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RESEARCH ARTICLE

Wnt/β-catenin signaling was activated in supporting cells during exposure of the zebrafish lateral line to cisplatin

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ABSTRACT

Zebrafish lateral line neuromasts are composed of central hair cells surrounded by supporting cells. Cisplatin is a common anticancer drug, with hair cell disruption being a frequent side effect of this drug. In our study, we observed complete functional hair cell loss after six hours of cisplatin insult in neuromasts, as demonstrated by anti-parvalbumin 3 immunofluorescence staining or YO-PRO1 vital dye staining. Time course analysis of neuromast hair cell regeneration showed that regenerated hair cells first appeared between 12 and 24 h after damage, and the abundance of these cells increased stepwise with recovery time. After 72 h, 90% of the hair cells were regenerated, and after 84 h, the number of regenerated hair cells was comparable to the number of neuromast hair cells before treatment. The expression pattern of *slc17a8* also showed that hair cells were regenerated after cisplatin exposure. Meanwhile, peripheral supporting cells moved toward the center of the neuromasts, as shown by the in situ expression pattern of *sox21a*. Increased hair cell progenitor formation was also observed, as demonstrated by the in situ expression pattern of *atoh1a*. Furthermore, we detected increased expression of *wnt2*, *wnt3a*, and *ctnnb1* in sorted supporting cells from the *sqet10* transgenic line, which labels neuromast supporting cells specifically. In situ hybridization analysis also showed decreased expression of *dkk1a* and *dkk2*. Regenerated hair cells were inhibited by early inhibition of Wnt/β-catenin signaling. Taken together, the results presented here showed that Wnt/β-catenin signaling was activated in supporting cells during cisplatin exposure earlier than expected. Our results also indicated that supporting cells enabled hair cell regeneration via Wnt/β-catenin signaling during cisplatin exposure.

1. Introduction

Hair cell regeneration requires nonsensory progenitor differentiation and sensory progenitor formation (Cruz et al., 2015; Groves et al., 2013). However, when and how hair cell progenitor formation occurs remain unknown. In mammals, hair cell loss is permanent. In lower vertebrates, such as zebrafish, hair cells exhibit robust regenerative ability after toxicant exposure (Kniss et al., 2016; Lush and Piotrowski, 2014; Pisano et al., 2014). Cisplatin is one of the most commonly used anticancer drugs, with the most frequent side effect being hearing disability, with 18% of adult patients had severe to profound hearing loss after cisplatin (Frisina et al., 2016) and severe ototoxicity ranged from 7% to 22% in children (Knight et al., 2017). It has been previously observed that cisplatin damaged zebrafish lateral line hair cells in a dose-dependent manner (Giari et al., 2012; Ou et al., 2007; Thomas et al., 2015). Ou et al. indicated that six hours of cisplatin exposure resulted in almost complete mature hair cell loss in neuromasts (Ou et al., 2007). It is also known that hair cells can regenerate fully after cisplatin-induced hair cell loss (Mackenzie and Raible, 2012). Nevertheless, the cellular and molecular mechanisms that regulate the hair cell progenitor population during the regeneration process remain unclear.

The zebrafish lateral line has another mechanosensory organ in addition to ears. The posterior lateral line ultimately develops 10–12 hair cells that are surrounded by supporting cells in the periphery in each neuromast in larvae five days post fertilization (Villegas et al., 2012). Lateral line neuromast hair cells share genetic and organ system homology with mammalian vestibular hair cells (Barbazuk et al., 2000; Goldsmith and Jobin, 2012). Additionally, zebrafish lateral line neuromast hair cells are easily visible and accessible, facilitating the study of the cellular and molecular mechanisms underlying hair cell regeneration.

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It is well known that regenerated hair cells originate from neuromast stem cells (Lopez-Schier and Hudspeth, 2006; Williams and Holder, 2000). Supporting cells are interposed between hair cells in the lateral line neuromasts and can enter the mitotic S-phase and become hair cell progenitors (Williams and Holder, 2000; Presson et al., 1996; Presson et al., 1995). Proliferating supporting cells may move inwards after drug-induced hair cell death. Additionally, regeneration of chicken and amphibian hair cells can occur from dividing or transdifferentiating supporting cells (Adler and Raphael, 1996; Baird et al., 1996). Previous studies have revealed that the *soxB1* and *soxB2* genes play essential roles in maintenance of the embryonic stem cells in an undifferentiated state (Bylund et al., 2003; Hernandez et al., 2007; Lan et al., 2011). Differentiation of hair cell progenitors requires expression of the transcription factor *atoh1a* (Sarrazin et al., 2006). Moreover, no data have demonstrated the behavior of neuromast supporting cells during the occurrence of cisplatin-induced hair cell damage. We set out to define the changes in gene expression in neuromast supporting cells at high resolution during hair cell death and to investigate the changes in gene expression in the hours before hair cell regeneration. We demonstrated that Wnt/β-catenin signaling was activated early in neuromast supporting cells, and this activation was necessary but not sufficient for hair cell regeneration. Our study indicated that early Wnt/β-catenin signaling activation in supporting cells is essential for hair cell progenitor formation.

2. Materials and methods

2.1. Ethics statement

The use of animals was in accordance with the Guidelines on the Handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare (UFAW). The experimental protocol was in accordance with the principles outlined in the Declaration of Helsinki.

2.2. Fish preparation and cisplatin treatment

The wild-type fish used in this study were Tübingen fish. *Sqet10* and *sqet4* transgenic fish were used to label neuromast supporting cells and hair cells, respectively (Parinov et al., 2004). Larval fish were generated by paired mattings. For cisplatin treatment, 1 mM cisplatin solution was freshly prepared before use by dissolving cisplatin powder (Sigma, 33422) in larval fish rearing medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4). Five days post fertilization (dpf), larval fish were incubated in 1 mM cisplatin medium for six hours at 28.5 °C.

2.3. In situ hybridization

In situ probes for *sox21a*, *atoh1a*, *dkk1a*, *dkk2*, and *slc17a8* were synthesized according to standard procedures. In brief, to generate *sox21a*, *atoh1a*, *dkk1a*, *dkk2*, and *slc17a8* antisense probes, PCR was performed on cDNA from 5 dpf larval fish using the primers listed in the following section. PCR products were then ligated with the Ez-T vector to obtain plasmids. Linearized plasmids were used as templates to synthesize DIG-labeled antisense probes. In situ hybridization procedures were performed as described previously (Thisse and Thisse, 2008). Images were obtained with a Zeiss Axio A1 inverted microscope. The following primers were used to clone these probes:

sox21a (Fw 5′-GCACGAGCAGGTCACTTCA-3′ and Rv 5′-CGGAATAATGCGGGAAAG-3′), *atoh1a* (Fw 5′-GCCACAAACTTTCCTCC-3′ and Rv 5′-GAATGTTCCGTCCTCGT-3′), *dkk1a* (Fw 5′-CATCCAGCCCGAGTGA-3′ and Rv 5′-GTTGAGCACAAAGCGAGT-3′), *dkk2* (Fw 5′-GTGCGGATGGGAGAAACT-3′ and Rv 5′-TCTGGAGAATGAGGTGGC-3′), and *slc17a8* (Fw 5′-TCTGAAGGAAAAGCTCAACCCGGGG-3′ and Rv 5′-GCACCGCTGACAAAATGCCCACCTT-3′).

2.4. Neuromast hair cell staining

In this study, neuromast hair cells were stained by immunofluorescence or vital dye staining. The immunofluorescence staining protocol was performed according to Harris et al. (2003). In brief, 10–15 fish larvae were fixed overnight at 4 °C with 4% PFA and then treated with acetone and blocked with PBDT plus 2% normal goat serum. The larvae were incubated in rabbit anti-parvalbumin 3 (1:500, Sigma) or rabbit anti-myosin VI (1:500, Proteus BioSciences) overnight at 4 °C. Then, the larvae were washed again with PBDT and incubated with Alexa Fluor 488-labeled goat

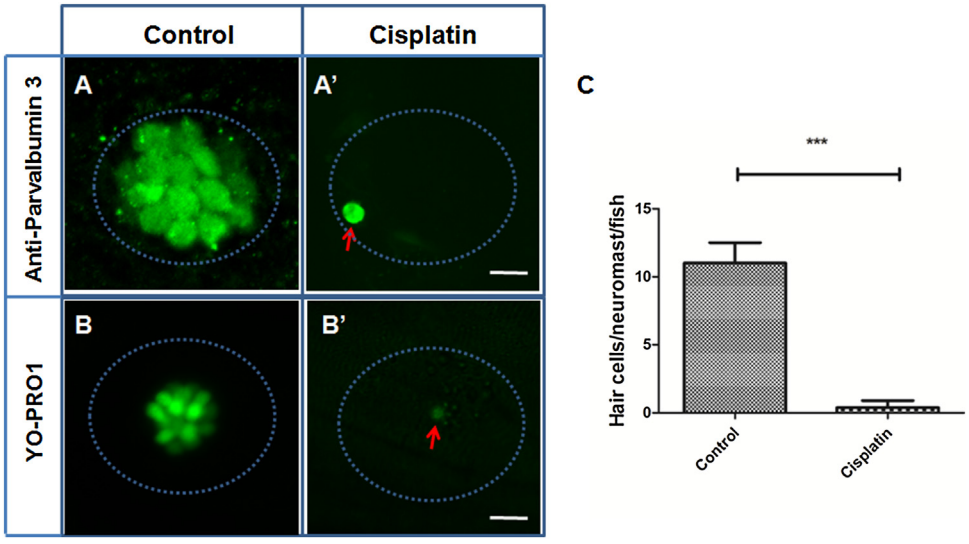

Fig. 1. Cisplatin-induced hair cell loss in zebrafish lateral line. (A and A') Anti-parvalbumin 3 antibody staining showed only one abnormal hair cells, with rounded body, remained after six hours treatment (red arrow). Scale bar, 5 μm. (B and B') YO-PRO1 live dye staining showed only one rounded-hair cells remained after treatment (red arrow). Scale bar, 10 μm. (C) Quantification of functional hair cells after six hours cisplatin insult. ***, $P < 0.0001$, 11 fish in each group, the first three posterior lateral line neuromasts hair cells were counted. The experiment was repeated at least three times. (For interpretation of the references to colour in the figure legend, the reader is referred to the web version of this article.)

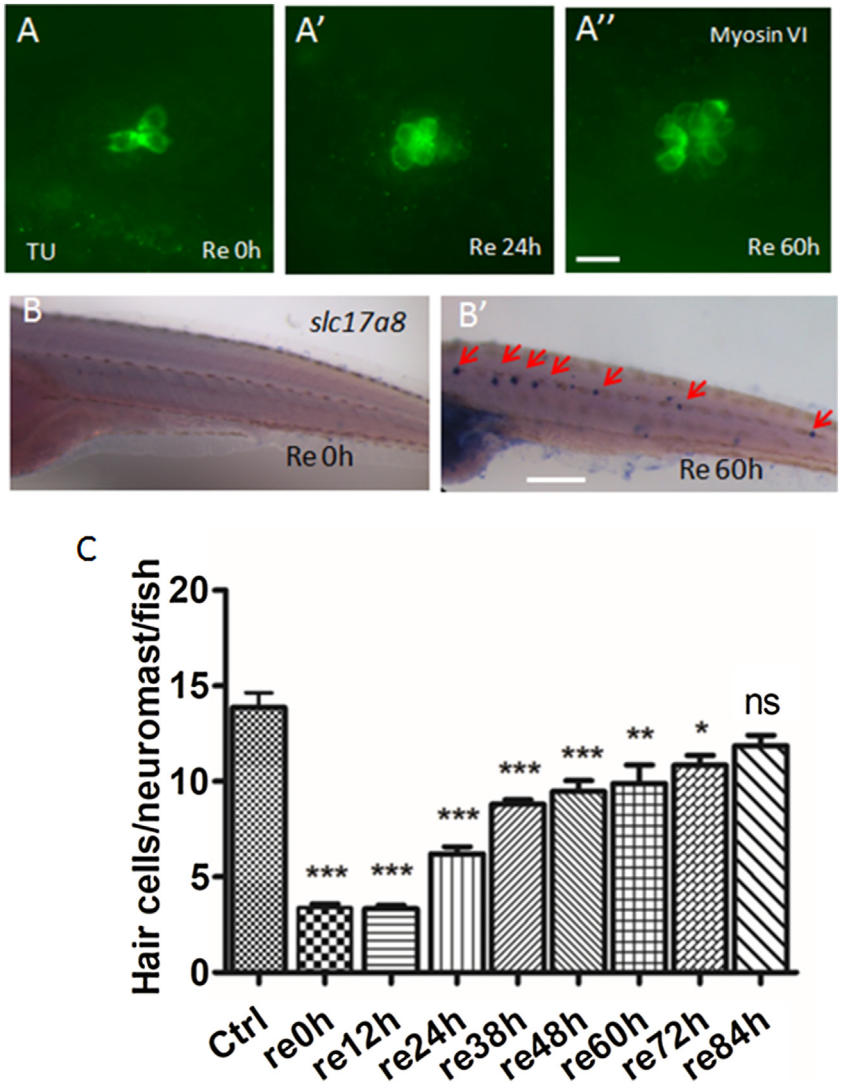

Fig. 2. Time course of hair cell regeneration following cisplatin insult. After cisplatin removal, fish larvae were incubated in fish medium to allow regeneration. (A, A' and A") In situ anti-myosin VI antibody staining showed regenerated hair cells at 0, 24 and 60 h after treatment. Scale bar, 10 μm. (B and B') In situ hybridization using anti-sense probe *slc17a8* showed regenerated hair cells in neuromasts at 60 h post treatment. Red arrow, regenerated hair cells. Scale bar, 20 μm. (C) Quantification of regenerated hair cells. Myosin VI-positive hair cells were quantified, with 12 h interval, during recovery time. Comparisons between each group were made by student *t* test. ***, $P < 0.0001$; ns, $P > 0.05$. 12 fish in each group. The experiment was repeated at least three times. (For interpretation of the references to colour in the figure legend, the reader is referred to the web version of this article.)

anti-rabbit IgG (H + L) (1:200, Invitrogen) for six hours at room temperature. Labeled cells in neuromasts were counted with a Zeiss Axio A1 inverted fluorescence microscope using a 40× objective. Vital dye staining of neuromast hair cells was performed by immersion in 2 μM YO-PRO1 (Invitrogen, Y3603) in embryo medium for 30 min (Santos et al., 2006).

2.5. Fluorescence activated cell sorting (FACS)

One thousand 5 dpf untreated (control) or cisplatin-treated *sqet10* larvae were harvested to remove head tissue and collected in 1.5-ml tubes. Then, 1 ml of Ringer's solution was added, and the mixture was gently pipetted to remove the yolk. Then, 1 ml of 0.25% trypsin-EDTA (Gibco, MB3379) was added, and the mixture was gently pipetted every five minutes at 28.5 °C for 20–30 min to dissociate the tissues into single cells. Finally, 10% FBS and 1 mmol/l $CaCl_2$ were added to stop the enzymatic reaction. The cells were centrifuged at 1000 rpm for 5 min and resuspended in PBS to obtain dissociated cells. Cells were sorted with a BD Aria II with a nozzle size of 90 μm, and GFP + cells were sorted directly into 200 μl of TRIzol reagent (Invitrogen, 15596026) and stored at −80 °C until further use.

2.6. RNA extraction, RT-PCR, QPCR

Approximately 10^6 sorted GFP + cells from several sorting experiments were gathered and mixed for total RNA extraction following the manufacturer's instructions. cDNA was synthesized using an M-MLV kit (Invitrogen, C28025) and served as a template for RT-PCR and QPCR. QPCR was performed using the SYBR® Green Quantitative RT-qPCR Kit (Invitrogen, A25780). Total RNA from each sample was normalized to β-actin.

2.7. Chemical compounds

For Wnt/β-catenin signaling inhibition, 0.1 μM FH535 (Merck, 219330) or 25 μM XAV939 (Selleck, S1180) was added to the fish medium (Huang et al., 2009; Jacques et al., 2012). For Wnt/β-

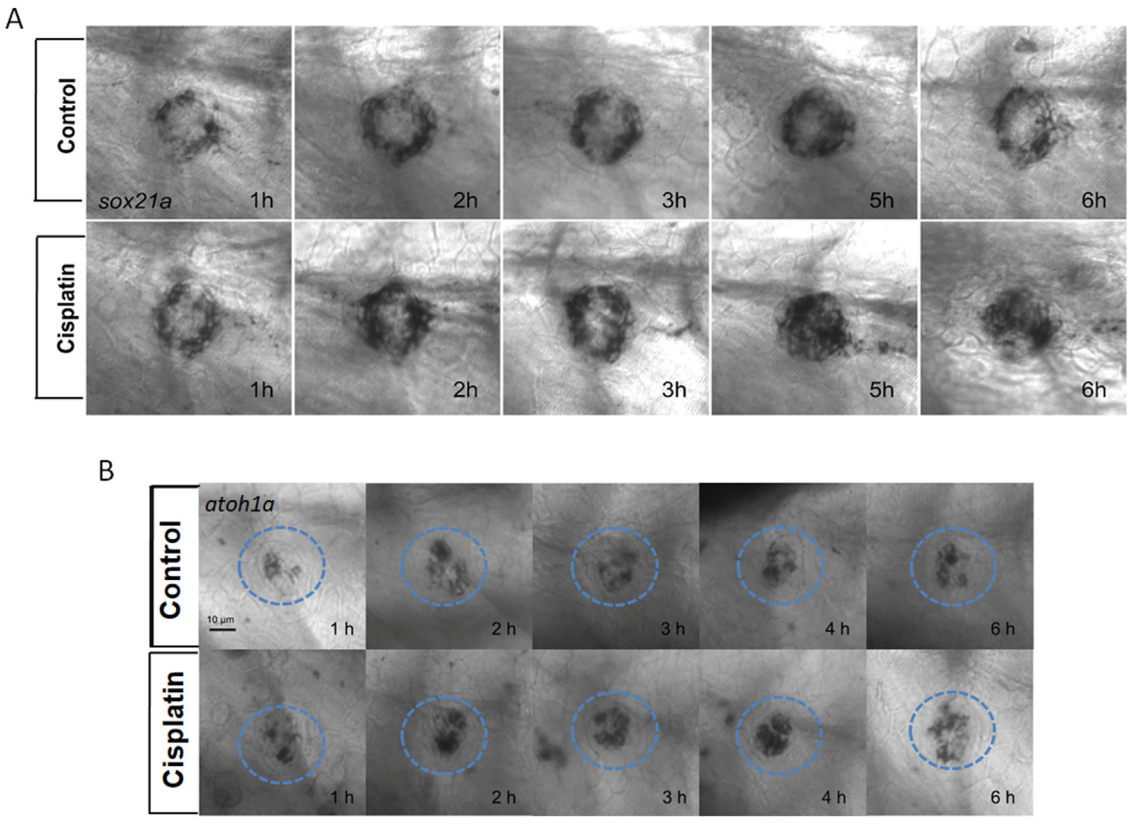

Fig. 3. The expression patterns of *sox21a* and *atoh1a*. Fish larvae were sampled one-hour interval during cisplatin insult regimen and in situ hybridization using anti-sense probe *sox21a* or *atoh1a* were made. (A) The expression pattern of *sox21a*. *sox21a* positive supporting cells moved gradually from the periphery to the center of neuromasts upon cisplatin exposure. Scale bar, 10 μm. The picture showed a representative neuromast. At least 10 fish larvae were observed in each experiment. Each experiment repeated thrice. (B) The expression pattern of *atoh1a*. *atoh1a* positive cells increased from two hours to four hours post treatment in neuromasts. Scale bar, 10 μm. Above panel, control group incubated in fish medium without cisplatin. Below panel, experimental group incubated in fish medium with cisplatin. A representative neuromast was shown. At least 10 fish larvae were observed in each group. The experiment was repeated at least three times.

catenin signaling activation, 1 μM BIO (Sigma, 361550) was added to the fish medium (Atilla-Gokcumen et al., 2006).

2.8. Statistical analysis

To provide easy access to hair cells and reduce variability in the number of hair cells found in different neuromasts, we counted the first three neuromast hair cells from the posterior lateral line in all experiments. All values were expressed as the mean ± SD, and statistical analyses were performed using Student's *t*-test. Differences were considered significant if $P < 0.05$. Each experiment was repeated three times.

3. Results

3.1. Neuromast hair cells regenerated following cisplatin insult in the zebrafish lateral line

To induce complete hair cell loss and rapid and full hair cell regeneration in zebrafish lateral line neuromasts, six hours of exposure to 1 mM cisplatin was used to damage the hair cells according to studies by Ou et al. (2007). Immunofluorescence staining using an anti-parvalbumin 3 antibody showed that occasionally one abnormal rounded-body hair cell remained after treatment (Fig. 1A and A'), which indicated that cisplatin induced almost complete mature hair cell death. Additionally, live fish staining with the vital dye YO-PRO1, which specifically labels neuromast hair cells (Santos et al., 2006), demonstrated that cisplatin treatment induced nearly complete mechanotransducive hair cell damage, albeit one abnormal hair cell undergoing the death process was occasionally observed (Fig. 1B and B'). Quantification of the remaining hair cells after cisplatin treatment is shown in Fig. 1C. Taken together, these results show that a six-hour cisplatin-induced damage regimen could induce complete functional hair cell loss in lateral line neuromasts.

The time course of hair cell regeneration following cisplatin-induced damage was analyzed. Immunofluorescence staining using anti-myosin VI revealed all the hair cells (both nascent and mature hair cells) in the neuromasts (Seiler et al., 2004). Quantification of hair cells using immunofluorescence staining with anti-myosin VI showed that hair cell regeneration occurred between 12 and 24 h after cisplatin removal and increased overtime. After 72 h, more than 90% of the hair cells were regenerated, and after 84 h, the regenerated hair cells in the neuromasts were comparable to the neuromast hair cells before treatment (Fig. 2A–A", and C). *Slc17a8* is the gene that encodes vesicular glutamate transporter 3 (VGluT3), which is a marker of neuromast hair cells in zebrafish and inner hair cells in mammals (Trapani and Nicolson, 2011). In situ hybridization using the antisense probe of *slc17a8* also demonstrated hair cell regeneration after cisplatin treatment (Fig. 2B and B'). Additionally, we observed no significant difference in the number of control neuromast hair cells during recovery (Fig. S1). Moreover, when 50 μM cisplatin was applied to the lateral line for 24 h, hair cell regeneration was delayed and started after 24 h post treatment (Fig. S2). Taken together, these results showed that a six-hour cisplatin treatment regimen can induce complete hair cell loss followed by rapid and complete hair cell regeneration in zebrafish lateral line neuromasts.

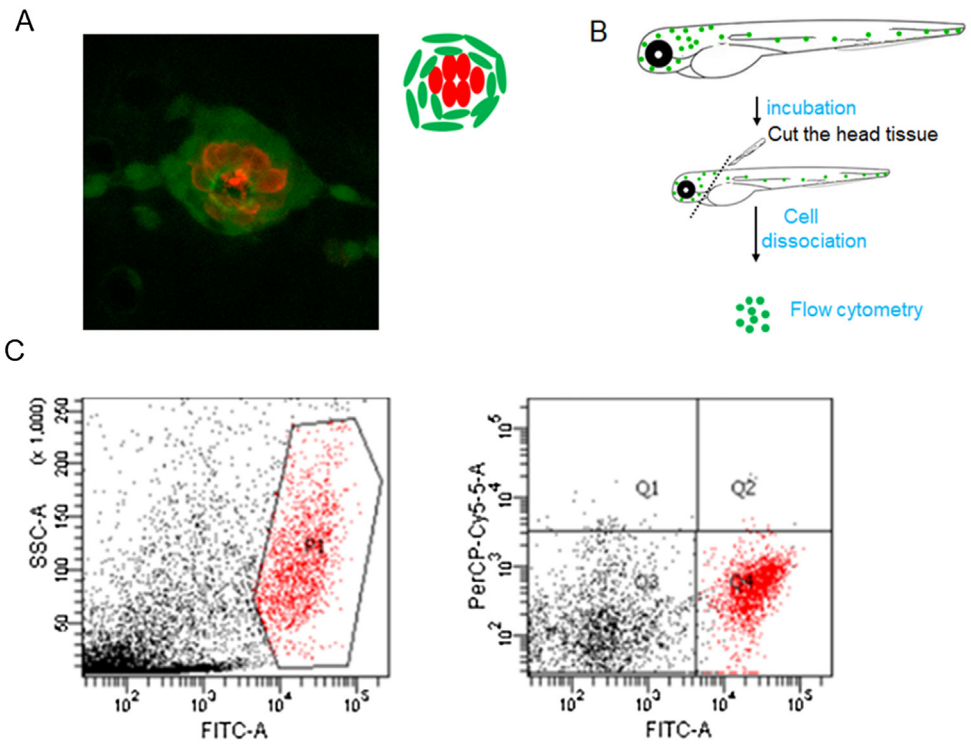

Fig. 4. Fluorescence activated cell sorting of neuromasts supporting cells. (A) A representative neuromast from transgenic line *sqet10*. Only supporting cells were labeled by GFP, hair cells were differentiated by red vital dye staining. Right is a scheme to show cell types in a neuromast. (B) Outline of FACS protocol. (C) Flow cytometry of GFP positive supporting cells. Left, FITC staining, P1 indicated the GFP positive supporting cells that were sorted. Right, perCP-Cy5-5-A staining, Q3 + Q4 (99%) indicated the alive supporting cells after sorting. (For interpretation of the references to colour in the figure legend, the reader is referred to the web version of this article.)

3.2. Neuromast supporting cells moved toward the center of the neuromasts during cisplatin exposure

To observe dynamic changes in neuromast supporting cells during cisplatin exposure, in situ hybridization of the antisense probe of *sox21a* was used to observe the dynamic patterns of the supporting cells (Lan et al., 2011). The expression pattern showed that *sox21a* was usually expressed in the peripheral supporting cells in normal neuromasts (Fig. 3A, control panel). After three hours of cisplatin treatment, *sox21a*-positive supporting cells moved toward the center of the neuromasts (Fig. 3A, cisplatin panel). We also observed that after hair cell regeneration, the expression of *sox21a* reverted to the periphery of the neuromasts (Fig. S3). This finding indicated that dynamic changes in supporting cells may occur in response to hair cell loss upon cisplatin insult.

The expression pattern of *atoh1a* showed that several *atoh1a*-positive cells were present among the supporting cells in normal larval developmental neuromasts (Fig. 3B, control panel). After cisplatin incubation, the abundance of *atoh1a*-positive cells increased from two hours to four hours post treatment (Fig. 3B, cisplatin panel; Fig. S4). These data demonstrated that the abundance of potential hair cell progenitors increased during hair cell death, suggesting that there were more supporting cells that exhibited hair cell differentiation potential at an early stage than expected.

3.3. Wnt/β-catenin signaling was activated in neuromast supporting cells during hair cell death

To examine changes in gene expression in supporting cells at a high resolution, fluorescence activated cell sorting (FACS) was used to sort the supporting cells in neuromasts during cisplatin exposure. In the *sqet10* transgenic line, supporting cells but not hair cells were specifically labeled by GFP (Fig. 4A). Five dpf *sqet10* larvae were treated with cisplatin, and after three hours of cisplatin exposure, fish larvae were harvested and head tissue was removed to exclude the anterior lateral line neuromast supporting cells (Fig. 4B). GFP-positive supporting cells were sorted according to the FITC gate, and the activity of the sorted cells was confirmed by perCP-Cy5-5-A dye staining (Fig. 4C and D).

We observed that cisplatin induced caspase 3 activation in neuromast hair cells (Fig. S5B), and previous studies revealed that caspase 3 induced Wnt/β-catenin signaling to regenerate the lost tissues (Bergmann and Steller, 2010). The Wnt/β-catenin signaling pathway is triggered by the binding of Wnt ligand to the LRP-5/6 receptors and Frizzled receptors. Dickkopfs (Dkks) serve as an extracellular Wnt inhibitor by disrupting the formation of Wnt/LRP/Frizzled complex (Mao et al., 2001). To measure the changes in expression of Wnt/β-catenin signaling genes, we first applied RT-PCR to screen the changes in factors involved in Wnt signaling. RT-PCR data showed that the expression of *wnt2* and *wnt3a* increased in cisplatin-treated supporting cells compared to the control group (Fig. 5A). We also observed slightly decreased expression of *wnt7bb* in the cisplatin-treated group (Fig. 5A). Furthermore, QPCR was used to quantify the relative changes in gene expression. The results showed that the expression of *wnt2* and *wnt3a* were increased significantly in the cisplatin-treated group, but the expression of *wnt7aa* was not significantly reduced in the cisplatin-treated supporting cells compared to the control (Fig. 5B). Additionally, in situ hybridization of *dkks* showed that *dkk1a* and *dkk2* levels were reduced in neuromast supporting cells during cisplatin exposure (Fig. 5C). Taken together, these data suggest that Wnt/β-catenin signaling is activated in supporting cells during cisplatin-induced hair cell death in neuromasts.

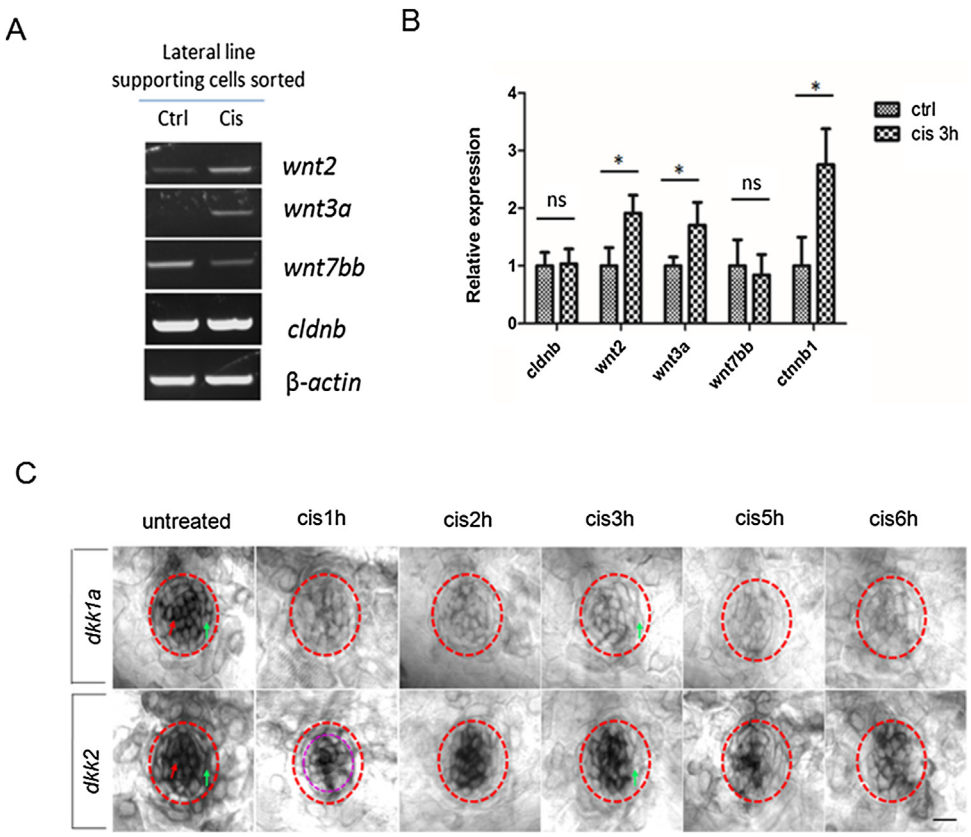

Fig. 5. Wnt/β-catenin signaling activated in neuromasts supporting cells. (A) RT-PCR in sorted supporting cells showed increased expression of *wnt2* and *wnt3a* in cisplatin-treated group compared to control group. Slightly decreased expression of *wnt7bb* was observed in cisplatin-treated group. *Cldnb* served as supporting cells "housekeep" gene. *β-actin* used as an internal control. (B) Quantification of expression changes in sorted supporting cells. *wnt2*, *wnt3a* and *ctnnb1* had significantly increased expression in cisplatin-treated group, while *wnt7bb* had not significantly reduced expression. *Cldnb* had the same expression level between two groups. *β-actin* used as an internal control. *, $P < 0.05$. ns, no significance. (C) Whole mount in situ hybridizations of *dkk1a* and *dkk2*. *dkk1a* and *dkk2* expressed in neuromasts hair cells and adjacent supporting cells in untreated fish larvae. The significantly reduced expressions were observed during cisplatin incubation time window. Above panel, *dkk1a* expression panel; below panel, *dkk2* expression panel. Red circles indicated the range of a neuromast. Purple circles indicated hair cells location. Red arrow showed hair cells. Green arrow indicated supporting cells. Scale bar, 10 μm. 10 fish in each group. The experiment was repeated at least three times. (For interpretation of the references to colour in the figure legend, the reader is referred to the web version of this article.)

3.4. Regenerated hair cells were inhibited by early Wnt/β-catenin signaling inactivation

To observe the effects of early Wnt/β-catenin signaling activation, we applied Wnt/β-catenin signaling inhibitors within the first few hours following cisplatin removal, to observe the effects on the number of regenerated hair cells. The data showed that incubation with FH535 for the first two hours significantly reduced hair cell regeneration at 48 h after cisplatin removal, and the first four hours of incubation with FH535 also led to significantly reduced hair cell regeneration compared to the control group. Additionally, we applied FH535 incubation for the first 12 h, and hair cell regeneration was significantly reduced compared to the control group (Fig. 6A and B). Moreover, XAV939, another Wnt/β-catenin signaling inhibitor, exhibited the same effect on hair cell regeneration (Fig. S6). However, addition of BIO, a specific GSK3 kinase inhibitor, did not influence the number of regenerated hair cells compared to the control group (Fig. 6A and C). These chemical compounds did not exhibit any toxicity toward the fish under the concentrations used in this study. Taken together, these findings demonstrated that early Wnt/β-catenin signaling activation is necessary but not sufficient for hair cell regeneration.

4. Discussion

Lateral line neuromast hair cell regeneration has been extensively studied after ototoxic drug treatment. However, there is little data regarding the changes in gene expression that occur during the treatment period. In our study, we subjected zebrafish lateral line neuromasts to six hours of cisplatin exposure to observe the changes in gene expression during the treatment period. We first used anti-parvalbumin 3 antibody staining and YO-PRO1 vital dye staining to demonstrate mature hair cell death in neuromasts, and we also used anti-myosin VI antibody staining to detect mature and nascent hair cells in neuromasts. Comparing the results from different labeling methods, we found that cisplatin damaged nearly all the mature hair cells but not nascent hair cells with our insult regimen. These data are consistent with a previous study that revealed that nascent hair cells were insensitive to cisplatin due to a lack of mechanotransduction (Mao et al., 2001).

Mackenzie and Raible showed that after 50 μM cisplatin treatment for 24 h, regeneration in larval zebrafish was significantly delayed and remained incomplete after 96 h (Mackenzie and Raible, 2012). In our study, after treatment with 1 mM cisplatin for six hours, larval zebrafish exhibited rapid hair cell regeneration after 12 h post treatment, and almost complete regeneration was observed after 84 h. Slattery and Warchol demonstrated that 10–20 μM cisplatin treatment for 24 h led to decreased supporting cell proliferation and transdifferentiation in cisplatin-damaged chicken basilar papilla (Slattery and Warchol, 2010). We speculated that a longer cisplatin treatment is not only toxic to hair cells but also damaged the regeneration capacity of supporting cells. In contrast to chronic cisplatin exposure, acute treatment with a high-

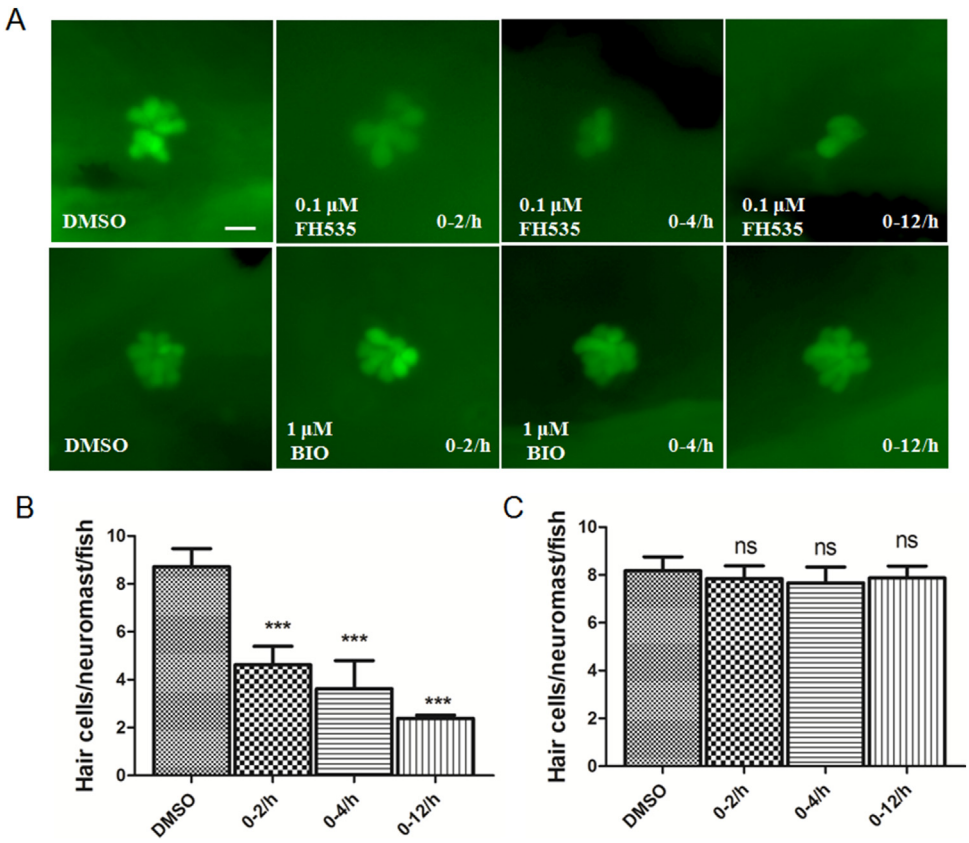

Fig. 6. Regenerated hair cells were reduced after early inhibition of Wnt/β-catenin signaling. Transgenic line *sqet4*, which labels hair cells specifically, was applied in this experiment. (A) Representative pictures showed regenerated hair cells after early hours of FH535 (inhibitor) or BIO (activator) addition during recovery time. Scale bar, 10 μm. 11 fish in each group. (B) Quantification of regenerated hair cells after FH535 addition. Comparisons were made between each group with control using student *t* test. ***, $P < 0.0001$. 11 fish in each group. (C) Quantification of regenerated hair cells after BIO addition. ns, no significance. 10 fish in each group. All experiments were repeated at least three times.

concentration cisplatin exposure regimen led to rapid and complete hair cell regeneration in the zebrafish lateral line.

A label retention assay from Cruz et al. demonstrated that differentiating supporting cells were located in the center of the neuromasts during regeneration (Cruz et al., 2015). In a study by Romero-Carvajal et al., proliferating cells and their progenies moved toward the center of neuromasts before mitosis and reverted to their original positions post mitosis in regenerating neuromasts (Romero-Carvajal et al., 2015). In our study, we observed that supporting cells moved toward the center of the neuromasts during hair cell death and back to the periphery after hair cell regeneration. We speculated that the supporting cells were prepared for hair cell regeneration upon hair cell loss. Additionally, we observed that the levels of *atoh1a*-labeled hair cell progenitors increased, which strongly supported the hypothesis that supporting cells undergo hair cell regeneration earlier than expected.

During sensory development, Wnt/β-catenin signaling governs prosensory domain specification and hair cell differentiation (Jacques et al., 2012). Jiang et al. found that Wnt/β-catenin signaling is not involved in regulating the onset of proliferation but governs proliferation at later stages of hair cell regeneration (Jiang et al., 2014). Suppression of the Wnt pathway reduced the number of regenerated hair cells and mitotic supporting cells in chick cochleae (Jiang et al., 2018). In our study, we found that the expression levels of the Wnt/β-catenin signaling ligands *wnt2* and *wnt3a* increased early during the cisplatin damage period but not those of *wnt10a*, *wnt10b* or *wnt4a*, as reported in previous studies (Blum and Begemann, 2012; Ramachandran et al., 2011). We speculated that cisplatin-induced hair cell death is a caspase-dependent mechanism that stimulates hair cell regeneration (Ding et al., 2012). Wnt/β-catenin signaling is widely involved in compensatory proliferation for tissue regeneration (Chera et al., 2009; Gurley et al., 2008; Petersen and Reddien, 2009). In Hydra head regeneration, Apoptosis is both necessary and sufficient to induce Wnt3 secretion (Chera et al., 2009). Our data and studies by Ou et al. have indicated that cisplatin damages neuromast hair cells in a caspase 3-dependent apoptotic manner (Fig. S5B) (Ou et al., 2007). We also found that the neomycin treatment regimen used by Jiang et al. did not induce caspase 3 activation (Fig. S5C). This finding might partially explain Jiang et al.'s observation that Wnt/β-catenin signaling is not involved in early stages of hair cell regeneration (Jiang et al., 2014). Previous studies have revealed that neomycin mainly induces perturbation of the mitochondrion (Owens et al., 2007).We speculated that the early Wnt/β-catenin signaling activation was a response to cisplatin-induced hair cell death, which stimulated hair cell regeneration. However, to make this claim, further research is needed. When we exposed the cells to high levels of copper, we did not observe caspase 3 activation or the accompanying early Wnt activation (Fig. S5 D–F). Our study indicated that early activation of Wnt/β-catenin signaling in supporting cells may be a unique response to caspase 3-dependent apoptotic hair cell death.

Previous studies have demonstrated that activation of Wnt/β-catenin signaling increases the number of supporting cells that return to the cell cycle in response to hair cell damage and the number of regenerated hair cells (Head et al., 2013; Jacques et al., 2014). In this study, we did not elucidate the impact of cisplatin on proliferating or transdifferentiating supporting cells, as we know that hair cell differentiation is also regulated in part by

Wnt/β-catenin signaling. We observed that both proliferating and transdifferentiating supporting cells were affected when Wnt/β-catenin signaling was inhibited (unpublished data). These results suggested that early Wnt/β-catenin signaling activation in supporting cells enabled hair cell regeneration in neuromasts after cisplatin-induced damage.

Mammals do not regenerate hair cell spontaneously but retain the potential. Bramhall et al. showed that Lgr5-positive supporting cells can serve as hair cell progenitors in the postnatal mouse cochlea following ototoxic damage (Bramhall et al., 2014). Li et al. demonstrated that Notch inhibition removes the brakes on Wnt/β-catenin signaling, thus promotes $Lgr5^{+}$ progenitor cells to mitotically regenerate new hair cells in mammalian cochlea (Li et al., 2015). Our study showed that an early Wnt activation in neuromasts supporting cells is necessary for hair cell regeneration following cisplatin insult. Our results also indicated that cisplatin-induced cell death triggers Wnt-dependent hair cell regeneration in zebrafish lateral line. Our findings will give a hint in the try to induce hair cell regeneration after cisplatin ototoxicity in mammals.

Taken together, these results demonstrated that a regimen of cisplatin exposure could induce complete hair cell loss followed by rapid and complete hair cell regeneration. We further evaluated the expression pattern of genes involved in neuromast supporting cells at high resolution by FACS. Wnt/β-catenin signaling was activated in supporting cells upon hair cell death induced by cisplatin. Early inhibition of Wnt/β-catenin signaling led to reduced hair cell regeneration, but early activation of Wnt/β-catenin signaling had no effect on the number of regenerated hair cells. Other signaling pathways, such as Notch and FGF, should be considered in future studies. Overall, we demonstrated that early Wnt/β-catenin signaling activation in supporting cells is necessary but not sufficient for hair cell regeneration. Our study will broaden our knowledge regarding the role of Wnt/β-catenin signaling in hair cell regeneration.

5. Conclusion

Studies on the gene expression patterns in supporting cells during hair cell death are scarce. Wnt/β-catenin signaling has been widely studied in late stages of hair cell regeneration. The results presented here demonstrate that Wnt/β-catenin signaling was activated in neuromast supporting cells during hair cell death earlier than expected. Early activation of Wnt/β-catenin signaling was found to be necessary for hair cell regeneration. This study broadens our knowledge regarding the role of Wnt/β-catenin signaling in hair cell regeneration.

Ethical statement

The use of animals was in accordance with the Guidelines on the Handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare (UFAW). The experimental protocol was in accordance with the principles outlined in the Declaration of Helsinki.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aanat.2019.07.001.

References

Adler, H.J., Raphael, Y., 1996. New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear. Neurosci. Lett. 205, 17–20, http://dx.doi.org/10.1016/0304-3940(96)12367-3.

Atilla-Gokcumen, G.E., Williams, D.S., Bregman, H., et al., 2006. Organometallic compounds with biological activity: a very selective and highly potent cellular inhibitor for glycogen synthase kinase 3. ChemBioChem 7, 1443–1450, http://dx.doi.org/10.1002/cbic.200600117.

Baird, R.A., Steyger, P.S., Schuff, N.R., 1996. Mitotic and nonmitotic hair cell regeneration in the bullfrog vestibular otolith organs. Ann. N. Y. Acad. Sci. 781, 59–70, http://dx.doi.org/10.1111/j.1749-6632.1996.tb15693.x.

Barbazuk, W.B., Korf, I., Kadavi, C., et al., 2000. The syntenic relationship of the zebrafish and human genomes. Genome Res. 10, 1351–1358, http://dx.doi.org/10.1101/gr.144700.

Bergmann, A., Steller, H., 2010. Apoptosis, stem cells, and tissue regeneration. Sci. Signal. 3, re8, http://dx.doi.org/10.1126/scisignal.3145re8.

Blum, N., Begemann, G., 2012. Retinoic acid signaling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration. Development 139, 107–116, http://dx.doi.org/10.1242/dev.065391.

Bramhall, N.F., Shi, F., Arnold, K., Hochedlinger, K., Edge, A.S., 2014. Lgr5-positive supporting cells generate new hair cells in the postnatal cochlea. Stem Cell Rep. 2, 311–322, http://dx.doi.org/10.1016/j.stemcr.2014.01.008.

Bylund, M., Andersson, E., Novitch, B.G., et al., 2003. Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nat. Neurosci. 6, 1162–1168, http://dx.doi.org/10.1038/nn1131.

Chera, S., Ghila, L., Dobretz, K., et al., 2009. Apoptotic cells provide an unexpected source of Wnt3 signaling to drive hydra head regeneration. Dev. Cell 17, 279–289, http://dx.doi.org/10.1016/j.devcel.2009.07.014.

Cruz, I.A., Kappedal, R., Mackenzie, S.M., et al., 2015. Robust regeneration of adult zebrafish lateral line hair cells reflects continued precursor pool maintenance. Dev. Biol. 402, 229–238, http://dx.doi.org/10.1016/j.ydbio.2015.03.019.

Ding, D., Allman, B.L., Salvi, R., 2012. Review: ototoxic characteristics of platinum antitumor drugs. Anat. Rec. (Hoboken) 295, 1851–1867, http://dx.doi.org/10.1002/ar.22577.

Frisina, R.D., Wheeler, H.E., Fossa, S.D., et al., 2016. Comprehensive audiometric analysis of hearing impairment and tinnitus after cisplatin-based chemotherapy in survivors of adult-onset cancer. J. Clin. Oncol. 34, 2712–2720, http://dx.doi.org/10.1200/JCO.2016.66.8822.

Giari, L., Dezfuli, B.S., Astolfi, L., et al., 2012. Ultrastructural effects of cisplatin on the inner ear and lateral line system of zebrafish (*Danio rerio*) larvae. J. Appl. Toxicol. 32, 293–299, http://dx.doi.org/10.1002/jat.1691.

Goldsmith, J.R., Jobin, C., 2012. Think small: zebrafish as a model system of human pathology. J. Biomed. Biotechnol. 2012, 817341, http://dx.doi.org/10.1155/2012/817341.

Groves, A.K., Zhang, K.D., Fekete, D.M., 2013. The genetics of hair cell development and regeneration. Annu. Rev. Neurosci. 36, 361–381, http://dx.doi.org/10.1146/annurev-neuro-062012-170309.

Gurley, K.A., Rink, J.C., Sanchez Alvarado, A., 2008. Beta-catenin defines head versus tail identity during planarian regeneration and homeostasis. Science 319, 323–327, http://dx.doi.org/10.1126/science.1150029.

Harris, J.A., Cheng, A.G., Cunningham, L.L., et al., 2003. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). J. Assoc. Res. Otolaryngol. 4, 219–234, http://dx.doi.org/10.1007/s10162-002-3022-x.

Head, J.R., Gacioch, L., Pennisi, M., et al., 2013. Activation of canonical Wnt/beta-catenin signaling stimulates proliferation in neuromasts in the zebrafishposterior lateral line. Dev. Dyn. 242, 832–846, http://dx.doi.org/10.1002/dvdy.23973.

Hernandez, P.P., Olivari, F.A., Sarrazin, A.F., et al., 2007. Regeneration in zebrafish lateral line neuromasts: expression of the neural progenitor cell marker sox2 and proliferation-dependent and-independent mechanisms of hair cell renewal. Dev. Neurobiol. 67, 637–654, http://dx.doi.org/10.1002/dneu.20386.

Huang, S.M., Mishina, Y.M., Liu, S., et al., 2009. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461, 614–620, http://dx.doi.org/10.1038/nature08356.

Jacques, B.E., Puligilla, C., Weichert, R.M., et al., 2012. A dual function for canonical Wnt/beta-catenin signaling in the developing mammalian cochlea. Development 139, 4395–4404, http://dx.doi.org/10.1242/dev.080358.

Jacques, B.E., Montgomery, W.H.t., Uribe, P.M., et al., 2014. The role of Wnt/beta-catenin signaling in proliferation and regeneration of the developing basilar papilla and lateral line. Dev. Neurobiol. 74, 438–456, http://dx.doi.org/10.1002/dneu.22134.

Jiang, L., Romero-Carvajal, A., Haug, J.S., et al., 2014. Gene-expression analysis of hair cell regeneration in the zebrafish lateral line. Proc. Natl. Acad. Sci. U. S. A. 111, E1383–92, http://dx.doi.org/10.1073/pnas.1402898111.

Jiang, L., Xu, J., Jin, R., et al., 2018. Transcriptomic analysis of chicken cochleae after gentamicin damage and the involvement of four signaling pathways (Notch,

FGF, Wnt and BMP) in hair cell regeneration. Hear. Res. 361, 66–79, http://dx.doi.org/10.1016/j.heares.2018.01.004.
Knight, K.R., Chen, L., Freyer, D., et al., 2017. Group-wide, prospective study of ototoxicity assessment in children receiving cisplatin chemotherapy (ACCL05C1): a report from the children's oncology group. J. Clin. Oncol. 35, 440–445, http://dx.doi.org/10.1200/JCO.2016.69.2319.
Kniss, J.S., Jiang, L., Piotrowski, T., 2016. Insights into sensory hair cell regeneration from the zebrafish lateral line. Curr. Opin. Genet. Dev. 40, 32–40, http://dx.doi.org/10.1016/j.gde.2016.05.012.
Lan, X., Wen, L., Li, K., et al., 2011. Comparative analysis of duplicated sox21 genes in zebrafish. Dev. Growth Differ. 53, 347–356, http://dx.doi.org/10.1111/j.1440-169X.2010.01239.x.
Li, W., Wu, J., Yang, J., Sun, S., Chai, R., Chen, Z.Y., Li, H., 2015. Notch inhibition induces mitotically generated hair cells in mammalian cochleae via activating the Wnt pathway. Proc. Natl. Acad. Sci. U. S. A. 112, 166–171, http://dx.doi.org/10.1073/pnas.1415901112.
Lopez-Schier, H., Hudspeth, A.J., 2006. A two-step mechanism underlies the planar polarization of regenerating sensory hair cells. Proc. Natl. Acad. Sci. U. S. A. 103, 18615–18620, http://dx.doi.org/10.1073/pnas.0608536103.
Lush, M.E., Piotrowski, T., 2014. Sensory hair cell regeneration in the zebrafish lateral line. Dev. Dyn. 243, 1187–1202, http://dx.doi.org/10.1002/dvdy.24167.
Mackenzie, S.M., Raible, D.W., 2012. Proliferative regeneration of zebrafish lateral line hair cells after different ototoxic insults. PLoS One 7, e47257, http://dx.doi.org/10.1371/journal.pone.0047257.
Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., Niehrs, C., 2001. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature 411, 321–325, http://dx.doi.org/10.1038/35077108.
Ou, H.C., Raible, D.W., Rubel, E.W., 2007. Cisplatin-induced hair cell loss in zebrafish (*Danio rerio*) lateral line. Hear. Res. 233, 46–53, http://dx.doi.org/10.1016/j.heares.2007.07.003.
Owens, K.N., Cunningham, D.E., MacDonald, G., et al., 2007. Ultrastructural analysis of aminoglycoside-induced hair cell death in the zebrafish lateral line revealsan early mitochondrial response. J. Comp. Neurol. 502, 522–543, http://dx.doi.org/10.1002/cne.21345.
Parinov, S., Kondrichin, I., Korzh, V., et al., 2004. Tol2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes in vivo. Dev. Dyn. 231, 449–459, http://dx.doi.org/10.1002/dvdy.20157.
Petersen, C.P., Reddien, P.W., 2009. A wound-induced Wnt expression programcontrols planarian regeneration polarity. Proc. Natl. Acad. Sci. U. S. A. 106, 17061–17066, http://dx.doi.org/10.1073/pnas.0906823106.
Pisano, G.C., Mason, S.M., Dhliwayo, N., et al., 2014. An assay for lateral line regeneration in adult zebrafish. J. Vis. Exp. 86, http://dx.doi.org/10.3791/51343.
Presson, J.C., Smith, T., Mentz, L., 1995. Proliferating hair cell precursors in the ear of a postembryonic fish are replaced after elimination by cytosine arabinoside. J. Neurobiol. 26, 579–584, http://dx.doi.org/10.1002/neu.480260410.
Presson, J.C., Lanford, P.J., Popper, A.N., 1996. Hair cell precursors are ultrastructurally indistinguishable from mature support cells in the ear of a post-embryonic fish. Hear. Res. 100, 10–20, http://dx.doi.org/10.1016/0378-5955(96)00109-8.
Ramachandran, R., Zhao, X.F., Goldman, D., 2011. Ascl1a/Dkk/beta-catenin signaling pathway is necessary and glycogen synthase kinase-3beta inhibition is sufficient for zebrafish retina regeneration. Proc. Natl. Acad. Sci. U. S. A. 108, 15858–15863, http://dx.doi.org/10.1073/pnas.1107220108.
Romero-Carvajal, A., Navajas Acedo, J., Jiang, L., et al., 2015. Regeneration of sensory hair cells requires localized interactions between the notch and Wnt pathways. Dev. Cell 34, 267–282, http://dx.doi.org/10.1016/j.devcel.2015.05.025.
Santos, F., MacDonald, G., Rubel, E.W., et al., 2006. Lateral line hair cell maturation is a determinant of aminoglycoside susceptibility in zebrafish (*Danio rerio*). Hear. Res. 213, 25–33, http://dx.doi.org/10.1016/j.heares.2005.12.009.
Sarrazin, A.F., Villablanca, E.J., Nunez, V.A., et al., 2006. Proneural gene requirement for hair cell differentiation in the zebrafish lateral line. Dev. Biol. 295, 534–545, http://dx.doi.org/10.1016/j.ydbio.2006.03.037.
Seiler, C., Ben-David, O., Sidi, S., et al., 2004. Myosin VI is required for structural integrity of the apical surface of sensory hair cells in zebrafish. Dev. Biol. 272, 328–338, http://dx.doi.org/10.1016/j.ydbio.2004.05.004.
Slattery, E.L., Warchol, M.E., 2010. Cisplatin ototoxicity blocks sensory regene-ration in the avian inner ear. J. Neurosci. 30, 3473–3481, http://dx.doi.org/10.1523/JNEUROSCI.4316-09.2010.
Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protoc. 3, 59–69, http://dx.doi.org/10.1038/nprot.2007.514.
Thomas, A.J., Wu, P., Raible, D.W., et al., 2015. Identification of small molecule inhibitors of cisplatin-induced hair cell death: results of a 10,000 compound screen in the zebrafish lateral line. Otol. Neurotol. 36, 519–525, http://dx.doi.org/10.1097/MAO.0000000000000487.
Trapani, J.G., Nicolson, T., 2011. Mechanism of spontaneous activity in afferent neurons of the zebrafish lateral-line organ. J. Neurosci. 31, 1614–1623, http://dx.doi.org/10.1523/JNEUROSCI.3369-10.2011.
Villegas, R., Martin, S.M., O'Donnell, K.C., et al., 2012. Dynamics of degeneration and regeneration in developing zebrafish peripheral axons reveals a requirement for extrinsic cell types. Neural Dev. 7, 19, http://dx.doi.org/10.1186/1749-8104-7-19.
Williams, J.A., Holder, N., 2000. Cell turnover in neuromasts of zebrafish larvae. Hear. Res. 143, 171–181, http://dx.doi.org/10.1016/S0378-5955(00)00039-3.