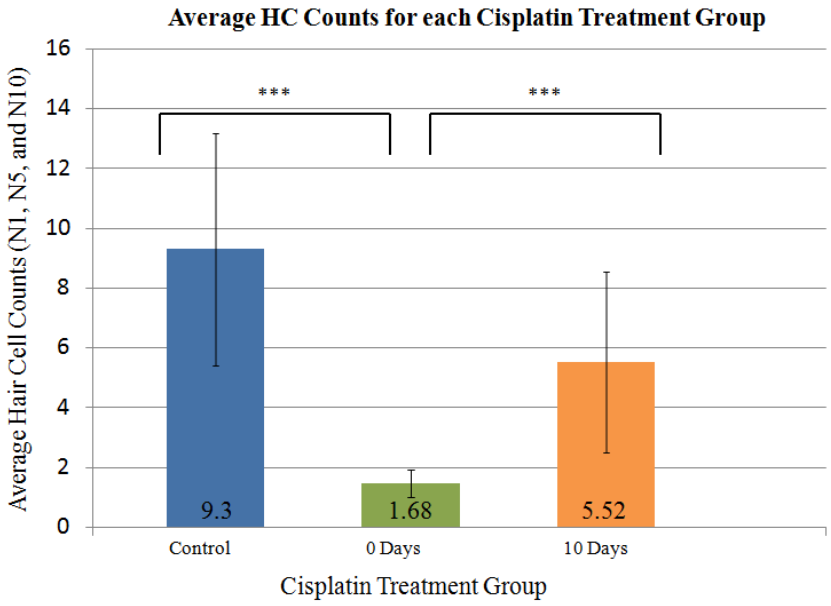

Figure 6. Quantification of average hair cell counts of N1, N5, and N10 of cisplatin treated neuromasts after 0 days (n=105) and after 10 days (n=21) compared to control neuromasts at 0 days (n=96). Average hair cell counts revealed a statistically significant decrease after cisplatin treatment at 0 days after the exposure ($p<0.0001$). 10 days after cisplatin treatment, a statistically significant increase in hair cell count averages compared to the 0 day injury was observed ($p<0.0001$).

cultures of the chick inner ear showed an eradication of regenerative abilities following cisplatin ototoxicity (Slattery & Warchol 2009). In order to determine whether cisplatin also blocks regeneration *in vivo*, zebrafish were maintained for 1-, 3-, 5-, 7-, and 10-day recovery intervals following the initial cisplatin injury (Fig. 7). A significant increase in average hair cell counts was observed after 1 day of recovery ($p<0.005$). After 10 days of recovery, average hair cells counts within N1, N5, and N10 had enhanced dramatically, to a value of 5.5 ± 3.0. These numbers were statistically significant when compared to fish in the 0-day treatment group, which

had an average hair cell count of 1.7±0.5 (Fig. 6 $p<0.0001$). These data demonstrate that the zebrafish lateral line can regenerate after cisplatin; however, this restoration is still incomplete by

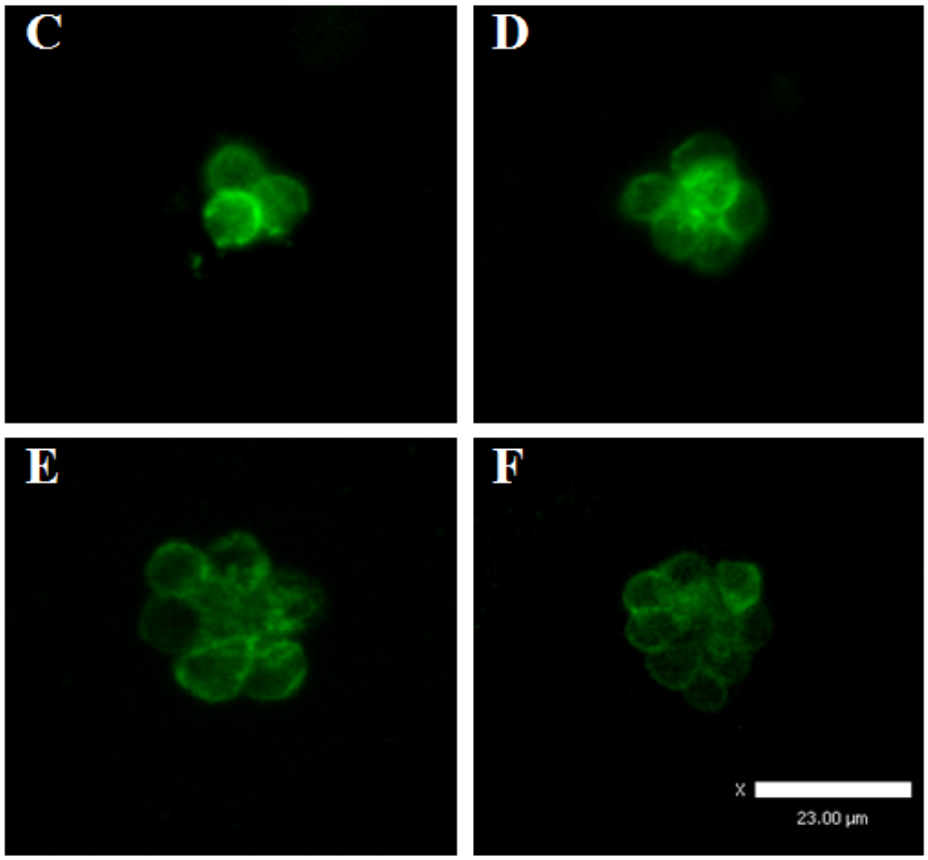

Figure 7. Zebrafish neuromast hair cells labeled with Alexa 488 and sustained for 3 day (**A**), 5 day (**B**), 7 day (**C**), and 10 day (**D**) recovery intervals, demonstrating that the zebrafish lateral line can regenerate after cisplatin; however, this restoration is still incomplete by 10 days, only approximating roughly 60% of the control groups.

10 days, reaching approximately 60% of the hair cell numbers in the control group.

To study possible differences in hair cell differentiation in the posterior vs. anterior neuromasts during hair cell recovery, the mean numbers of hair cells in three most posterior neuromasts (N1, N2, N3) were again compared to hair cell numbers in the three most anterior neuromasts (N8, N9, N10). These comparisons were conducted at each recovery time point. After 7 days of recovery, the posterior neuromasts yielded an overall average of 2.4±2.2 hair

cells, whereas the anterior neuromasts yielded an overall average of 4.5±3.5 hair cells. A statistically significant difference in the degree of regeneration was observed between these anterior and posterior data sets ($p<0.0001$; Fig. 8C). A lesser, but still significant difference was also seen in the 10-day recovery group ($p<0.05$; Fig. 8D). At this recovery period, the three most posterior neuromasts contained 5.0±2.1 hair cells, while the three most anterior neuromasts contained 6.9±3.5 hair cells. No significant difference was found in any of the earlier 1-, 3-, and 5-day recovery groups. Furthermore, no significant difference existed in the corresponding control groups for any of the 1-10 day recovery periods.

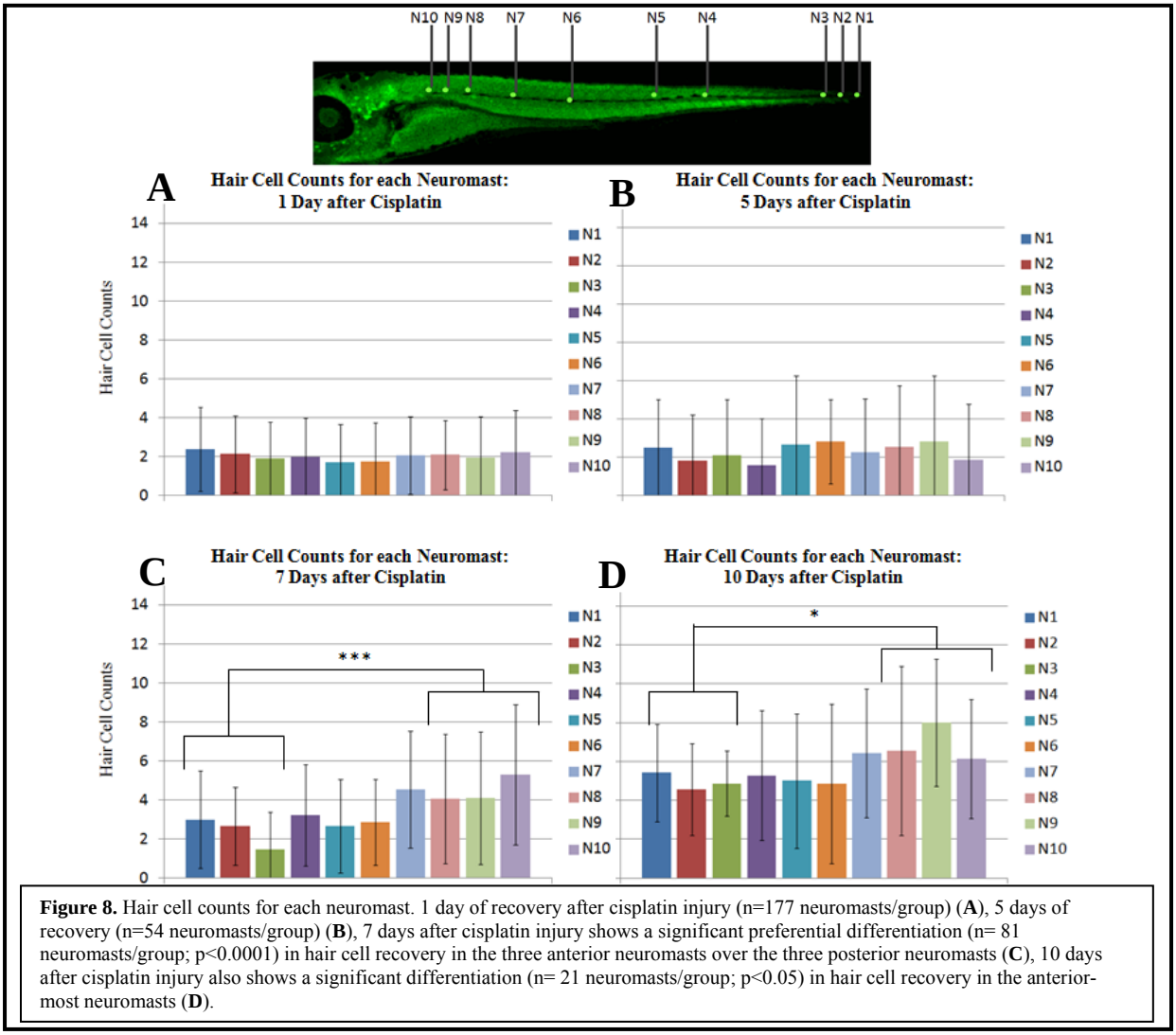

Figure 8. Hair cell counts for each neuromast. 1 day of recovery after cisplatin injury (n=177 neuromasts/group) (**A**), 5 days of recovery (n=54 neuromasts/group) (**B**), 7 days after cisplatin injury shows a significant preferential differentiation (n= 81 neuromasts/group; $p<0.0001$) in hair cell recovery in the three anterior neuromasts over the three posterior neuromasts (**C**), 10 days after cisplatin injury also shows a significant differentiation (n= 21 neuromasts/group; $p<0.05$) in hair cell recovery in the anterior-most neuromasts (**D**).

Inhibition of Notch Signaling

During inner ear development and regeneration, hair cell differentiation is mediated by the Notch signaling pathway (Bermingham *et al.* 1999; Woods *et al.* 2005). Previous evidence indicates that disruption of Notch signaling through inhibition of □-secretase results in enhanced

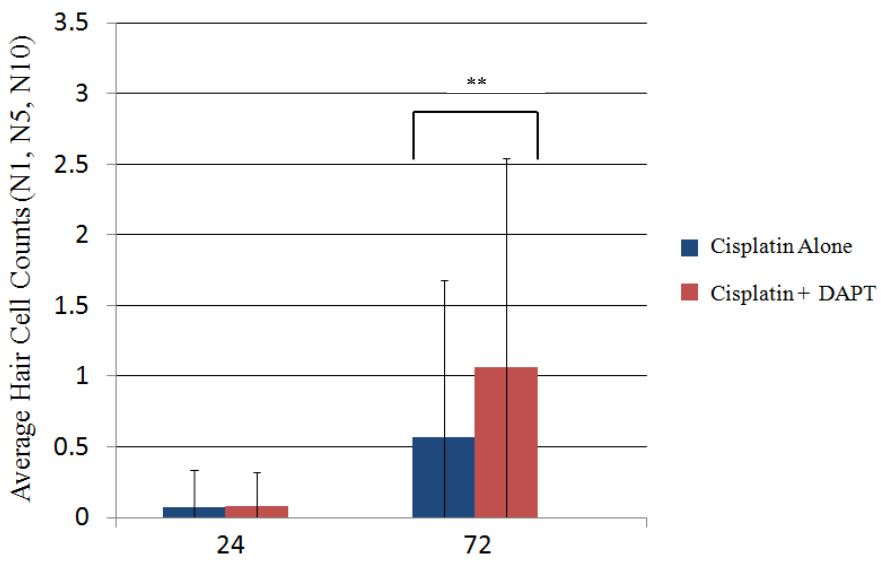

Figure 9. Quantification of average hair cell counts (N1, N5, and N10) of cisplatin-alone treated neuromasts after 24 hours (n=42) and after 72 hours (n=102); in addition to groups treated with cisplatin and DAPT after 24 hours (n=24) and after 72 hours (n= 174).

regeneration (Ma *et al.* 2007; Lin *et al.* 2011). Since the preceding analyses revealed a moderate degree of hair cell regeneration in the lateral line neuromasts following cisplatin injury, it was next determined whether Notch pathway inhibition would increase hair cell recovery following cisplatin treatment. Zebrafish were treated with 1000 μM of cisplatin, followed by incubation in 50 μM of DAPT. Fish were maintained in DAPT for either 24 hour or 72 hour recovery periods. Following fixation and processing, the numbers of neuromast hair cells were quantified. Control fish also received cisplatin but were maintained in 0.2% DMSO for identical recovery periods.

After 24 hours of recovery, the cisplatin alone control group yielded an average hair cell count of 0.1±0.3, and the cisplatin with DAPT group yielded an average of 0.08±0.2 (Fig. 9). There was no significant difference between these two groups ($p = 0.879$). After 72 hours of recovery, however, neuromasts of the control fish contained 0.6±1.1 hair cells, while neuromasts of the DAPT-treated fish contained 1.1±0.9 hair cells (Fig. 9). This difference was statistically significant ($p=0.002$; Student's *t*-test), indicating that Notch inhibition through DAPT treatment moderately enhances regeneration following cisplatin injury on the zebrafish lateral line.

DISCUSSION

Cisplatin is Ototoxic to the Posterior Lateral Line

This investigation aimed to evaluate cisplatin ototoxicity and subsequent regeneration in the zebrafish lateral line. It was found that a 1000 μM dose of cisplatin for 4 hours was adequate to kill the vast majority of lateral line hair cells. These results are similar to those obtained by Ou and colleagues, who reported that a 1000 μM concentration for < 4 hours created an extensive hair cell lesion on the zebrafish lateral line (Ou *et al.* 2007).

This study also analyzed whether cisplatin toxicity differed among hair cells within the anterior vs. posterior neuromasts of the lateral line. A significant difference in hair cell loss was observed in the posterior vs. anterior neuromasts immediately after cisplatin exposure. The three most anterior neuromasts (N8, N9, N10) displayed a higher susceptibility to cisplatin, compared to their posterior neuromast counterparts. These results were unexpected, since all of the neuromast hair cells on the posterior lateral line are external, in which a continuous concentration of cisplatin was assumed. In the mammalian cochlea, however, cisplatin is known to target the outer hair cells at the basal end of the organ of Corti, presenting as a high-frequency sensorineural hearing loss (Budnick, Kopelman, Sessions, Kramer, & Wong 1988; Berg, Spitzer, & Garvin 1999). These differences in the zebrafish could reflect variances in the cisplatin uptake in the anterior vs. posterior neuromasts, or metabolic differences in hair cells along the anterior-posterior axis of the fish.

Neuromast Hair Cells Regenerate on the Posterior Lateral Line

Having established that cisplatin is toxic to hair cells in the zebrafish lateral line, the

regenerative abilities following cisplatin injury were then analyzed. Hair cell regeneration following cisplatin treatment was observed as early as 24 hours of recovery; however, hair cell numbers returned to approximately 60% of their control groups after 10 days. This delayed and incomplete regeneration after cisplatin in the lateral line is quite different from that which occurs after neomycin ototoxicity (Ma *et al* in 2008). Those authors exposed 5 dpf zebrafish larvae to neomycin, which killed nearly all lateral line hair cells; however, they observed nearly complete neuromast hair cell regeneration after 3 days of recovery (Ma *et al.* 2008). The authors proposed the existence of two independent subpopulations of supporting cells residing in the zebrafish

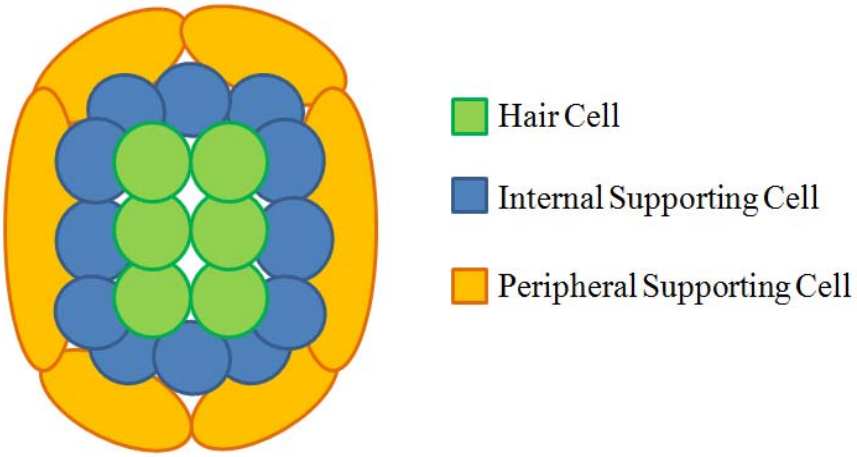

Figure 10. Schematic of the zebrafish neuromast hair cells (green) that are surrounded by the internal supporting cells (blue) that are thought to be responsible for creating new hair cells. The peripheral supporting cells (orange) may give rise to new progenitors that are responsible for replacing the internal supporting cells.

neuromast: internal and peripheral supporting cells (Fig. 10). The internal supporting cells are thought to be responsible for creating new hair cells, whereas the peripheral supporting cells may give rise to new progenitors that are responsible for replacing the internal supporting cells. It is possible that the administration of cisplatin may eradicate the internal supporting cell population, and that these cells are then replaced by the peripheral supporting cells which lack susceptibility to cisplatin.

A prior study demonstrated a lack of regeneration in the chick inner ear following cisplatin treatment, suggesting that cisplatin is toxic to both hair cells and supporting cells (Stattery & Warchol 2010). Notably, in the chick inner ear, there are no peripheral cells that may mediate the replacement of internal supporting cells and promote new hair cell differentiation. Additionally, the dosage of cisplatin that is sufficient to kill hair cells on the zebrafish lateral line may not cause an equivalent injury to supporting cells. As a result, the supporting cells of the chick may be more directly targeted than in the zebrafish lateral line.

Differences were also observed in recovery between the posterior-most (N1, N2, N3) and anterior-most (N8, N9, N10) neuromast groups. Specifically, hair cell recovery in the posterior neuromasts lagged behind their anterior counterparts at 7 days of recovery, but recovery in the two groups became nearly equal by day 10. Beginning at approximately 3-4 dpf, it has been shown that secondary neuromasts begin to develop between the eight primary posterior lateral line neuromasts in a head-to-tail sequence (Ledent 2002). Although neuromast counts varied between fish in each experimental group, 10 neuromasts were accurately identified in each fish at 5 dpf and were counted in a posterior to anterior (N1-N10) fashion for each experiment. Unaffected hair cells in the anterior portion of the fish may have been developing in conjunction with other recovering neuromast hair cells. At 7 dpf these most recently developed secondary anterior neuromasts may also undergo faster cell division than their primary posterior neuromast counterparts.

Inhibition of the Notch Signaling Pathways Enhances Regeneration

When Notch signaling was inhibited by treatment with 50 μM of the □-secretase inhibitor DAPT, an increase in hair cell differentiation was observed after 72 hours of recovery. Again

the zebrafish model differs from the chick inner ear, where DAPT treatment does not lead to hair cell transdifferentiation after cisplatin (Slattery & Warchol 2010). These results, however, are in agreement with previous research that examined inhibition of Notch signaling in the zebrafish after neomycin injury (Ma *et al.* 2008). If a sufficient number of supporting cells are capable of surviving the ototoxic injury and can produce new hair cells, perhaps this process can be enhanced through DAPT. Additionally, these observations reconfirm the role of *Notch signaling* in the determination of hair cell differentiation during regeneration.

CONCLUSION

This study provided a preliminary analysis of hair cell death and regeneration in the zebrafish posterior lateral line following cisplatin treatment. It was established that a 1000 μM dose of cisplatin for 4 hours was adequate to kill the majority of lateral line hair cells. Additionally, hair cell regeneration was first observed by one day of recovery. After 10 days of recovery, a further increase in hair cell numbers was observed, but the overall regenerative abilities were limited. Hair cell numbers did not return to those observed in the normal (control) animals. Lastly, an increase in hair cell counts after 3 days of recovery following cisplatin treatment was observed through the use of the □-secretase inhibitor DAPT, reconfirming the role of *Notch signaling* in hair cell differentiation during regeneration. Further investigation into the morphology of the lateral line hair cells and supporting cells during cisplatin administration will provide a greater understanding of the precise mechanisms of cisplatin ototoxicity and its effect on regenerative abilities.

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