5-2012

The Time-Course of the Effects of Growth Hormone During Zebrafish (*DANIO RERIO*) Auditory Hair Cell Regeneration

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THE TIME-COURSE OF THE EFFECTS OF GROWTH HORMONE DURING ZEBRAFISH (*DANIO RERIO*) AUDITORY HAIR CELL REGENERATION

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Yajie Wang

May 2012

THE TIME-COURSE OF THE EFFECTS OF GROWTH HORMONE DURING ZEBRAFISH (*DANIO RERIO*) AUDITORY HAIR CELL REGENERATION

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I dedicate this thesis to my parents, Ding Wang and Junyue Niu, who are a great inspiration to me.

ACKNOWLEDGMENTS

I am extremely grateful to my professor Dr. Michael Smith for offering the opportunity for me to learn from him and all the help he has given to me in my graduate study. I also wish to thank Dr. Huifang Sun for assisting in the design and completion of my experiments. Dr. Huifang Sun also patiently trained me in the art of zebrafish inner ear dissection. I am also grateful to Amanda Webb for help with maintenance of fish used in this experiment, Chia-Hui Lin and Julie Schuck for doing the previous work which was the foundation of this research, and all the other members in the Smith lab for their support. Furthermore, I want to thank my parents for their support and love, so that I could finish my graduate study in the United States. This research was supported by NIH INBRE grant P20 RR-16481 and the WKU Biotechnology Center.

CONTENTS

LIST OF FIGURES

LIST OF TABLE

THE TIME-COURSE OF THE EFFECTS OF GROWTH HORMONE DURING ZEBRAFISH (*DANIO RERIO*) AUDITORY HAIR CELL REGENERATION

Yajie Wang May 2012 37 Pages

Directed by: Michael E. Smith, Nancy A. Rice, and Claire A. Rinehart

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Growth hormone (GH) was upregulated in the zebrafish inner ear following sound exposure in a previous study. To identify the specific role of GH in hair cell regeneration and the possible cellular mechanisms of this regeneration, groups of zebrafish were divided into baseline (no sound exposure, no injection), buffer-injected and GH-injected groups. Buffer- and GH-injected fish were exposed to a 150 Hz tone at a source level of 179 dB re 1 μPa root mean squared (RMS) for 36 h. Phalloidin-staining was used to assess the effects of GH on hair cell bundle density; BrdU-labeling was used to assess the effects of GH on cellular proliferation; TUNEL-labeling was used to assess the effects of GH on apoptosis in the zebrafish inner ear following acoustic trauma. The time-course of hair cell bundle density, cell proliferation, and apoptosis was established by combining data for baseline fishes and sound-exposed fishes at post-sound exposure day 1 (psed1), psed2, and psed3. GH-injected fish exhibited greater densities of hair cells than buffer-injected controls. In addition, GH-injected fish had higher levels of cell proliferation and lower levels of apoptosis than buffer-injected controls. This suggests that GH may play an important role in zebrafish inner ear hair cell regeneration by stimulating cellular proliferation and inhibiting cellular apoptosis.

Introduction

Deafness is a worldwide problem with tremendous societal costs. In 2004, over 250 million people worldwide had moderate to profound hearing impairment (Smith, 2004). But there still is no effective therapeutic which can successfully treat deaf patients. Hearing impairment can be caused by the loss of auditory hair cells; these are the sensory hair cells in the organ of Corti in the cochlea of inner ear. Auditory hair cells can be damaged by exposure to long or prolonged noise (Lim, 1976; Lindeman and Bredberg, 1972; Stockwell et al., 1969), ototoxic drugs (Lim, 1976; Theopold, 1977), infection, autoimmune disorders, and aging (Keithley and Feldman, 1982). In order to develop treatments and prevention for deafness, an intensive understanding of the process of auditory hair cell regeneration of lost hair cells must be established.

Noise-exposure can cause temporary loss of hearing ability, or a temporary threshold shift (Lonsbury-Martin et al., 1987). Permanent noise-induced hearing loss (NIHL), or a permanent threshold shift, is caused by exposure to very loud sounds or to sounds of lower intensity for a long period of time (Saunders et al., 1991). Long term exposure and high levels of sound have been shown to have negative impacts on all sound-detecting vertebrates (Busnel and Fletcher, 1978; Richardson et al., 1995).

In mammalian utricular hair cells exposed to acoustic trauma, new hair cells can be formed via mitosis *in vitro* (Warchol et al., 1993; Zheng et al., 1997), as well as via non-mitotic differentiation of supporting cells *in vivo* (Rubel et al., 1995). Unfortunately, cochlear hair cells cannot be replaced spontaneously following trauma in the organ of Corti (Richardson et al., 1995).

In contrast, non-mammalian vertebrates such as fish and birds can spontaneously regenerate hair cells after damage in both vestibular and auditory portions of the inner ear (Cotanche, 1987b; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Lombarte et al., 1993; Weisleder and Rubel, 1993; Smith et al., 2006). During evolution, the auditory sensory epithelium of the mammalian cochlea lost its capability of regeneration (Albert et al., 2008), even though limited hair cell regeneration has been reported in mammalian utricular hair cells (Warchol et al., 1993). In contrast, non-mammalian sensory tissues have retained the ability to regenerate hair cells. Such regeneration has been found in the lateral line and inner ear of fish and amphibians and in the avian vestibule and basilar papilla (Lombart et al., 1993; Cotanche et al., 1994; Song et al., 1995; Corwin and Oberholtzr 1997; Cotanche 1999) and also in the avian cochlea (Cotanche, 1999). The damaged hair cells can be replaced by regenerative proliferation of supporting cells, and by trans-differentiation of supporting cells into hair cells in anamniotes and in birds (Staecker and Van De Water, 1998; Corwin and Oberholtzer, 1977). Hair cell regeneration also has been observed in the saccule and lateral line of urodele amphibians (Jones and Corwin, 1996; Taylor and Forge, 2005).

In the fish auditory system, there are three semicircular canals which respond to changes in head position and three pairs of otolithic end organs (the utricle, lagena, and saccule). These organs respond to changes in the head position and, one or more of the end organs also serve as a sound detector (Popper et al., 2003). They contain sensory hair cells found in patches referred to as maculae (Fay and Potter, 2000), which are similar to those found in the ears of all vertebrates. Like urodele amphibians, hair cell regeneration has also been found in the goldfish saccule (Smith et al., 2006), oscar (*Astronotus*

ocellatus) utricle and lagena (Lombarte et al., 1993), and zebrafish (*Danio rerio*) lateral line (Harris et al., 2003). The short and long-term effects of noise exposure on goldfish have been examined. Acoustic trauma can cause noise-induced hearing loss in goldfish, but recovery of hearing was found after acoustic trauma (Smith et al., 2004a, 2004 b; Smith et al., 2006). After two days of noise exposure, significant saccular hair cell and hearing loss occurred, but significant recovery of both hair cells and hearing capabilities occurred after seven days. Schuck and Smith (2009) also found that the number of hair cell bundles recovered to control levels in the zebrafish saccule after 14 days post-noise exposure.

Because regeneration of hair cells after noise exposure happens spontaneously in fishes and birds, an understanding of the process of auditory hair cell regeneration must be established before it can be translated to human treatments for deafness. Non-human animal models are used to study this phenomenon, because their genetics can be controlled and humans cannot be exposed to controlled acoustic trauma. Birds are excellent models for studying noise-induced hearing loss, but because of the long generation times, husbandry difficulties, and a significant lack of genomic data, it is not an optimal animal model.

In contrast, zebrafish (*Danio rerio*) is an excellent animal model for vertebrate inner ear development and deafness (Whitfield, 2002) and genetic disease associated with hearing loss (Ernest et al., 2000). In zebrafish, the basic structure and function of the inner ear is similar to that of other vertebrates (Popper and Fay, 1999), and mammals share homologous genes with zebrafish which have been identified to affect the structure and function of the inner ear. In addition, zebrafish has readily accessible transparent

embryos which can be cloned and rapidly screened for mutation (Sprague et al., 2001). Many genetic mutations related to inner ear development have been identified (Malicki et al., 1996), and examples of genes that are related to inner ear development include *Foxi1*, *Atoh1*, and *MyosinVIIA*. *Foxi1* (aka Fkh10) expressed in otic precursor cells is necessary for normal inner ear development in both mice (Hulander et al., 1998, 2003) and zebrafish (Solomon et al., 2003); *Atoh1* (atonal homolog 1) known as *Math1* is a key regulator of differentiation of precursor cells that become hair cells in mice (Bermingham et al., 1999; Zheng and Gao, 2000). The *mariner* phenotype of the *circler* zebrafish mutant has been shown to be defective in myosin VIIA (Ernest et al., 2000). Thus, zebrafish is a useful animal model to observe human hereditary deafness and into the pathways of hair cell regeneration (Ernest et al., 2000).

Furthermore, details of structure and function of the auditory system in different vertebrate groups has been examined, and Fay and Popper (2000) found that the basic mechanisms for detection and processing sound are similar in all of these animals. Thus, understanding factors that damage and regenerate sensory cells of zebrafish may lead to a good understanding of the causes and potential remediation of hearing loss in humans.

Damaged hair cells can be replaced by regenerative proliferation of supporting cells, and by trans-differentiation of supporting cells into hair cells in anamniotes and in birds (Steacker and Van De Water, 1998; Corwin and Oberholtzer, 1997). Because the supporting cells in the inner ear epithelium play a role as the progenitors for the hair cells (Corwin and Cotanche, 1988; Balak et al., 1990; Raphael and Altschuler, 1991a; Weisleder and Rubel, 1992), the proliferation of the supporting cells is essential for the replacement of the damaged hair cells.

The trans-differentiation of some supporting cells into hair cells in both lower vertebrates (Adler and Raphael, 1996; Jones and Corwin, 1996) and mammals (Li and Forge, 1996), along with the proliferation of supporting cells in both avian (Cotanche et al., 1994) and mammalian inner ear epithelia (Warchol et al., 1993), both play important roles in hair cell regeneration. Understanding the proliferation process of the inner ear supporting cells may lead to potential treatments for hearing loss.

Hemopoietic and nervous systems growth factors are important in cell proliferation and differentiation (Anderson, 1989; Cattaneo and McKay, 1990; Gao et al., 1995; Ghosh and Greenberg, 1995; Vicario-Abejon et al., 1995). More recently, growth factors have also been found have the ability to promote proliferation in auditory cells following damage. Several fibroblast growth factor (FGF) family members, including insulin-like growth factors-1 and -2 (IGF-1, IGF-2), transforming growth factor-alpha (TGF-α), and epidermal growth factor (EGF), stimulate proliferation of mammalian utricular epithelial cells (Zheng et al., 1997). The function of some growth factors is to act as both a mitogen and a differentiating factor. Some growth factors function at regulating only one of the developmental steps; while the others may work in a sequential way to control the final cell phenotype. The determination of the possible influences of growth factors on proliferation and differentiation of progenitor cells is usually done in cell cultures.

Growth hormone (GH) is a secretory protein from the anterior pituitary gland (Tanner 1972; Okada and Kopchick, 2001). It is a member of the cytokine superfamily of polypeptide regulators (Bravo and Health, 2000) and affects growth, cellular

proliferation, differentiation, and metabolism (Isaksson and Jansson, 1982; Davidson, 1987; Casanueva, 1992).

GH can affect regeneration in a variety of tissue types. GH can promote liver regeneration (Pennisi et al., 2004), bone regeneration (Isaksson and Jansson, 1982) and muscle regeneration (Ullman et al., 1989). It has also been used to promote rat nerve regeneration (Kanje et al., 1988; Jung et al., 1998). Even though the effects of GH on specific tissues in fish have not been examined, GH is known to increase growth rate and muscle mass in zebrafish (Biga and Goetz, 2006).

Our previous RT-PCR and microarray analysis of inner ear tissues of noise-exposed zebrafish showed that growth hormone (GH) was strikingly upregulated during the process of hair cell regeneration induced by sound exposure, coincident with a peak of cellular proliferation. This result indicated that growth hormone may play a significant role in hair cell regeneration in zebrafish. The purpose of this research is to examine the time course of the effects of GH on auditory hair cell regeneration after acoustic trauma to the zebrafish inner ear.

Materials and Methods

Experimental animals:

One hundred and fourteen fish were acquired by commercial suppliers (Fishey Business, Bowling Green, KY) and maintained in 170-L flow-through aquarium under constant temperature (25 °C) and a 12 hours light/12 hours dark cycle. All experiments were done under the approval of the Institutional Animal Care and Use Committee of Western Kentucky University.

Experimental design

Six fish for each group were exposed to sound (see *Acoustic exposure* below) and then immediately injected intraperitoneally with either carp recombinant growth hormone (GH) (20 μg/gram of body mass) or buffer (0.1 M, pH 7.4 phosphate buffer) and then placed in a recovery tank. At 1, 2, or 3 days following acoustic exposure, inner ears were dissected from the fish. Saccular hair cell densities were quantified by counting phalloidin-labeled stereocilia bundles at five specified locations (5%, 25%, 50%, 75% and 90% along the rostral-causal axis of the saccule). At 1, 2, 3 days post- sound exposure, cellular proliferation in the saccule, lagena and utricle was tested by BrdU-labeling to assess the effects of GH on inner ear proliferation post-acoustic trauma. The three time points were chosen since our previous studies showed a peak in cell proliferation in the zebrafish saccule at 2 days post-trauma (Schuck and Smith, 2009) and near-complete hearing recovery occurs between 7 and 14 days post-trauma (Smith et al., 2004a, Smith et al., 2006). At 1 and 2 days post-sound exposure, cellular apoptosis was tested by TUNEL-labeling. The reason that we did not perform TUNEL labeling on PSED3 (post sound exposure day 3) was that TUNEL labeling in the treated group returned to baseline levels at the second day post sound exposure. A group of 6 fish without sound exposure and any injection were dissected and the saccules were subjected to phalloidin, BrdU and TUNEL staining. The results of this group were used as a non-sound exposed baseline control.

Growth hormone injection

Carp recombinant growth hormone (GH) powder (Pro-Spec-Tany Technogen Ltd., Israel) was dissolved into Nanopure water at a final concentration of 0.5 μg/μl. This GH

solution was injected to fish immediately after sound exposure (see *Acoustic exposure* below). A light dose (approximately 10μg in 1 ml tap water) of tricaine methanesulfonate (MS-222, Argent, Redmond, WA) was used to sedate fish and then each fish was injected intraperitoneally with 20 μg carp GH per gram of body mass. Fish were allowed to recover from injection in a small container containing fish tank water. After fish recovered to normal movement, they were put back to their normal aquaria until needed for specific experimental time points.

Acoustic exposure

Zebrafish were exposed to a 150 Hz tone at a source level of 179 dB re 1 μPa root mean squared (RMS). The tone was produced by a function generator (4017A, B&K Precision) attached to a 5.3 amp/200 watt Audiosource monoblock amplifier and an underwater speaker (University Sound UW-30). All fish were exposed for 36 hours at 25ºC and placed in a 19-L sound exposure chamber.

Determination of hair cell bundle density (phalloidin-labeling)

After sound exposure and injection with growth hormone (GH) or buffer, fish were euthanasized via excess tricaine methanesulfonate (MS-222, Argent, Redmond, WA) (n=6 per group). Fish heads were cut off from the bodies and fixed in 4% paraformaldehyde at 4°C overnight. After that, the fish heads were washed with 0.1 M pH 7.4 phosphate buffer (PB) for 3 X 10 minutes. Then the inner ear maculae (saccules, lagenaes, and utricles) were dissected out of the head and excess tissue was trimmed. All tissue was incubated in concavity wells with 1:100 fluorescein phalloidin (F432, Invitrogen) in phosphate buffer (PB) at room temperature in a dark box for 1 hour. After incubation, end organs were transferred to glass slides and mounted with Prolong Gold

Antifade DAPI mount reagent (P36931, Invitrogen) and then cover-slipped. Low power images (10 X objectives) of the saccule, lagenae and utricle were viewed under FITC and DAPI filters of a Zeiss Axioplan 2 epifluorescent microscope and photographed with an AxioCam MRm camera. The phalloidin-labeled hair cell stereocilia flouresced green under the FITC filter. A cell nucleus was visualized under DAPI staining (blue color). Hair cell number counts were obtained from 5 preselected locations of the saccular epithelium as in previous studies (Figure 1). These locations were at 5%, 25%, 50%, 75% and 90% of the distance along the rostral-causal axis of saccule, and were an area of 30X30 µm. Numbers and types of hair cells were counted in each of these pre-selected areas. The boxes presenting 30X30 µm were placed over digital images in Photoshop for counting purposes and consistency of area of epithelia counted. Four types of hair cells were categorized by the status of the stereocilia. Intact hair cells were cells with standard numbers and lengths of stereocilia. Damaged hair cells were the cells with few, fractured, or fused stereocilia. Bundleless hair cells (or cuticular plates) were cells without any stereocilia where only the cuticular plate was left. Newly formed hair cells were the cells with compact, well-ordered, but much shorter stereocilia compared to intact hair cells.

Determination of cell proliferation (BrdU-labeling)

Following acoustic trauma and injection of GH or buffer, fish were placed in the tank to recover for 8 hours. According to the standard protocol of Amersham Cell Proliferation Kit (RPN20LR, GE Healthcare), after 8 hours the fish were taken out to be intraperitoneally injected with Bromodeoxyuridine (BrdU). BrdU is a synthetic thymidine analog and can be incorporated into cellular DNA during S-phase, thus allowing to detection of cell proliferation. Sixteen hours later (at PSED 1), fish were

euthanized with an excess of MS-222 and then their heads were removed and their inner ears were immediately dissected out and fixed in 4% paraformaldehyde for 1 hour. After washing with 0.1 M PB, the excess tissue was trimmed off and the intact saccules, lagenaes, and utricles were placed on adhesive poly L-Lysine coated slides and then incubated for 1 hour at room temperature in mouse monoclonal anti-BrdU antibody. After washing 3 X 5 minutes again in 0.1 M PB, tissues were incubated with 1:200 Alexa Fluor 488- conjugated goat anti-mouse IgG (A11001, Invitrogen) for 1 hour at room temperature in a dark box. Lastly, the tissues were washed with 0.1 M PB for 3 X 5 minutes, then these end organs were mounted with Prolong Gold Antifade reagent with DAPI (P36931, Invitrogen) and then cover-slipped. Low (10X objective) and high (100X objective) power images of the saccule, lagenae and utricle were viewed under FITC and DAPI filter of a Zeiss Axioplan 2 epifluorescent microsecope and merged pictures were photographed by the AxioCam MRm camera. The qualitative evaluation of cell proliferation was determined by counting Alexa Fluor 488-labeled cells in the whole saccule, laganea, and utricle under 10X objective lens. A 100X objective lens was used to show the nuclear co-localization of the BrdU labeling and DAPI staining.

Determination of cell death (TUNEL-labeling)

To detect apoptotic hair cell following acoustic trauma, treated fish were euthanized with an overdose of MS-222 (n=6 per group), and the heads were cut off. Their inner ears were dissected out and immediately fixed in 4% paraformaldehyde and processed for TUNEL-labeling. After washing with 0.1 M PB, the intact saccules, lagenaes, and utricles were then trimmed out from the ears and placed on adhesive poly L-Lysine coated slides and following the standard instruction of ApopTag Fluorescein In

Situ Apoptosis Detection Kit (S7710, Millipose). Tissues were post-fixed in pre-chilled ethanol: acetic acid 2:1 for 6 minutes at -20°C and then washed 3 X 5 minutes with 0.1 M PB. Then the tissue was incubated with equilibration buffer on the slide and incubated at room temperature for at least 10 seconds. Then TdT (terminal deoxyribonucleotidyl transfer) enzyme was added on the slide and incubated in a humidified incubator at 37°C for 1 hour. Stop/Wash Buffer was applied, agitated for 15 seconds, and then incubated at room temperature for 10 minutes. After washing 3 X 5 minutes by 0.1 M PB, Anti-Digocigenin Conjugate (Fluorescein) was added to the slides and incubated in a humidified dark container at room temperature for 1 hour. Lastly, the slides were washed with 0.1 M PB 3 X 5 minutes again and mounted with the Prolong Gold Antifade reagent with DAPI (P36931, Invitrogen) and then cover-slipped. Labeled cells were counted manually under a Zeiss compound microscope at 10X objective lens. A 100X objective lens was utilized to capture the details of the apoptotic cells, especially the nuclear co-localization of DAPI and TUNEL.

Data analysis

Analysis of variance (ANOVA) was used to examine differences between buffer- and GH-injected fish for all the quantified results. Separate ANOVAs were done for each location along the rostral-caudal axis for hair cell bundle counts, for each hair cell morphotype in each location, and for each endorgan (saccule, lagena, and utricle) for BrdU- and TUNEL-labeled cells.

Table 1. Experimental design. Sample sizes for the experiments performed at three time points: post-sound exposure day 1 (PSED1), PSED2, and PSED3. Three endpoints were examined: phalloidin-labeled hair cell bundles for examining hair cell density, BrdU-incorporation to quantify cell proliferation, and TUNEL-labeling to measure cellular apoptosis in the zebrafish inner ear.

Treatment (day)	**Experiment**		
	Phalloidin	**BrdU**	**TUNEL**
Baseline (no sound)	6	6	6
Buffer (PSED1)	6	6	6
GH (PSED1)	6	6	6
Buffer (PSED2)	6	6	6
GH (PSED2)	6	6	
Buffer (PSED3)	6	6	
GH (PSED3)	6	6	
Total fish =	42	42	30

Results

GH accelerates hair cell bundle replacement:

Under normal conditions, the number of hair cell bundles in the saccule is at a high level (approximately 17-20 hair cells/900 μm^2). Immediately after sound exposure, the hair cell bundles were damaged greatly, with some of the hair cell bundles being lost (approximate density of 6-12 hair cells/900 μm^2); some hair cells lost all stereocilia leaving only cuticular plates visible (Figure 1A). When the hair cell bundles are compared between the buffer groups and GH groups at PSED 1, less damaged hair cell bundles are found in GH groups ($P<0.001$). After this time point, in both the GH group and the buffer group, the numbers of hair cell bundles began to increase back toward control levels. The GH group hair cell bundles recovered faster than that of the buffer group- at PSED 2 the hair cell bundles in GH group had already recovered to baseline levels, but the buffer group still had not recovered to the normal levels by PSED 3 (Figure 1B, C D). The hair cell bundles at 25%, 50% and 75% location along the rostral-caudal axis of the saccules showed a similar trend. Compared the hair cell bundles at 25% and 50% location, the hair cell bundles at 75% location were damaged greatest (i.e., had the least hair cells) at PSED1 (Figure 1D).

Furthermore, after sound exposure the number of new hair cell bundles in the GH group increased at PSED 2 compared to the buffer group. The quantity of newly formed hair cell bundles was highest level at PSED 2 in the GH group, but was highest at PSED 3 in the buffer group but was still less than that in the GH group at PSED 2. The newly formed hair cell bundles at 25%, 50% and 75% showed similar results (Figure 2). Following sound exposure at all three time points, there were more intact hair cell

bundles in the GH-injected group than in the buffer-injected group (Figure 3A, B, C). This may indicate that GH inhibits the loss of hair cell bundles, or that GH promotes the formation of new hair cells rapidly.

GH promotes cellular proliferation:

For the cell proliferation assays, BrdU-labeling was co-localized with DAPI in the nuclei of the cells as expected (Figure 4A). Buffer-injected fish exhibited significantly greater BrdU-labeled cells in the saccule, lagena, and utricle, compared to baseline fish ($P<0.01$), suggesting that proliferation was a natural response to acoustic trauma. With injection of GH, the saccule, lagena, and utricle showed much more BrdU-labeled cells after sound exposure at PSED 1 ($P<0.001$; Figure 4B, 5). At PSED 1 the BrdU-labeled cells reached a level which was at least five to ten times greater than buffer-injected controls in all three endorgans. After PSED 1, BrdU-labeled cells in the GH group decreased and returned to baseline levels by PSED 3 (Figure 4C, 5B, 5C). In the buffer group, numbers of BrdU-labeled cells peaked at PSED 2 instead of at PSED 1 as in the GH group. Therefore, these results suggest that GH may accelerate and promote more auditory cell proliferation.

GH suppresses sound exposure-induced apoptosis

For the apoptosis assays, TUNEL-labeling was also co-localized with DAPI in the nuclei of the cells (Figure 6A). Buffer-injected fish had greater numbers of TUNEL-labeled cells in the saccule, lagena, and utricle at PSED 1, compared to either baselines or GH-injected fish ($P<0.001$; Figure 6B, 7A). With the injection of GH, the saccule, lagena, and utricle showed minimal TUNEL-labeled cells following sound exposure. At PSED 1, the number of TUNEL-labeled cells reached the highest level in both buffer and GH

groups, but were five or ten times greater in the buffer compared to the GH- injected group. At PSED 3, the TUNEL-labeled cells in the GH group decreased to baseline levels, but in the lagena, the number of TUNEL-labeled cells were still higher than baseline levels (Figure 6C, 7B, 7C). These results indicate that GH may inhibit sound-induced cell apoptosis in the zebrafish inner ear.

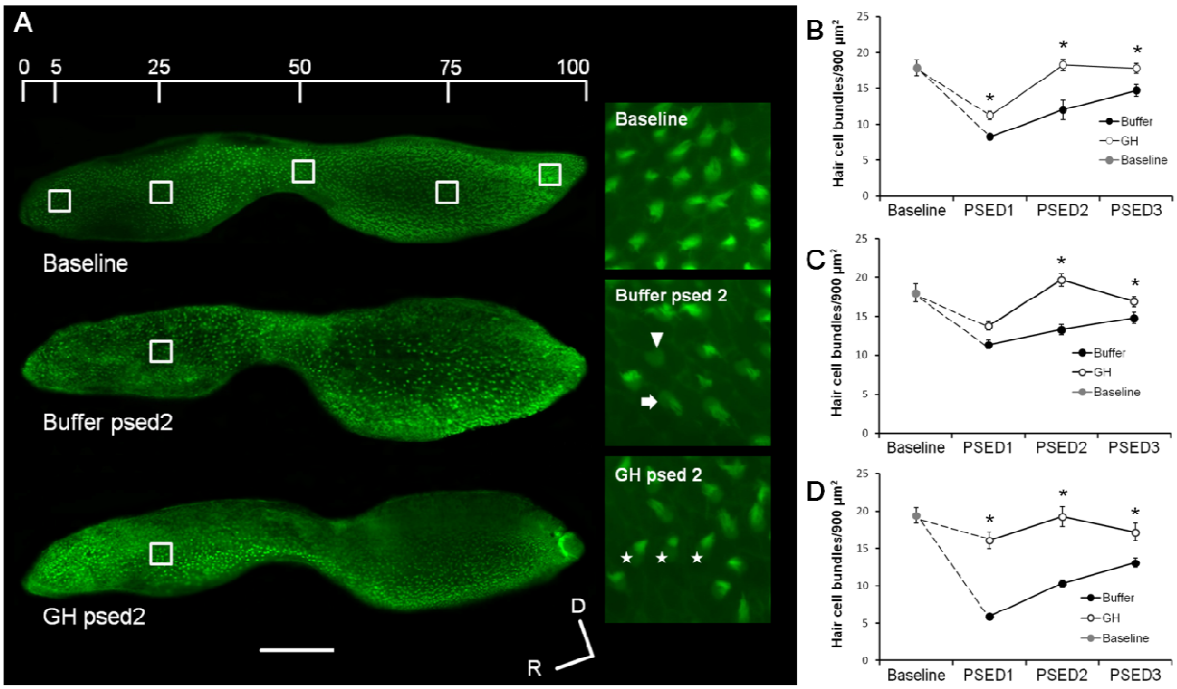

Figure 1. Effects of growth hormone on hair cell bundle density

(A) Phalloidin-labeled saccular epithelia of baseline, buffer-injected, and GH-injected zebrafish at post-sound exposure day 2 (PSED2). The six boxes show locations of hair cell counts along the rostral-caudal axis of the saccule. The enlarged images to the right of the saccules are representative 100X images of saccules at 25% along the rostral-caudal axis. Time course of hair cell bundle density at (B) 25%, (C) 50% and (D) 75% locations along the rostral-caudal axis of the saccule of baseline and buffer- or GH-injected zebrafish. Scale bar = 100 µm, D = dorsal, R = rostral, triangle = cuticular plate, arrow =scar formation characteristic of hair cell loss, star= presumed newly formed hair cell bundles in GH group. N=6; * P<0.05.

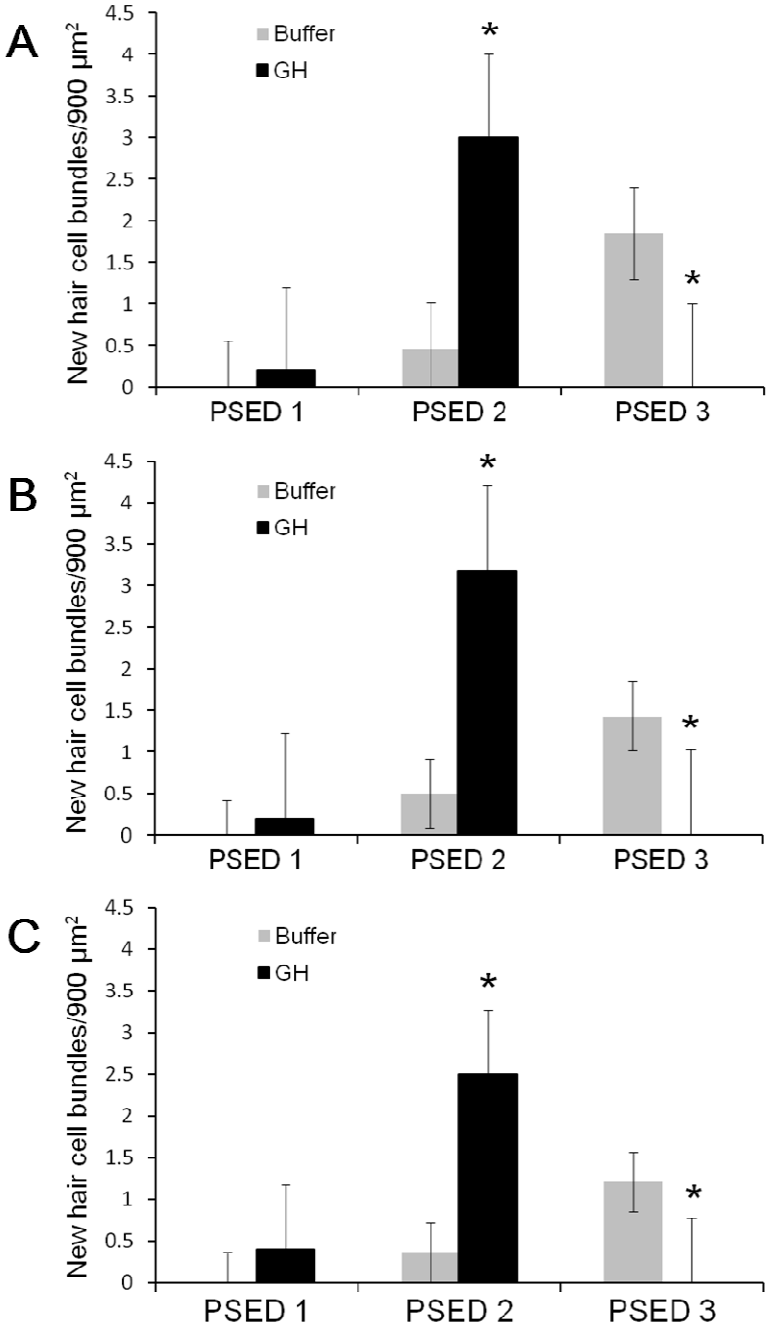

Figure 2. Effects of growth hormone on newly formed hair cell bundles

Time course of newly grown hair cell bundles at (A) 25%, (B) 50% and (C) 75% along the rostral-caudal axis of the saccule of buffer- or GH-injected zebrafish at post-sound exposure days (PSED) 1, 2, and 3. N=6; * P<0.001.

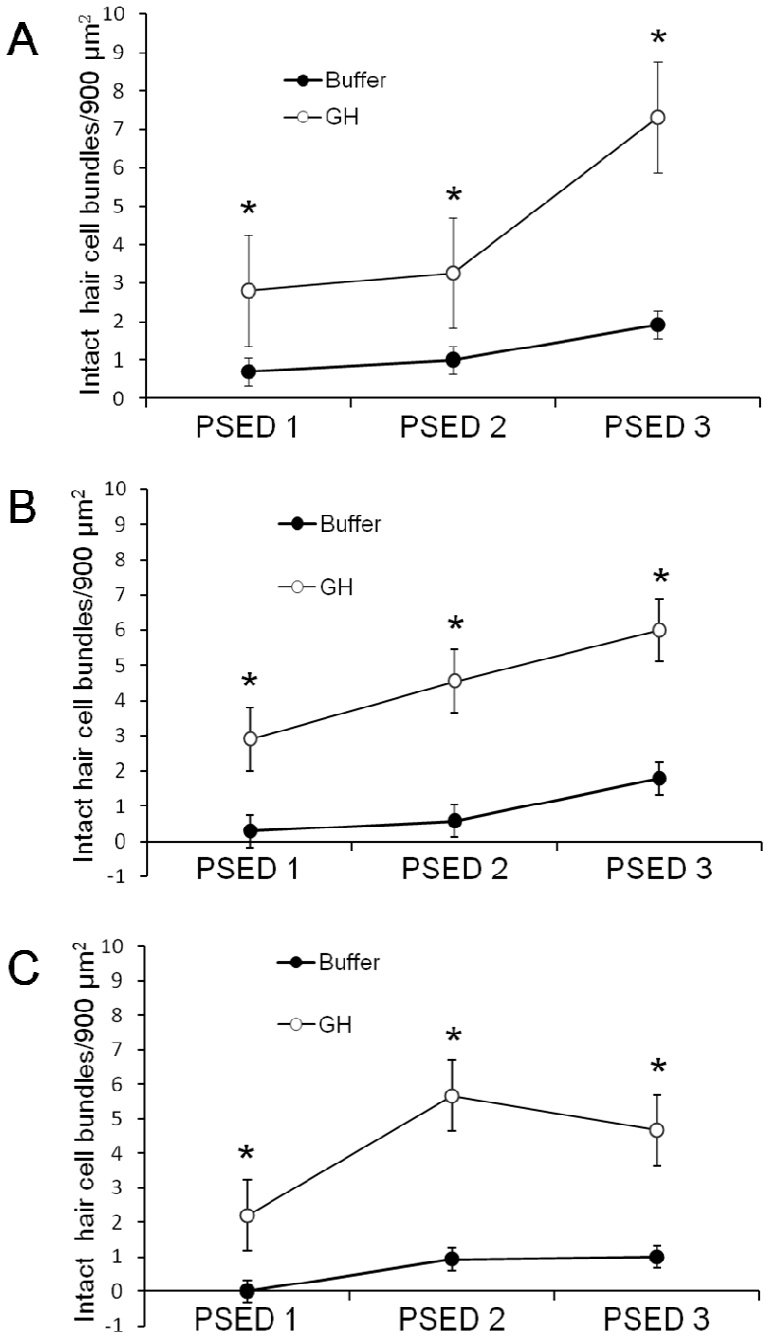

Figure 3. Effects of GH on intact hair cells

Time course for the number of intact hair cell bundles at (A) 25%, (B) 50% and (C) 75% along the rostral-caudal axis of the saccule of buffer- or GH-injected zebrafish at post-sound exposure days (PSED) 1, 2, and 3. N=6; * P<0.001.

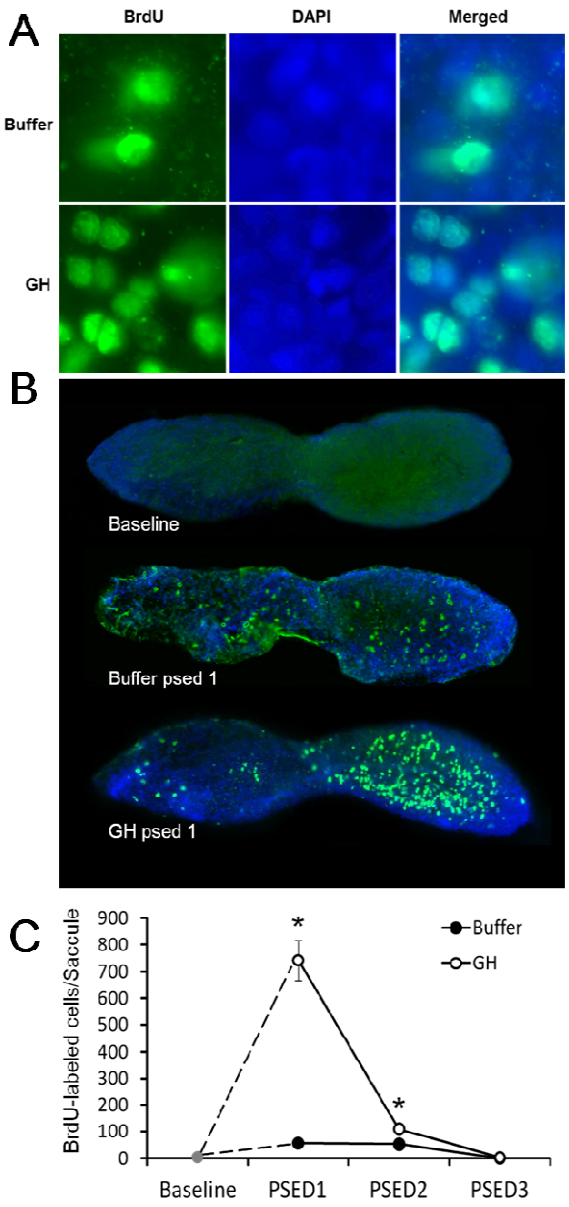

Figure 4. Effects of GH on cell proliferation in saccule

(A) 100X images of BrdU- and DAPI- labeling in the saccule of buffer- and GH-injected zebrafish. (B) BrdU-labeling in the saccules of baseline, buffer- or GH-injected zebrafish at post-sound exposure day 1 (PSED1). (C) Time course of BrdU-labeled cells in the saccules of baseline and buffer- or GH-injected zebrafish. N = 6; * $P<0.001$. Rostral-caudal orientation is the same as Figure 1A.

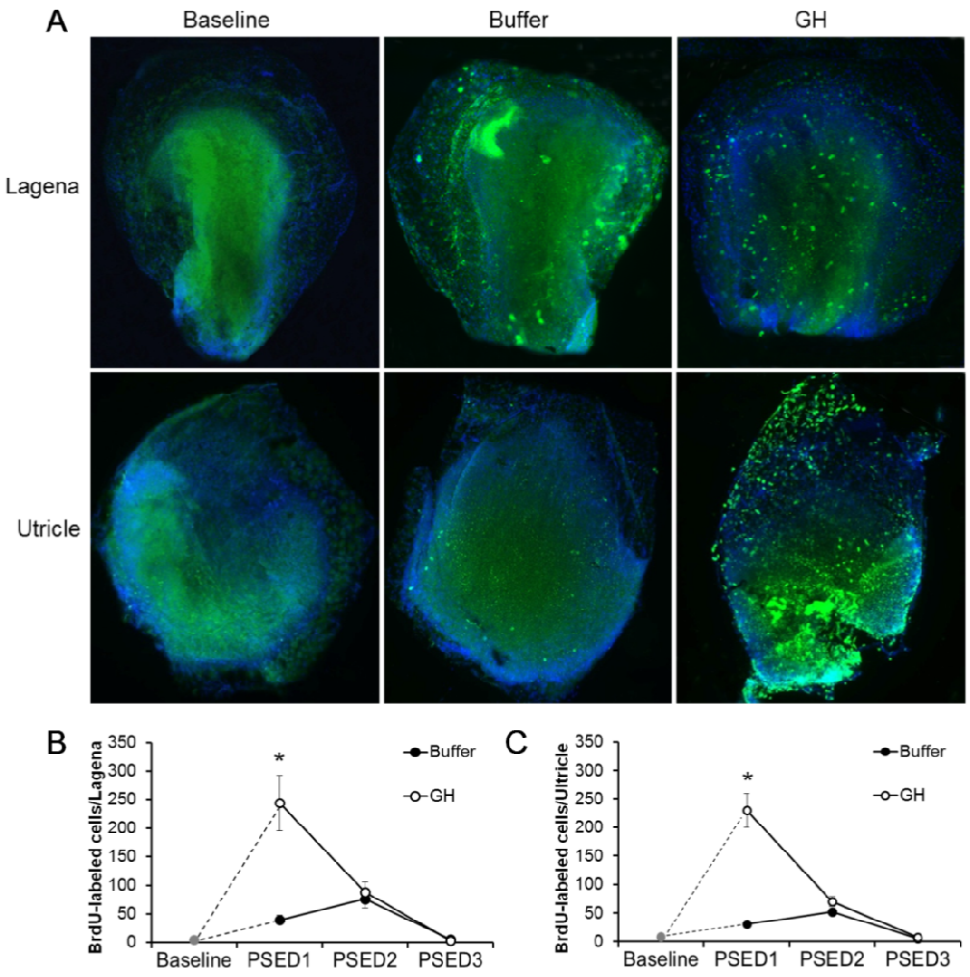

Figure 5. Effects of GH on cell proliferation in lagena and utricle

(A) BrdU-labeling in the lagena and utricle of baseline, buffer- or GH-injected zebrafish at post-sound exposure day 1 (PSED1). Time course of BrdU-labeled cells in the lagena (B) and utricle (C) of baseline and buffer- or GH-injected zebrafish. N=6; * P<0.001.

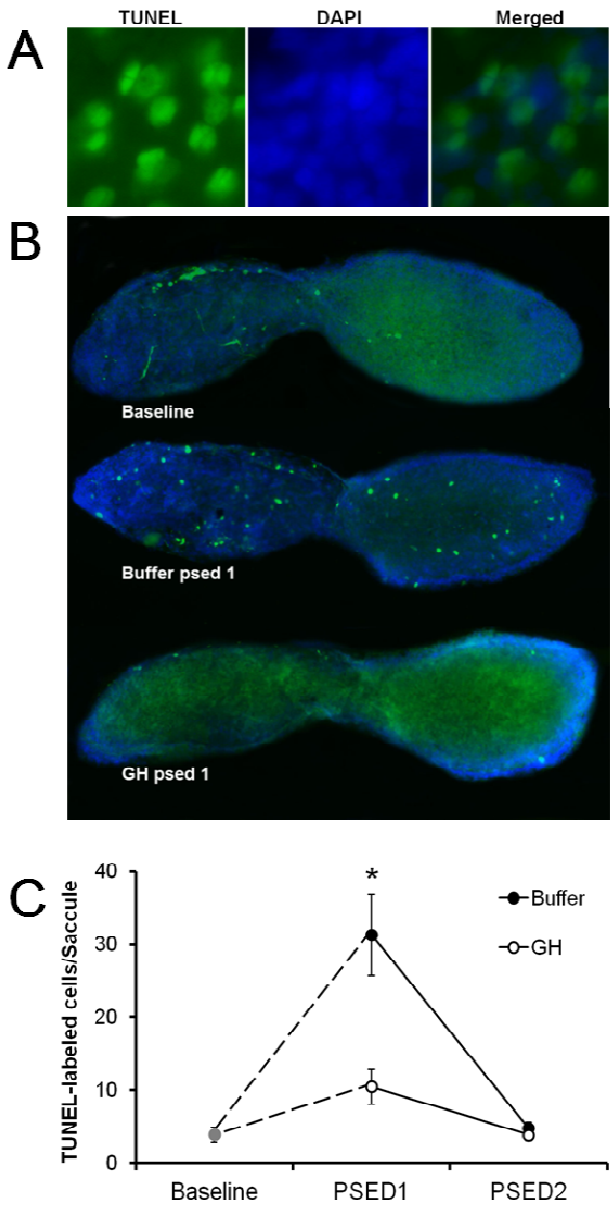

Figure 6. Effects of GH on apoptosis in saccule

(A) 100X images of TUNEL and DAPI labeling in the saccules of buffer- and GH-injected zebrafish. (B) TUNEL-labeling in the saccules of baseline, buffer- or GH-injected zebrafish at post-sound exposure day 1 (PSED1). (C) Time course of TUNEL-labeled cells in the saccules of baseline and buffer- or GH-injected zebrafish. N = 6; *P<0.001. Scale bar = 100 µm. Rostral-caudal orientation is the same as Figure 1A.

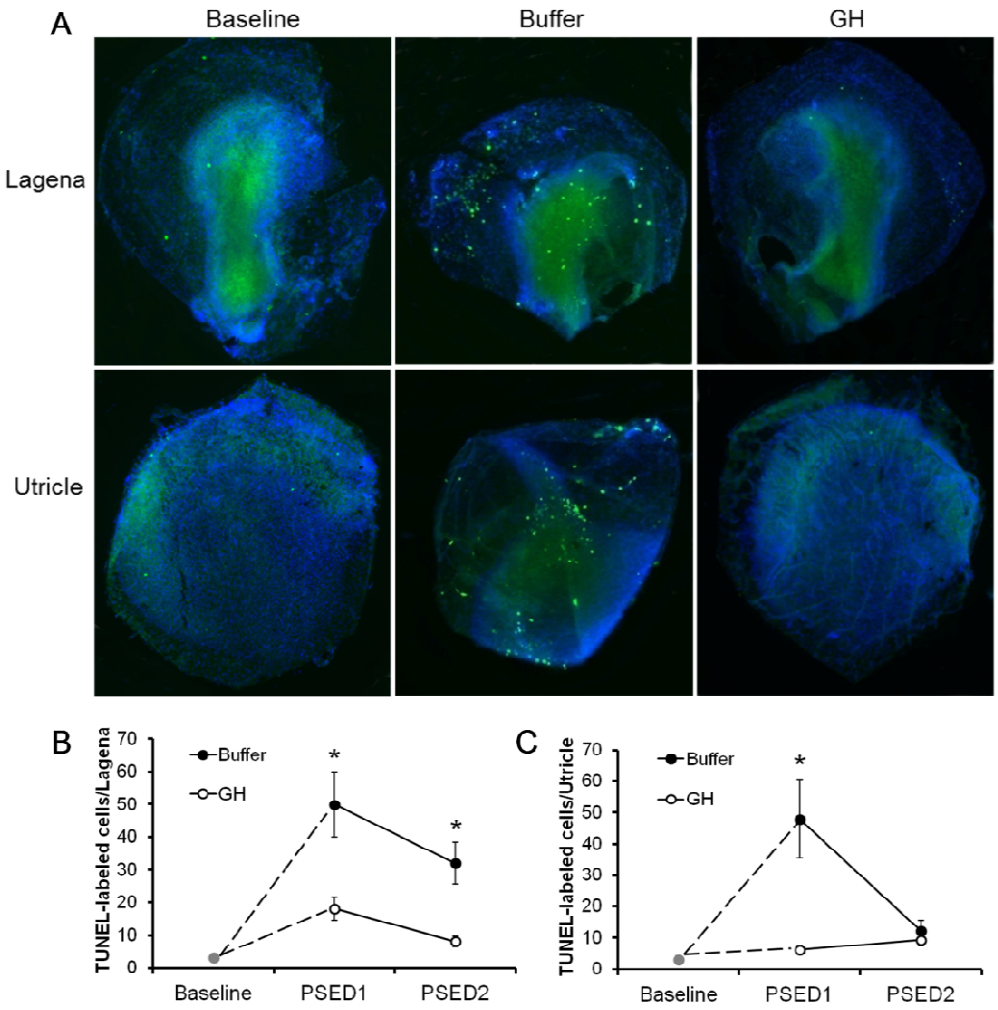

Figure 7. Effects of GH on apoptosis in lagena and utricle

(A) TUNEL-labeling in the lagena and utricle of baseline, buffer- or GH-injected zebrafish at post-sound exposure day 1 (PSED1). Time course of TUNEL-labeled cells in the lagena (B) and utricle (C) of baseline and buffer- or GH-injected zebrafish. N=6; * $P \leq 0.01$.

Discussion

GH promotes auditory hair cell regeneration

In the current study, we found that following injection of GH into zebrafish, the recovery of hair cell bundle density following acoustic trauma was much higher and more rapid than that of the control group; and there were less damaged hair cells and more intact hair cells than that of the buffer group. This suggests that GH may either prevent hair cell loss and/or promote the formation of new hair cells.

One possible scenario explaining why there might be more hair cells in GH-injected fish following acoustic trauma is that hair cells could be damaged by acoustic trauma, but GH may inhibit the hair cells from undergoing apoptosis and being ejected out of the epithelia as they normally would. Instead they could exhibit self-repair of their stereocilia bundles. Self-repair of hair cell stereocilia has been reported in the inner ear epithelia of other organisms, for example in the rat utricle (Zheng et al., 1999). This problem can be studied in future experiments using double-labeling of BrdU and hair cell markers (such as parvalbumin or calretinin) to identify whether the newly-formed hair cell bundles exist in non-mitotic zebrafish hair cells. If the results show the locations of the BrdU-labeled cells and the cell markers match, the newly formed hair cell bundles will be the results of proliferation of surrounding supporting cells; if they do not match, the newly formed hair cell bundles will be the results of direct transdifferentiation of supporting cells or via self-repair.

Alternatively, GH may play a role in promoting the production of new hair cells. Following sound exposure, at PSED 2, the hair cell density in the GH treated group had already recovered to control levels; but for the buffer-injected group, even at PSED 3, the

density still had not returned to the control levels (Figure 1B, C, D). Furthermore, the peak of newly formed hair cell bundles happened at PSED2 in GH-injected fishes, but at PSED 3 or later in buffer-injected fishes, and the peak in the GH group was much higher than that of the buffer group (Figure 2). Also, the intact hair cell bundles in the GH group recovered much more quickly than that in the buffer group (Figure 3). All these results indicate that hair cell recovery could potentially be accelerated by GH.

Comparing this time course with previous time course studies of hair cell bundle regeneration in goldfish (Smith et al., 2006), we found that GH greatly shortened the recovery time. Following acoustic trauma, hair cell bundles regenerated to control levels in the goldfish saccule after eight days (Smith et al., 2006), and in the zebrafish saccule after seven days (Schuck and Smith, 2009). My data shows that the hair cell density had already recovered to control levels after only two days in GH-injected zebrafish following acoustic trauma (Figure 1). In summary, GH can increase hair cell bundle density although it is unclear whether this is by promoting new hair cell bundle formation or preventing normal hair cell bundle loss (Figure 2 and 3).

Following sound exposure, rostral and caudal tip regions of the saccule at the 5% and 90% locations along the axis from rostral tip of the saccule showed minimal hair cell loss. While there were significant numbers of hair cells lost in the central region of saccule (25%, 50% and 75% of the total distance from rostral tip), suggesting that this region is more susceptible to trauma induced by my low frequency sound stimulus. Moreover, the region at 75% location is the most susceptible region in saccule, with the most hair cells being lost in this region after sound exposure (Figure 1D). This result is similar to previous studies in which a loud 100 Hz tone exposure for two days

significantly decreased hair cell bundle density in the central rostral and caudal area in goldfish (Smith et al., 2006) and zebrafish (Schuck and Smith, 2009). In 75% along the R-C (rostral-caudal) axis of saccule, the intact hair cells recover more quickly than in the 25% and 50% areas in both GH group and buffer group (Figure 3). One possible reason may be that the cells in this area are more sensitive to GH than other areas of saccule, and that this sensitivity may be related to inflammation and immune responses to more damaged tissues. In the current study, after GH injection, there was a significant increase in hair cells in this same central area compared to the buffer group. It is likely that at least some of this increase is the result of newly-formed hair cells. This is supported by the fact that hair cell bundle numbers reach control levels at PSED 2 in the GH group, at which point newly formed hair cell numbers are also the highest, while the buffer group is highest at PSED 3 at which point their newly formed hair cell numbers are higher than at PSED 2.

GH promotes auditory cell proliferation

This study demonstrated that after acoustic stimuli, with the injection of GH, a significant increase in cellular proliferation was observed in the saccule, lagena, and utricle, compared to the buffer group. This supports the hypothesis that GH may play an important role in cell proliferation in the inner ear that could lead to the regeneration of hair cells following trauma.

At PSED 1, obvious cell proliferation was observed, indicating that GH can promote cell proliferation in the zebrafish inner ear, potentially speeding up the regeneration process since some of these proliferating cells could become new hair cells. The ability of GH to promote cellular proliferation has been found in mammary and

endometric tissues (Kaulsy et al., 2001; Pandey et al., 2008; Brunet-Dunand et al., 2009). However, the potential effect of GH on zebrafish inner ear proliferation with acoustic trauma, or any fish tissue for that matter, had not been examined previously. This study found that the cellular proliferation in saccule, lagena and utricle was greatly increased by GH compared with buffer group (Figure 4 and 5).

In our previous study, Schuck and Smith (2009) found that after 36 hours of sound exposure (100 Hz tone, 179 re 1 mPa), cell proliferation peaked in the zebrafish saccule two days following trauma, with an average of 53 BrdU- positive cells per saccule. In the current study, the average number of BrdU-positive cells was similar to the previous study, but at PSED 1 the hair cell proliferation in the saccule, lagena and utricle reached the highest level. This is one day earlier post-trauma than the condition without GH. This supports the idea that GH expedites the process of post-trauma cell proliferation.

Even though the current study focused on examining the time course of hair cell proliferation post-acoustic trauma, we still do not know the molecular mechanisms of how GH effects hair cell proliferation. In a previous study, Schuck et al. (2011) injected zebrafish that were not exposed to sound with salmon GH. They found that GH induced cell proliferation in the zebrafish inner ear, although this effect was only significant in the utricle, a vestibular portion of the ear (Schuck et al., 2011). In contrast, in the current study the effect of GH on cell proliferation was greatest in the zebrafish saccule, which is thought to be important for hearing and is known to be damaged by acoustic stimuli (Schuck and Smith, 2009). Although numbers of BrdU-labeled cells cannot be directly compared between the two studies, since different concentrations and sources of GH

were used, the fact that GH induced cell proliferation in the saccule of sound-exposed fish suggests that acoustically-damaged tissues may be more sensitive to GH signaling pathways.

GH inhibits cellular apoptosis

This study demonstrated that growth hormone can suppress programmed cell death in the zebrafish inner ear after sound exposure. Without injection of GH, the cellular apoptosis increased significantly at PSED 2 in all inner ear end organs, but in the GH-injected group, apoptosis was inhibited relative to buffer-injected controls. This suggests that GH plays an important role of not only controlling cell proliferation, but also suppressing cellular apoptosis.

However, the effect of GH on apoptosis has not been extensively studied, but it has been shown to have an effect in a few non-auditory tissues. For example, Svensson et al. (2008) found human recombinant growth hormone (rhGH) was able to counteract the effects of morphine-induced cell damage and apoptosis in mouse embryo hippocampal neurons in the brain. Decker et al. (2005) found that GH can reduce apoptosis in neutrophils of post-surgical patients. The cellular mechanisms and molecular pathways by which GH produces these effects on apoptosis are currently unknown. If GH can inhibit apoptosis in the zebrafish inner ear, then it may also be able to protect hair cells from acoustically-induced damage and loss. Thus GH may have potential prophylactic benefits that may help prevent hearing loss.

Summary and Future Research

In summary, the results of this time course study suggest that GH can both promote auditory cell proliferation and suppress hair cell apoptosis, ultimately increasing hair cell density following acoustic trauma. Thus, GH appears to play an important role in the process of hair cell regeneration. Exactly how GH affects the hair cell and the surrounding supporting cells, and the cellular pathways involved has not yet been identified. In the future, Next Generation Sequencing experiments will be used to analyze the mRNA of zebrafish inner ear tissues post-acoustic trauma. The goal will be to examine gene expression patterns during the process of hair cell regeneration to find which genes are involved in GH-mediated hair cell regeneration.

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