Fucoidan Promotes Mechanosensory Hair Cell Regeneration following Aminoglycoside induced Cell Damage

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A Dissertation
Submitted to the Department of Medical Science
and the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In Seok Moon

August 2011

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The Graduate School
Yonsei University
August 2011

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ABSTRACT

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Lateral line system of the zebrafish is a useful model for study of hair cell toxicity and regeneration. We found that low molecular weight fucoidan (LMWF) stimulated the regeneration of mechanosensory hair cells after neomycin induced cell death in zebrafish lateral line. In the past decade, anticoagulant, antiviral, antitumor, anti- complement, immunomodulatory, anti-inflammatory, and antioxidant activities of fucoidan have been reported. Fucoidan is also known to promote tissue regeneration. In this study, we found that LMWF enhanced hair cell regeneration after neomycin-induced ototoxicity in both wild-type and transgenic zebrafish larva. The aims of this study were to quantify the regenerative effects of LMWF and determine their relationship to the Notch and FGF signaling pathways.

Wild-type zebrafish and three different transgenic zebrafish lines (Pou4f3::GFP, scm1::GFP, and ET20::GFP) were used. At 4.5 to 6 days post-fertilization, lateral-line hair cells of larvae were eliminated using neomycin (500 μM). Larvae were then treated with LMWF. Neuromasts were observed using confocal microscopy. Stereocilia

morphology was observed using scanning electron microscopy, and the location and status of regeneration was assessed using 5-bromo-2-deoxyuridine (BrdU) incorporation.

Hair cells damaged by neomycin treatment regenerated faster in wild-type and Pou4f3::GFP larvae treated with LMWF (50 μg/ml) than in untreated controls. LMWF also enhanced the regeneration of supporting cells in scm1::GFP and ET20::GFP larvae. Increased numbers of BrdU- labeled cells were found after LMWF treatment in neuromast regions corresponding to internal and peripheral supporting cells. The effect of LMWF was mimicked by the Notch-signaling inhibitor N-[N- (3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT), but the effects of LMWF and DAPT were not additive.

LMWF enhances the regeneration of hair cells damaged by neomycin. The mechanism may involve the Notch signaling pathway. LMWF shows promise as a therapeutic agent for hearing and balance disorders.

Key words: fucoidan, hair cell, cell death, regeneration, zebrafish

INTRODUCTION

Hair cell death as a result of noise, drugs, or aging is the primary cause of deafness and balance disorders that affect more than 600 million people worldwide (Schuknecht et al., 1993; Owens et al., 2008). Hair cell loss in humans and other mammals is irreversible (Ruben, 1967, Rubel et al., 1995), but many hair cells are added throughout life in the ears of various non-mammalian vertebrates (Stone and Rubel, 2000). Research into the mechanisms of hair cell regeneration and efforts to discover chemicals that promote hair cell regeneration in vertebrates offer the potential for future therapies for humans with hearing loss.

The zebrafish (Danio rerio) offers advantages as a screening system for ototoxicity and chemical prevention of hair cell death (Harris et al., 2003). Neuromasts in lateral line of zebrafish are sensory organs composed of neuroepithelial clusters of hair cells, supporting cells, and integrated neurons (Fig. 1). Although these organs are specific to fish and amphibians, they are essentially identical in ultra-structure to the neuroepithelium of the mammalian inner ear (Whitfield TT, 2002; Nicolson T, 2005). Zebrafish lateral line hair cells selectively take up fluorescent vital dyes, facilitating the in vivo assessment of hair-cell status (Chiu et al., 2008). The pharmacological effects of various natural and synthetic compounds on neuromasts can be assessed by their application to the surrounding water at graded concentrations. Several groups have reported on the use of the zebrafish lateral line system to screen for drugs that prevent

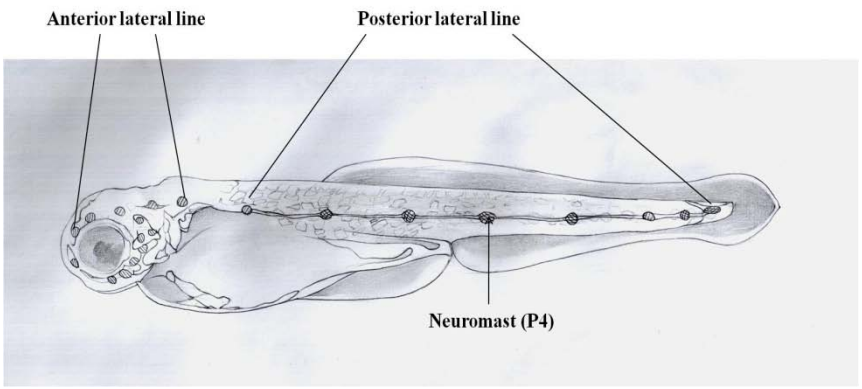

Fig. 1. Zebrafish lateral line system. Anterior lateral line system include 18 neuromasts on each side and posterior lateral line system include 9 neuromast in each sides. Neuromasts are sensory organs composed of neuroepithelial clusters of hair cells, supporting cells, and integrated neurons.

aminoglycoside-induced hair cell death (Harris et al., 2003; Ton and Parng, 2005; Chiu et al., 2008).

The zebrafish lateral line system is also an attractive model for studying hair cell regeneration, because the zebrafish mechanosensory cells have the potential to regenerate following aminoglycoside-induced toxicity. In addition, transgenic zebrafish lines expressing cell type-specific green fluorescent protein (GFP) provide direct insight into the development and regeneration of neuromasts (Parinov et al., 2004; Xiao et al., 2005; Behra et al., 2009). From a clinical viewpoint, a therapeutic agent that could promote hair-cell regeneration would be most desirable, because patients usually seek care only after damage has occurred. We have used an established zebrafish larva model (Harris et al. 2003) for the screening of the compounds which stimulate hair-cell regeneration. In the course of screening for hair cell regeneration, we discovered that low molecular weight fucoidan (LMWF), a purified natural compound enriched in seaweed, has the potential to promote hair cell regeneration. Here, we report the effects of LMWF on hair cell regeneration and describe a possible underlying mechanism in a zebrafish model.

MATERIALS AND METHODS

Animals and embryo media

Wild-type adult breeder zebrafish were obtained from a commercial supplier. Three transgenic zebrafish lines expressing GFP on specific neuromast cells were also used. The GFP-tagged lines were: pou4f3::GFP, which display GFP on hair cells (Xiao et al., 2005); scm1::GFP line, which express GFP on the internal neuromast supporting cells (Behra et al., 2009); and ET20::GFP, which express GFP on peripheral neuromast supporting cells (Parinov et al., 2004) (Fig. 2). Adult fish stocks were maintained according to standard protocols (Westerfield, 2000). Embryos obtained from group mating of wild-type adults and the three transgenic lines were raised at 28.5°C in E3 embryo medium (see below) at a density of 50 larvae per 10-cm Petri dish. All experiments were performed using larvae at 4.5 to 6 days post-fertilization (dpf). Baseline E3 embryo medium contained 1 mM MgSO4, 120 μM KH2PO4, 74 μM Na2HPO4, 1 mM CaCl2, 500 μM KCl, 15 μM NaCl, and 500 μM NaHCO3 in deionized H2O, with the pH adjusted to 7.2.

Screening for hair cell regeneration

We screened 470 compounds provided by Korea Research Institute of Chemical Technology (Daejeon, Korea, http://www.chembank.org/) (Kang et al., 2009; Kim et al, 2006) and 10 purified natural compounds (fucoidan, Keumsa Linteusan, saponin, beta-glucan extract , polyhexosamine extract,

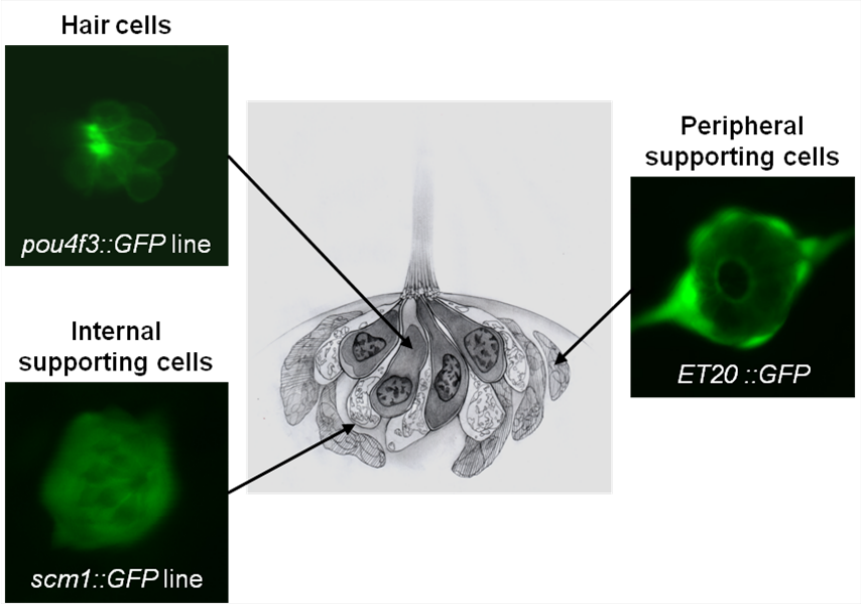

Fig. 2. Expression of fluorescence in transgenic zebrafish. Transgenic pou4f3::GFP larvae displayed GFP on hair cells; scm1::GFP larvae, on the internal neuromast supporting cells; and ET20::GFP larvae, on peripheral neuromast supporting cells.

chito-oligosaccharide extract, terpene extract, flavonoid extract, alkaloid ectract, and lignins) from Kyoungpook National University (Daegu, Korea). Wild-type zebrafish larvae at 4.5 dpf were treated with 500 μM neomycin for 1 hour and rinsed quickly five times. The larvae were then distributed to 48-well plates and natural compounds were added individually to wells at a concentration of 25 μg/ml. The concentration of neomycin was determined from previous reports (Harris et al., 2003) and our pilot study. Twelve hours following neomycin treatment, hair cells of free-swimming larvae of wild-type zebrafish were labeled by a 20-minute immersion in 2 μM YO-PRO1 (Invitrogen Molecular Probes) or 3 μM FM1-43 (Invitrogen Molecular Probes), followed by three rinses in fresh E3 medium. The zebrafish larvae were anesthetized with 0.1% tricaine (3-amino benzoic acid ethyl ester, also called ethyl 3-aminobenzoate, Sigma) and mounted in 3% methylcellulose (Sigma) in a depression slide. The average hair cell number in three posterior neuromasts (P1, P7, and P8) was counted under a fluorescent microscope. These posterior neuromasts were selected because they exhibit low variability in the number of hair cells, definite difference in rate of regeneration, and they produce clear confocal images due to the thin surrounding tissue. If the average number of regenerated hair cells was at least 25% more than in the group treated with neomycin alone, that compound was selected as a candidate compound.

Low molecular weight fucoidan (LMWF)

We used LMWF which is more soluble than high molecular

weight fucoidan, hence it to facilitate absorption and bioavailability. LMWF was prepared by acid hydrolysis of high molecular weight fucoidan extracts (Haewon Biotech, Korea) derived from brown seaweed, as previously described (Jung et al., 2008; Jung et al., 2007). Briefly, high molecular weight fucoidan (0.1 to 20%, w/v; preferably 0.1 to 6%, w/v) were hydrolyzed by treating with 2N pyruvic acid and 2N acetic acid (1:4) for 5 hours at 75°C. The hydrolyzed product of high molecular weight fucoidan was filtered through an ultra-filtering membrane to afford a constant molecular weight hydrolysis product (1,000 to 10,000 Da cutoff value). The filtrate was concentrated under reduced pressure to remove water and organic acids, and was then washed with ethanol, decolorized, and dried to afford the final LMWF product as a powder
with the following characteristics: average molecular mass 3 ± 0.5 kDa (polydispersity 1.9), 6
fucose 38.3% (w/w), galactose 17.1% (w/w), uronic acid 3% (w/w), sulfate 28% (w/w), protein 5.4% (w/w), moisture 3.2% (w/w), and ash 5% (w/w). Slight decoloriztion was induced by using ethanol during hydrolyzation. The light brownish-white powder was stored in a desiccator to protect it from light and humidity.

Analysis of the effects of LMWF

To investigate the mechanism of hair cell regeneration by LMWF, transgenic zebrafish larvae (pou4f3::GFP; scm1::GFP; and ET20::GFP) at 4.5 dpf were incubated in neomycin- containing medium for 1 hour at 28.5°C and then rinsed quickly five times in

fresh media. The larvae were then maintained in E3 media in the presence or absence of LMWF (50 μg/ml), and the numbers and shapes of hair cells within three posterior trunk neuromasts (P1, P7, and P8) were evaluated at 4, 8, 12, 16, and 24 hours after the neomycin treatment. In some experiments, larvae were co-treated with the Notch signaling inhibitor N-[N-(3, 5- difluorophenacetyl) - L-alanyl]-S-phenylglycine t-butyl ester (DAPT; CalBiochem), or the fibroblast growth factor (FGF) inhibitor 3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl) methylene]-2-indolinone (SU5402; CalBiochem). The zebrafish larvae were anesthetized with 0.1% tricaine and placed on a depression slide for viewing with confocal microscopy (Zeiss, LSM510).

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to investigate the loss or regeneration of stereocilia. Larvae were fixed overnight in 2.5% glutaraldehyde in phosphate buffered saline (PBS; pH 7.2) at 4°C. Larvae were washed three times (5 minutes per wash) in distilled water and dehydrated through serial 10-minute exposures to graded concentrations of ethanol solutions (25, 50, 70, 80, 95, and 100%). Samples were further dehydrated through serial 10 minute exposures to graded concentrations of isoamyl acetate solutions (25, 50, 75, and 100%) in ethanol. The specimens were then dried using a critical point dryer and were sputter-coated twice with carbon using an evaporator (MED010; Baltec, Hudson, NH). All of the L1 neuromasts were observed by scanning electron microscopy (S800; Hitachi, Tokyo, Japan) at 5 or 7 kV.

BrdU incorporation for cell proliferation analysis

S-phase cells in lateral-line neuromasts were identified using a pulse-fix protocol labeling with 5-bromo-2-deoxyuridine (BrdU; Sigma) in transgenic zebrafish larvae. Larvae were collected at 4, 8, 12, 16, and 24 hours after treatment, and cells were labeled by adding 10 mM BrdU in 1% dimethylsulfoxide (DMSO) to the medium for 2hours at 28.5°C before collection. Larvae were anesthetized in tricaine, and fixed with 4% paraformaldehye in PBS for 2 hours at room temperature (RT). BrdU incorporation was detected by immunohistochemistry. The fixed larvae were washed in PBS with 1% DMSO and 0.1% Tween 20 (PBDT), and were placed in methanol at -20°C for 1 hour. Larvae were then rehydrated in a graded methanol series and washed in PBDT for 20 minutes. Following rehydration, all larvae were digested with proteinase K (10 μg/ml; Roche) for 20 minutes, washed in PBDT, and fixed again in 4% paraformaldehye for 20 minutes. After another washing in PBDT, the larvae were placed into 2N HCl at RT for 1 hour. Larvae were washed again in PBST, and non-specific binding was blocked in 5% normal goat serum in PBDT for 1 hour at RT. The larvae were incubated with mouse anti-BrdU in blocking serum (1:100; Sigma, #2531) overnight at 4°C. Larvae were washed for 1 hour in PBST and incubated in Rhodamine Red-X goat anti-mouse IgG at RT for 6 hours (1:200; Invitrogen). The larvae were whole mounted under a cover glass. BrdU-labeled cells in neuromasts were counted on a confocal microscope, using a 20× objective, and by confocal microscopy (Zeiss, LSM510). Red dots having a shape identical to that of a hair cell or supporting cell and corresponding to the

exact location of a neuromast were counted. These values for P1 and terminal neuromasts (P7, P8) of were analyzed for five larvae in each group.

Statistical analysis

All experiments were performed independently at least 3 times using more than 10 larvae in each group for each experiment. Statistical analysis was performed using SPSS v.16.0.
Tukey corrected one-way ANOVA (BrdU incoporation) and Bonferroni-corrected independent t-tests (others) were used for analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Identification of fucoidan as an enhancer for hair cell regeneration

Neomycin (50 to 1,000 μM) produced dose-dependent loss of hair cells in lateral line neuromasts. Hair cells labeled with YO-PRO-1 is disappeared regardless of their position 4 hours after treatment with 500 mM neomycin. Hair cell regeneration was evident 12 hours after neomycin exposure, 3.01 ± 0.48 hair cells per neuromast were observed. Among the 480 compounds screened, 20 compounds apparently enhanced hair cell regeneration, as defined by more than 25% regeneration of hair cells observed 12 hours after neomycin treatment (Fig. 3). The effect of LMWF was the most prominent and consistent, and it was studies in greater detail. As shown in Figure 4A and 4B, zebrafish larva maintained in LMWF (25 μg/ml) expressed significantly greater numbers of hair cells (6.74 ± 1.60 hair cells) than untreated control cells 12 hours after neomycin treatment ($p < 0.001$, $n = 10$ each respectively). Regenerated hair cells were also stained with FM1-43, which can enter hair cells through functioning mechanotransduction channels at the stereociliary tips of hair cells (Fig. 4C). SEM showed a loss of stereocilia 4 hours after neomycin treatment, and 24 hours after neomycin treatment the stereocilia bundles of the hair cells remained severely damaged. However, stereocilia bundles of the hair cells in larvae maintained with LMWF (50 μg/ml) after neomycin treatment

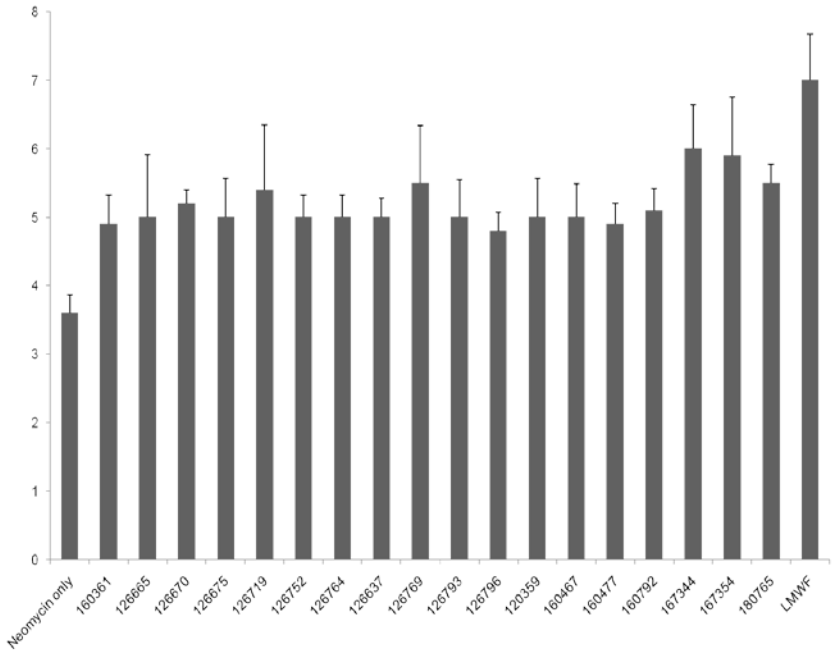

Fig. 3. Screening results for hair cell regeneration. Twenty compounds apparently enhanced hair cell regeneration as defined by more than 25% hair cells (more than 4.5 cells) observed 12 hr after neomycin treatment. The result is shown the tables below. Other 460 compounds did not show more than 25% enhancement of regeneration. Unfortunately, we could not describe the characteristics of the chemicals that demonstrated effects on hair cell regeneration in our primary natural compound screening because the analysis of these chemicals are still on-going.

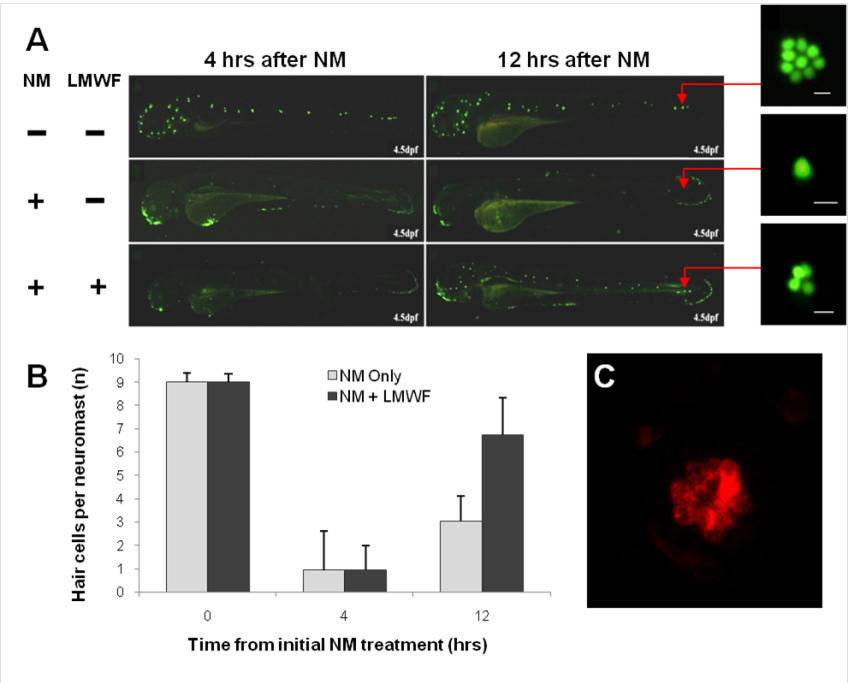

Fig. 4. The effect of LMWF on hair cell regeneration. (A) Hair cells in the lateral line labeled with YO-PRO-1were eliminated in zebrafish 4 hours after neomycin (500 μM) exposure. The number of trunk neuromasts and hair cells per neuromast were markedly increased in the LMWF treatment group 12 hours after neomycin exposure. Insets are close views of P8 neuromasts. (B) Summary data from 10 independent experiments ($p < 0.001$) compared to neomycin only. (C) A regenerated hair cell stained with FM1-43. (NM; Neomycin, LMWF; low molecular weight foucoidan, *; $p<0.05$, scale bar; 10μM)

showed significant recovery 24 hours after neomycin treatment (Fig. 5).

The effect of fucoidan in POU4f3::GFP line To investigate the mechanism of hair cell regeneration by LMWF without vital-dye staining, we used transgenic POU4f3::GFP zebrafish, which display GFP on neuromast hair cells. Hair cells in POU4f34::GFP larvae were also sensitive to neomycin, and only small numbers of hair cells were observed in neuromasts 4 hours after neomycin (250 to 1,000 μM) treatment (Fig. 6). Because of the general toxicity of neomycin at higher concentrations, a concentration of 500 μm was used in subsequent experiments. The effect of LMWF on hair cell regeneration after neomycin treatment in POU4f3::GFP larvae was similar to that in wild-type larvae. LMWF, at concentrations above 50 μg/ml, produced an enhancement of hair cell regeneration 12 hours after neomycin treatment ($p < 0.001$, $n = 10$; Fig. 7A and B). We also assessed the time course of LMWF-induced hair cell regeneration, using a fixed concentration of 50 μg/ml. LMWF significantly enhanced hair cell regeneration 12 hours (neomycin only, 3.16 ± 1.42 hair cells; LMWF, 7.63 ± 1.38 hair cells; $p < 0.001$, $n = 10$) and 16 hours (neomycin only, 3.95 ± 1.40 hair cells; LMWF, 8.05 ± 1.47 hair cells; $p < 0.001$, $n = 10$) after neomycin treatment. In zebrafish larvae not exposed to neomycin, LMWF (50 μg/ml) alone had no effect on hair-cell numbers (Fig. 7C). Because fucoidan is known to inhibit apoptotosis in various cell types, we also investigated whether LMWF prevented neomycin-induced hair cell death. We found, however, no significant difference in neomycin- induced hair cell death between untreated larvae and

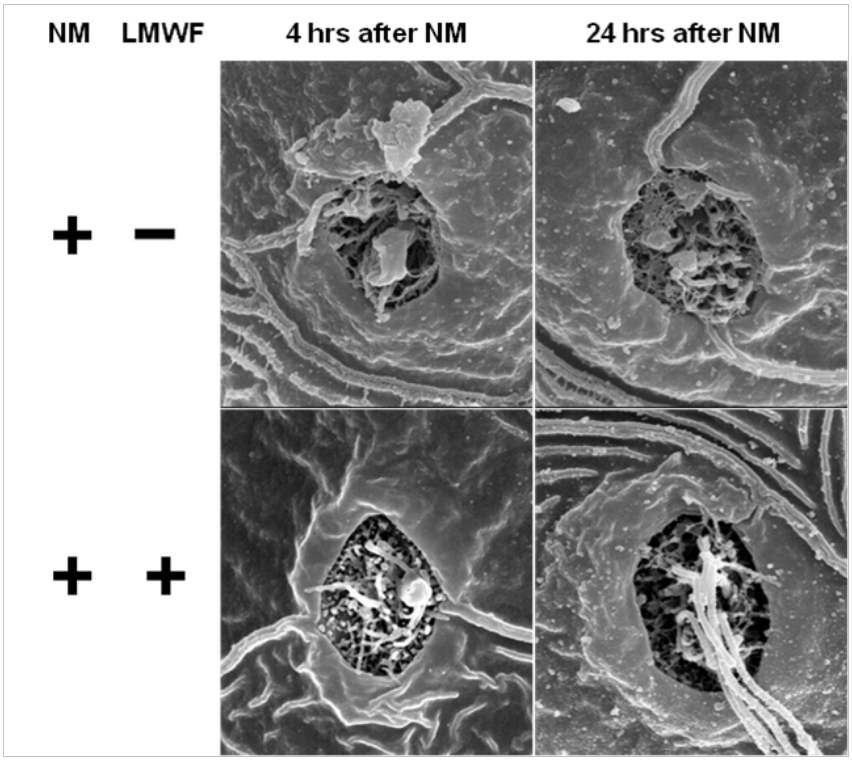

Fig. 5. Scanning electron microscopy of hair cells in neuromasts. Stereocilia bundles on hair cells were still destroyed 24 hours after neomycin (500 μM) treatment, but regenerated in the presence of LMWF 24 hours after neomycin treatment (x5,000)

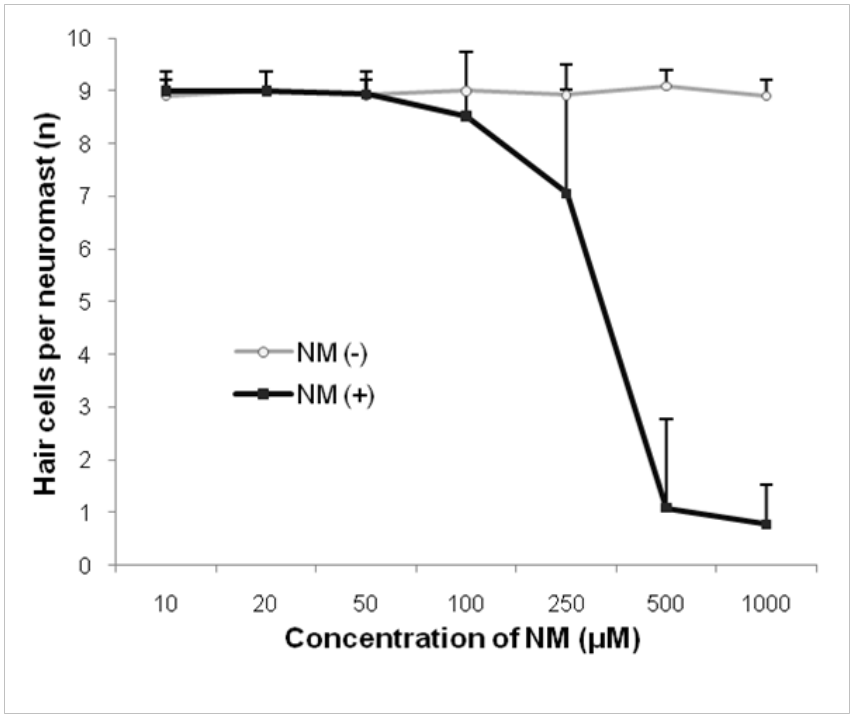

Fig 6. The effect of neomycin in *POU4f3::GFP* larvae.

Neomycin induced hair cell death in a dose-dependent manner.

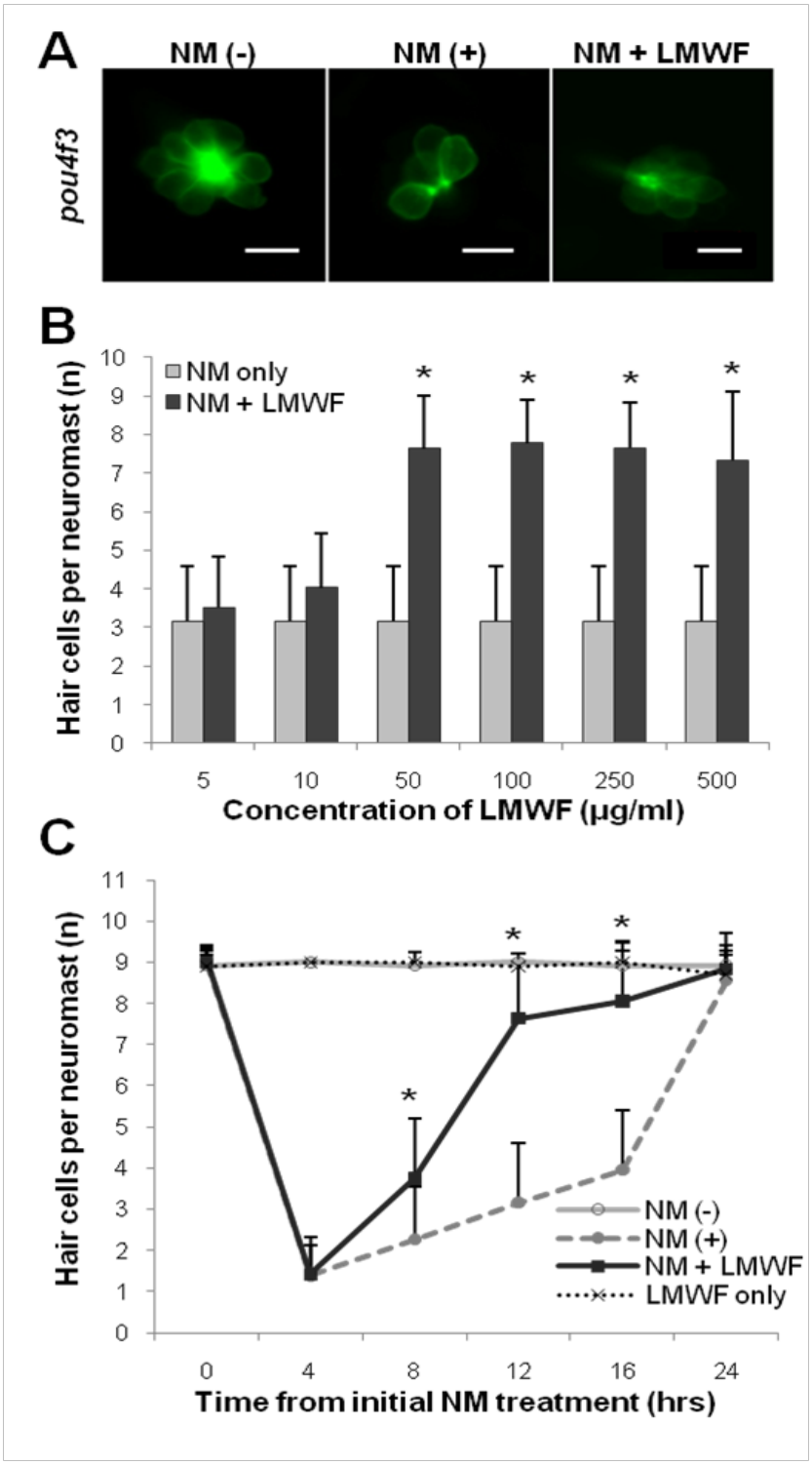
A
NM (-)
NM (+)
NM + LMWF
pou4f3
B
NM only
NM + LMWF
Hair cells per neuromast (n)
Concentration of LMWF (µg/ml)
5
10
50
100
250
500
C
Hair cells per neuromast (n)
Time from initial NM treatment (hrs)
NM (-)
NM (+)
NM + LMWF
LMWF only

Fig. 7. The effect of LMWF on hair regeneration after neomycin-induced ototoxicity in POU4f3::GFP larvae. (A) A greater number of hair cells was observed in the neuromasts (P8) of LWMF-treated larvae 12 hours after neomycin treatment. (B) Summarized data from six independent experiments. LMWF (50 μg/ml) significantly enhanced hair cell regeneration 12 and 16 hours after neomycin treatment. (C) Concentrations of LMWF above 50 μg/ml had similar effects on hair cell regeneration ($p < 0.001$ compared to neomycin only).

those treated with LMWF (50 μg/ml) for 2 hours before exposure to neomycin (p=0.115 to 1.000, n=10 respectively; Fig. 8).

Fucoidan stimulates supporting cells proliferation

To investigate the mechanism of action of LMWF, we investigated its effect on neuromast supporting cells using transgenic zebrafish larvae. The internal supporting cells in scm1::GFP larvae and peripheral supporting cells (mantel cells) in ET20::GFP larvae decreased 4 hours after treatment with neomycin (500 μM) and showed partial recovered after 12 hours. In the LMWF-treated group, both internal supporting cells and peripheral supporting cell also more rapidly recovered (Fig. 9). BrdU incorporation was used to identify neuromast cells undergoing active cell division.

The rate of entry of neuromast cells into the S-phase was significantly higher at 8, 12, and 16 hours in the presence of LMWF ($p < 0.001$, $n = 10$; Fig. 10A and B). Most BrdU-labeled cells were located at the periphery of hair cells in POU4f3::GFP larvae, and overlapped with or were centered on mantle cells in ET20::GFP larvae, corresponding to the regions of the internal supporting cells and peripheral supporting cells (Fig. 10C). The patterns of BrdU incoporation were similar in wild-type larvae to POU4f3::GFP larvae (data not shown).

Inhibition of Notch signaling mimics the fucoidan effect on the hair cell regeneration

As a next step, we tested the role of FGF and Notch signaling pathways in LWMF- induced hair cell regeneration. Both signaling pathways are known to play important roles in hair cell

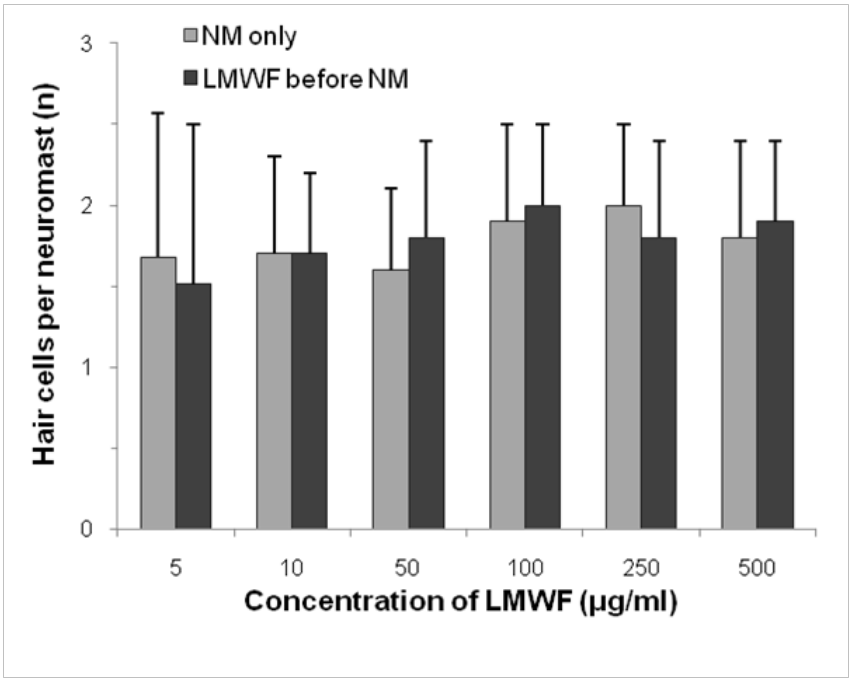

Fig 8. The effect of LMWF-pretreatment of larvae on neomycin-induced ototoxicity. LMWF did not prevent neomycin induced hair cell death (p=0.115 to 1.000, *n* = 10).

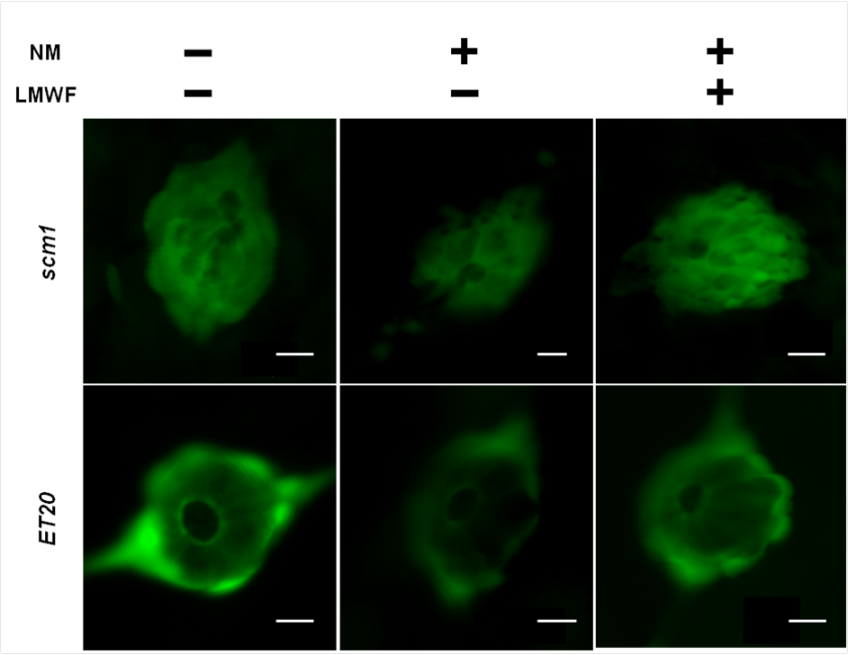

Fig. 9. The effect LMWF on the proliferation of supporting cells. Greater numbers of internal supporting cells in scm1::GFP larvae and peripheral supporting cells in ET20::GFP larvae were observed after LMWF treatment (quantification was not done). Each figure shows P7 neuromasts 12 hours after neomycin (500 μM) treatment.

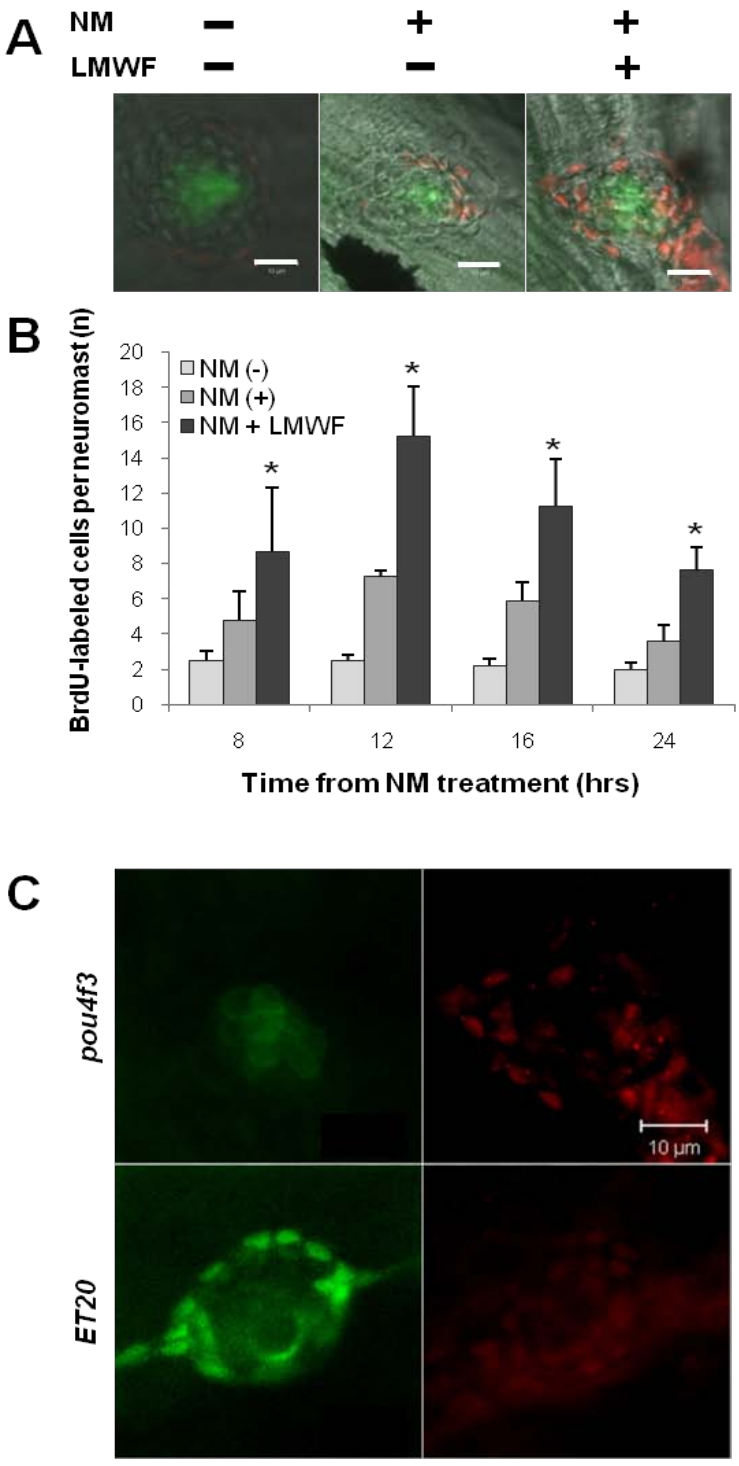
A
NM
LMWF
B
BrdU-labeled cells per neuromast (n)
NM (-)
NM (+)
NM + LMWF
Time from NM treatment (hrs)
C
pou4f3
ET20
10 µm

Fig. 10. BrdU incorporation after neomycin treatment in POU4f3::GFP larvae. (A) More cells are positive for BrdU (red color) in the LMWF group 12 hours after neomycin treatment. (B) Summary of data from six experiments. Neuromast cell entry into the S-phase increased 8, 12, 16, and 24 hours after neomycin treatment in LMWF treat group ($p < 0.001$, $n=10$; compared to neomycin only). (C) BrdU-labeled cells (red dots) are generally located at the periphery of hair cells (green color) in POU4f3::GFP larvae and are centered over the peripheral supporting cells in ET20::GFP larvae. (NM; Neomycin, LMWF; low molecular weight foucoidan, *; $p<0.05$)

development and regeneration. Inhibition of the Notch signaling pathway with DAPT enhanced hair cell regeneration 12 hours after neomycin treatment (6.23 ± 1.36 hair cells) compared to the neomycin-only group (2.27 ± 0.58 hair cells; $p < 0.05$), and the effect was similar to that seen in LMWF-treated larvae (6.53 ± 1.31 hair cells). Interestingly the effects of LMWF and DAPT were not additive: LMWF + DAPT, 6.67 ± 1.27 hair cells ($p > 0.1$, $n = 10$). In contrast, inhibition of the FGF signaling pathway by SU5402 resulted in delayed hair cell regeneration (2.56 ± 1.14 cells). However, LMWF still enhanced hair cell regeneration in the presence of the SU5402 (6.35 ± 0.71 hair cells; $p = 0.247$, $n = 10$; Fig. 11).

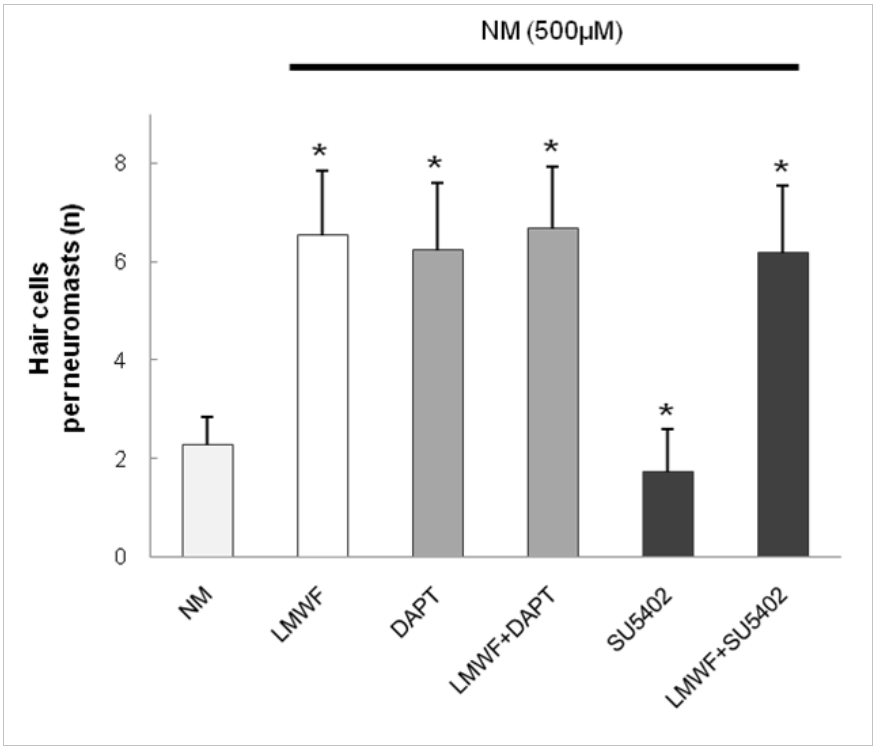

Fig. 11. Hair cells regenerated more rapidly in larvae treated with the Notch singling inhibitor DAPT after neomycin, compared to larvae treated with neomycin only ($p<0.001$, $n=10$ respectively). The rate of DAPT-induced hair cell regeneration was similar to that induced by LMWF ($p=0.382$, $n=10$) and LMWF had no additional effect in the presence of DAPT. ($n=10$; $p=0.690$ compared to LMWF after neomycin). Hair cell regeneration was stimulated by LMWF in the presence of the FGF inhibitor SU5402 ($n = 10$; $p=0.007$ compared to neomycin only).

DISCUSSION

In the past decade, fucoidans from different species have gained attention due to their numerous interesting biological activities. Anticoagulant, antiviral, antitumor, anti- complement, immunomodulatory, anti-inflammatory, and antioxidant activities have been reported (Wang et al., 2009; Changotade et al., 2008; Li et al., 2008). Fucoidan is also known to promote tissue regeneration. In a rat model of critical hind limb ischemia, fucoidan was found to promote therapeutic revascularization by potentiating FGF-2 activity (Luyt et al., 2003). Moreover, progenitor stem cell mobilization was promoted by fucoidan via increasing plasma SDF-1 in a monkey model (Sweeney et al. 2002). Fucoidan has also been shown to simulate bone formation and wound repair via mechanisms involving hepatocyte growth factor (Fukuta et al, 2008). In this study, we found that LMWF enhanced hair cell regeneration after neomycin-induced ototoxicity in both wild-type and transgenic zebrafish larva. A compound that directly enhances the regeneration of neuroepithelial cells, such as hair cells, is an interesting new finding. Hair cells in sensory epithelia might be replaced by both the proliferation of precursor cells (Lippe et al., 1991; Stone and Rubel, 2000) and via transdifferentiation of supporting cells (Baird et al., 1993; Adler and Raphael, 1996). Hair cell regeneration after neomycin toxicity is most likely to occur through the mitosis of proliferative progenitors, and the supporting cell may be the most likely candidate for that precursor cell (Ma et al., 2008). Our results also indicate that

LMWF enhances hair cell regeneration by stimulating the proliferation of supporting cells. The number of BrdU- positive cells was increased by LMWF in neomycin-treated larvae, and most of the BrdU- labeled cells were found at the periphery of the hair cells, in the region populated by supporting cells. However, the rate of BrdU incoporation was slightly different between transgenics, and this was especially notable in ET20::GFP larvae. Because fucoidan has shown anti-apoptotic effects in various cell types, we expected LMWF to protect against neomycin-induced hair cell death (Rybak and Ramkumar, 2007; Kim et al., 2008). Our results showed, however, that LMWF was not effective in preventing neomycin-induced hair cell death.

The mechanism by which LMWF stimulated the proliferation of supporting cells is unclear. During the development of mechanosensory neuromasts in zebrafish, FGF and Notch are the main signaling molecules for hair cell differentiation (Nechiporuk et al., 2008; Millimaki et al., 2007; Ma et al., 2007)(Fig. 12). We investigated the effects of these signal molecules in fucoidan-induced hair cell regeneration after neomycin ototoxicity. Inhibition of FGF by SU5402 had no significant effect on hair cell regeneration, nor did it modify the effects of LMWF. In contrast, the inhibition of Notch signaling with DAPT stimulated the regeneration of hair cells to the similar degree as LMWF, and there was no additional stimulatory effect of LMWF in DAPT-treated larvae. These results indirectly suggest that the promotion of hair cell regeneration after neomycin treatment is at least partially dependent on Notch signaling. Notch signaling is important for not only hair cell development, but also for hair cell

regeneration. Inhibition of Notch signaling has no effect on mature, undamaged neuromasts. However, inhibition of Notch signaling results in the overproduction of hair cells following hair cell damage (Ma et al., 2008; Fig 12B). The zebrafish mind bomb (mib) mutant, which has a defect in the Notch signaling pathway, is characterized by an overproduction of hair cells (Itoh and Chitnis, 2001). Recently, a similar result was reported for a mammalian ear. Notch signaling components were found to be upregulated in the epithelia of adult guinea pigs, and the administration of γ-secretase inhibitors led to the formation of ectopic auditory hairs (Hori et al., 2007). These data suggest that Notch signaling plays an important role in limiting hair cell number. Although our data indicate that the effects of LMWF on hair cell regeneration could overlap with the effects of Notch signal inhibition, the exact mechanism of fucoidan-induced hair cell regeneration needs to be clarified using various durgs and transgenic phenotypes of zebrafish. The zebrafish is a useful animal model for drug screening, and there are many reports regarding the use of zebrafish to screen for ototoxic drugs and chemicals to prevent ototoxicity (Harris et al., 2003; Ton and Parng, 2005; Chiu et al., 2008). Zebrafish model is also useful to screen for drugs that stimulate hair cell regeneration after ototoxicity (Harris et al., 2003). Larvae were treated first with the aminoglycoside neomycin to eliminate mechanosensory hair cells in lateral line, followed by treatment with the test compounds and an assessment of hair cell regeneration. Using this approach, we have identified additional compounds in our libraries that stimulate hair cell regeneration and are proceeding with their

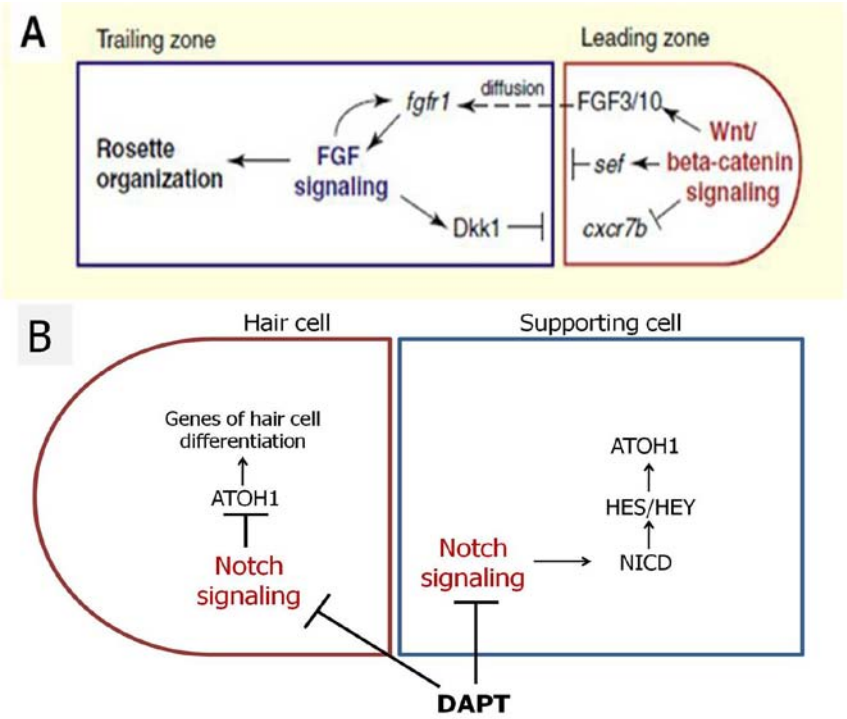

Fig. 12. FGF and Notch are the main signaling molecules for hair cell differentiation. (A) Schematic model showing interactions between the Wnt/b-catenin signaling pathway in the leading zone and FGF signaling in the trailing zone of the primordium (sourced from Ma et al., 2009). (B) Inhibitors of g-secretases such as (N-[N-(3,5-difluorophenacetyl-L-alanyl)]- S-phenylglycine t-butyl ester (DAPT) appear to interrupt Notch signaling by blocking the generation of the NICD. Inhibition of Notch signaling results in the overproduction of hair cells following hair cell damage (sourced from Collado et al., 2008)

characterization (unpublished observations). The use of transgenic larva for drug screening has several advantages. For example, their use facilitates the serial observation of hair cells in the same larva without staining, and transgenic larvae with fluorescent hair cells or supporting cells are useful in dissecting the mechanisms of hair cell regeneration.

In summary, we found that LMWF enhanced hair cell regeneration after ototoxicity by promoting the proliferation of supporting cells, and our evidence indicate involvement of the Notch signaling pathway in this process. Future studies with well-defined fucan structures will further advance our understanding of the biological role of LMWF in hair cell regeneration.

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국 문 요 약

Aminoglycoside 계통 항생제에 의해 유도된 세포 손상 후 재생과정 중 후코이단의 측선 유모세포 재생 촉진 효과 분석

노년인구가 증가하고 의료이용이 늘어남에 따라 항생제 또는 항암제 사용으로 인한 청각 유모세포의 손상이 증가하고 있다. 손상된 청각 유모세포를 재생시킬 수 있다면 난청의 유병률을 크게 낮출 수 있다.

제브라피쉬 모델은 초파리나 선충에서만 가능하던 대량 screening 이나 유전자변형이 가능한 거의 척추동물 모델로 인간과 유전적 정보가 유사하고 몸통의 외측면에 유모세포와 지지세포의 군집으로 이루어진 측선이 있다. 이러한 측선의 유모세포는 포유류와 달리 유모세포 손상 후 재생능력이 발달되어 있고 치어 상태에서는 투명한 몸통을 가지고 있어 유모세포의 손상과 재생 과정을 live 상태에서 관찰 가능하므로 이독성 난청 예방 및 치료 물질 발굴 연구에 매우 유용한 모델이다.

4 일에서 6 일째의 제브라피쉬 치어를 이용하여 항생제 이독성 치료 약물을 스크리닝하여 저분자량의 후코이단이 neomycin 에 의해 유도된 측선 유모세포 사멸 후 유모세포의 재생을 촉진함을 발견하였다. Neomycin 전처리 후 후코이단을

부가한 군과 부가하지 않은 군, 그리고 neomycin 처리를 하지 않은 대조군의 측선유모세포 재생과정을 live 상태 및 전자현미경으로 관찰한 결과 후코이단 부가군에서 재생이 현저히 촉진되었다. 후코이단의 재생 촉진 기전을 Pou4f3::GFP, scm1::GFP, ET20::GFP 등의 형질 변환 제브라피쉬에서 반복 실험 및 BrdU incoporation 을 시행한 결과, 내측 지지세포가 유모세포의 전구세포로 분열되고 유모세포로의 합성 및 분화가 되는 기존의 과정을 촉진하는 것이며 특히 Notch signal 과 연관되어 있음을 알 수 있었다.

핵심되는 말: 후코이단, 유모세포, 세포사멸, 재생, 제브라피쉬