Hearing Research xxx (2011) 1–7

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Research Paper

Fucoidan promotes mechanosensory hair cell regeneration following aminoglycoside-induced cell death ☆

In Seok Moon [a], Ju-Hoon So [b], Young-Mi Jung [c,1], Won-Sang Lee [d], Eun Young Kim [d], Cheol-Hee Kim [b,*], Jae Young Choi [d,*]

[a] Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Chung-Ang University, Seoul, South Korea
[b] Department of Biology and GRAST, Chungnam National University, Daejeon, South Korea
[c] Department of Genetic Engineering, College of Natural Science, Kyoungpook National University, Daegu, South Korea
[d] Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Yonsei University, Seoul, South Korea

ARTICLE INFO

Article history:
Received 28 December 2010
Received in revised form
7 June 2011
Accepted 15 July 2011
Available online xxx

ABSTRACT

Objective: 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. The aims of this study were to quantify the regenerative effects of LMWF and determine their relationship to the Notch and FGF signaling pathways.
Methods: Wild-type zebrafish and three different transgenic zebrafish lines (*Pou4f3*::GFP, *scm1*::GFP, and *ET20*::GFP) were used. At 4.5–6 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.
Results: 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.
Conclusion: 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.

1. 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 and Gacek, 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. 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, 2002; Nicolson, 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

☆ This study was supported by a grant of the Korean Healthcare Technology R&D Project for Health, Welfare & Family Affairs, Republic of Korea (Project Number A090496) and a Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (20100024645).
* Corresponding authors.
E-mail addresses: zebrakim@cnu.ac.kr (C.-H. Kim), jychoi@yuhs.ac (J. Y. Choi).
[1] Young-Mi Jung has the Korean Patent for bioavailable fucoidan and methods for preparing the same (2007-07059705).

0378-5955/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.heares.2011.07.007

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 amino glycoside-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 amino glycoside-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.

2. Materials and methods

2.1. 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. 1). 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 4.5–6 days post-fertilization (dpf). Baseline E3 embryo medium contained 1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 μM NaCl, and 500 μM $NaHCO_3$ in deionized H_2O, with the pH adjusted to 7.2.

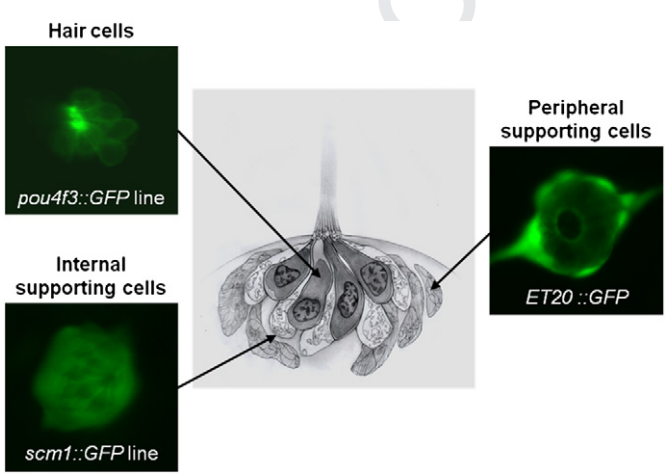

Fig. 1. Expression of fluorescence in transgenic zebrafish. Transgenic *pou4f3::GFP* larvae displayed GFP on hair cells; *scm1::GFP* larvae, on the internal neuromast supporting cells; and *ET20::GFP* larvae, on peripheral neuromast supporting cells.

2.2. 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., 2005) and 10 purified natural compounds (fucoidan, Keumsa Linteusan, saponin, beta-glucan extract, polyhexosamine extract, 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 h 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-min 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.

2.3. 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, 2007). Briefly, high molecular weight fucoidan (0.1–20%, w/v; preferably 0.1–6%, w/v) were hydrolyzed by treating with 2 N pyruvic acid and 2 N acetic acid (1:4) for 5 h 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 (1000 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), 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.

2.4. 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 h 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

Please cite this article in press as: Moon, I.S., et al., Fucoidan promotes mechanosensory hair cell regeneration following amino glycoside-induced cell death, Hearing Research (2011), doi:10.1016/j.heares.2011.07.007

three posterior trunk neuromasts (P1, P7, and P8) were evaluated at 4, 8, 12, 16, and 24 h 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 (SU5 402; CalBiochem). The zebrafish larvae were anesthetized with 0.1% tricaine and placed on a depression slide for viewing with confocal microscopy (Zeiss, LSM510)

2.5. 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 min per wash) in distilled water and dehydrated through serial 10-min exposures to graded concentrations of ethanol solutions (25, 50, 70, 80, 95, and 100%). Samples were further dehydrated through serial 10-min 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.

2.6. 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 h after treatment, and cells were labeled by adding 10 mM BrdU in 1% dimethylsulfoxide (DMSO) to the medium for 2 h at 28.5 °C before collection. Larvae were anesthetized in tricaine, and fixed with 4% paraformaldehye in PBS for 2 h 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 h. Larvae were then rehydrated in a graded methanol series and washed in PBDT for 20 min. Following rehydration, all larvae were digested with proteinase K (10 μg/ml; Roche) for 20 min, washed in PBDT, and fixed again in 4% paraformaldehye for 20 min. After another washing in PBDT, the larvae were placed into 2 N HCl at RT for 1 h. Larvae were washed again in PBST, and non-specific binding was blocked in 5% normal goat serum in PBDT for 1 h 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 h in PBST and incubated in Rhodamine Red-X goat anti-mouse IgG at RT for 6 h (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.

2.7. 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.

3. Results

3.1. Identification of fucoidan as an enhancer for hair cell regeneration

Neomycin (50–1000 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 h after treatment with 500 μM neomycin. Hair cell regeneration was evident 12 h after neomycin exposure, 3.01 ± 0.48 hair cells per neuromast were observed (Fig. 2). Among the 480 compounds screened, 20 compounds apparently enhanced hair cell regeneration, as defined by more than 25% regeneration of hair cells observed 12 h after neomycin treatment (Moon et al. unpublished observation). The effect of LMWF was the most prominent and consistent, and it was studies in greater detail. As shown in Fig. 2A and B, 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 h 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. 2C). SEM showed a loss of stereocilia 4 h after neomycin treatment, and 24 h 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 showed significant recovery 24 h after neomycin treatment (Fig. 3).

3.2. 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 h after neomycin (250–1000 μM) treatment. Because of the general

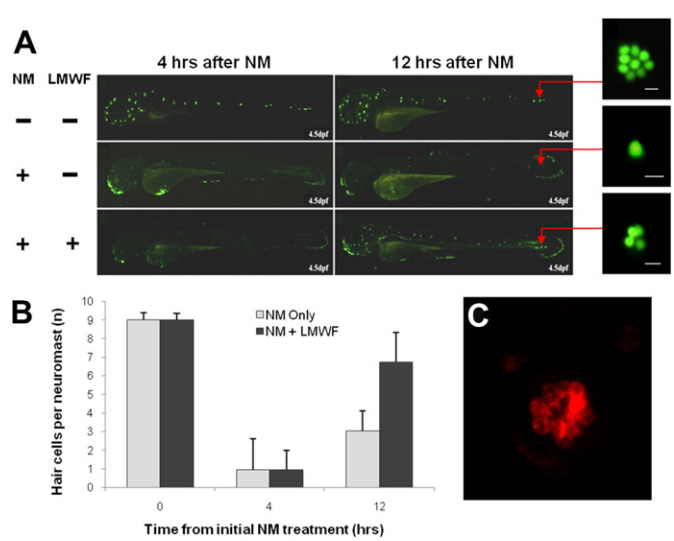

Fig. 2. The effect of LMWF on hair cell regeneration. (A) Hair cells in the lateral line labeled with YO-PRO-1were eliminated in zebrafish 4 h after neomycin (500 μM) exposure. The number of trunk neuromasts and hair cells per neuromast were markedly increased in the LMWF treatment group 12 h after neomycin 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; 10 μM).

Please cite this article in press as: Moon, I.S., et al., Fucoidan promotes mechanosensory hair cell regeneration following amino glycoside-induced cell death, Hearing Research (2011), doi:10.1016/j.heares.2011.07.007

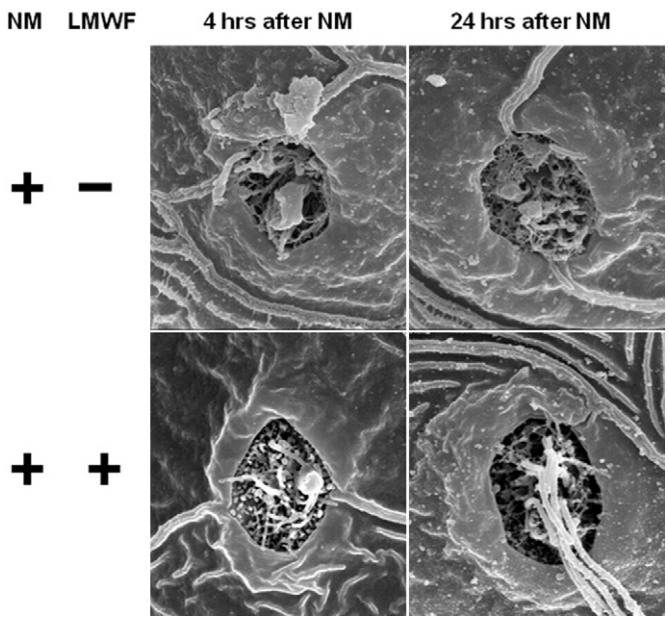

Fig. 3. Scanning electron microscopy of hair cells in neuromasts. Stereocilia bundles on hair cells were still destroyed 24 h after neomycin (500 μM) treatment, but regenerated in the presence of LMWF 24 h after neomycin treatment (×5000).

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 h after neomycin treatment ($p < 0.001$, $n = 10$; Fig. 4A 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 h (neomycin only, 3.16 ± 1.42 hair cells; LMWF, 7.63 ± 1.38 hair cells; $p < 0.001$, $n = 10$) and 16 h (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. 4C). 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 those treated with LMWF (50 μg/ml) for 2 h before exposure to neomycin ($p = 0.115$ to 1.000, $n = 10$ respectively).

3.3. 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 h after treatment with neomycin (500 μM) and showed partial recovered after 12 h. In the LMWF-treated group, both internal supporting cells and peripheral supporting cell also more rapidly recovered (Fig. 5).

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 h in the presence of LMWF ($p < 0.001$, $n = 10$; Fig. 6A 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

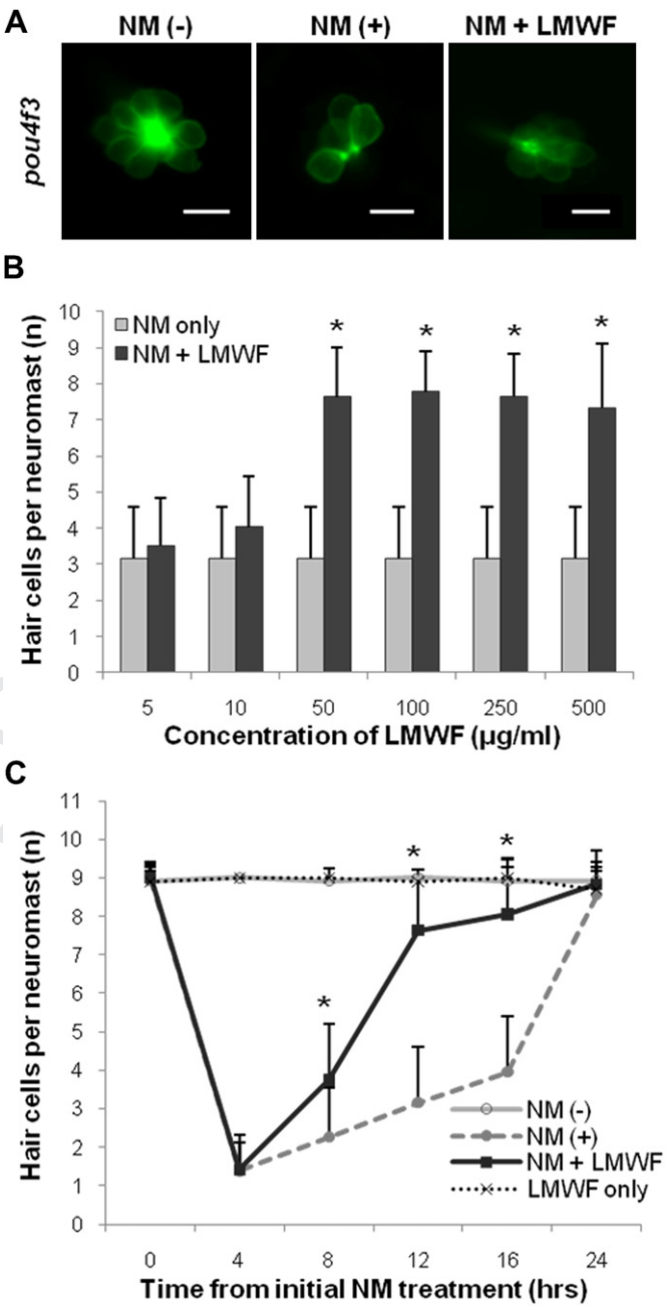

Fig. 4. The effect of LMWF on hair regeneration after neomycin-induced ototoxicity in *POU4f3*::*GFP* larvae. (A) A greater number of hair cells was observed in the neuromasts (P8) of LWMF-treated larvae 12 h after neomycin treatment. (B) Summarized data from six independent experiments. LMWF (50 μg/ml) significantly enhanced hair cell regeneration 12 and 16 h 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).

(Fig. 6C). The patterns of BrdU incoporation were similar in wild-type larvae to *POU4f3*::GFP larvae (data not shown).

3.4. 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

Please cite this article in press as: Moon, I.S., et al., Fucoidan promotes mechanosensory hair cell regeneration following amino glycoside-induced cell death, Hearing Research (2011), doi:10.1016/j.heares.2011.07.007

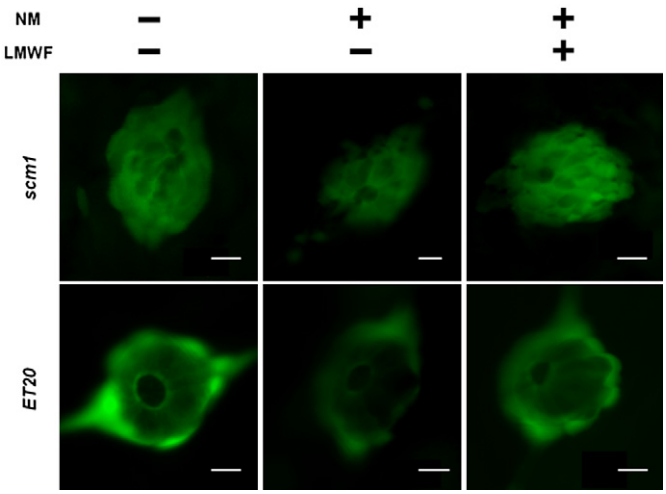

Fig. 5. The effect LMWF on the proliferation of supporting cells. Greater numbers of internal supporting cells in *scm1*::GFP larvae and peripheral supporting cells in *ET20*::GFP larvae were observed after LMWF treatment (quantification was not done). Each figure shows P7 neuromasts 12 h after neomycin (500 μM) treatment.

development and regeneration. Inhibition of the Notch signaling pathway with DAPT enhanced hair cell regeneration 12 h 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. 7).

4. 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 and Nakamura, 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

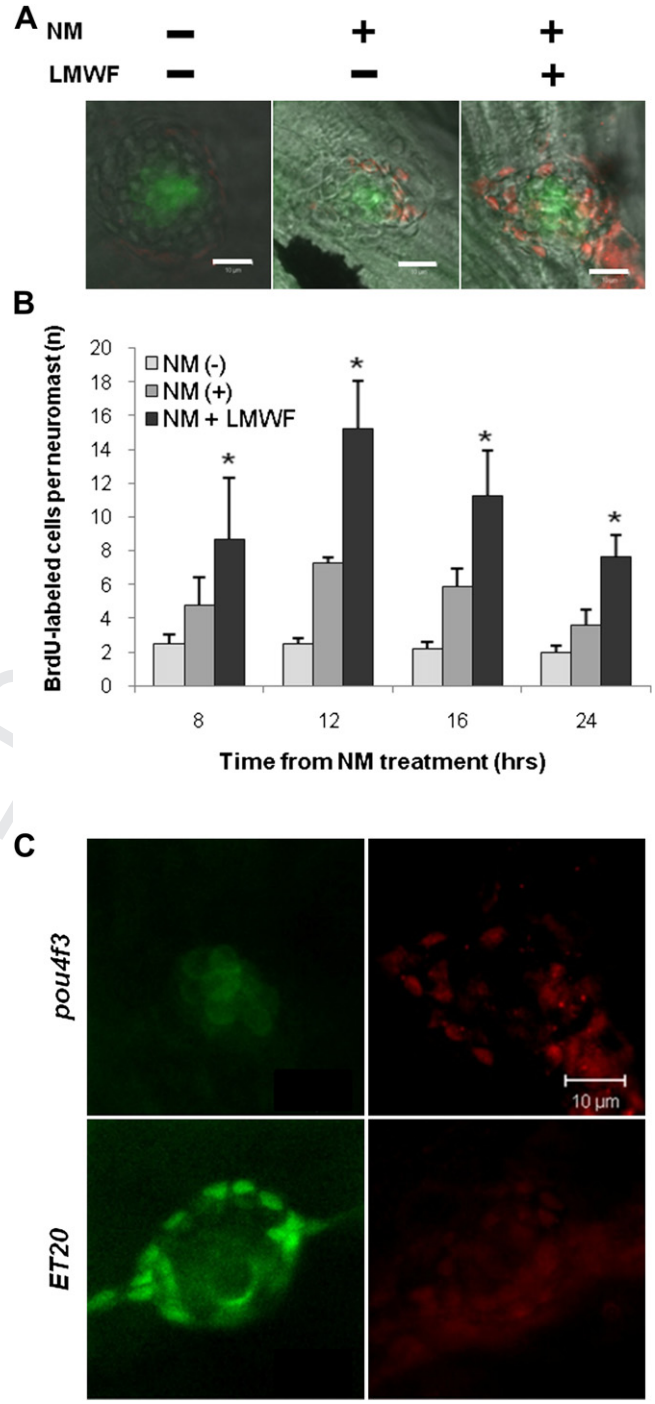

Fig. 6. BrdU incorporation after neomycin treatment in *POU4f3*::*GFP* larvae. (A) More cells are positive for BrdU (red color) in the LMWF group 12 h after neomycin treatment. (B) Summary of data from six experiments. Neuromast cell entry into the S-phase increased 8, 12, 16, and 24 h after neomycin 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, *; $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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.

Please cite this article in press as: Moon, I.S., et al., Fucoidan promotes mechanosensory hair cell regeneration following amino glycoside-induced cell death, Hearing Research (2011), doi:10.1016/j.heares.2011.07.007

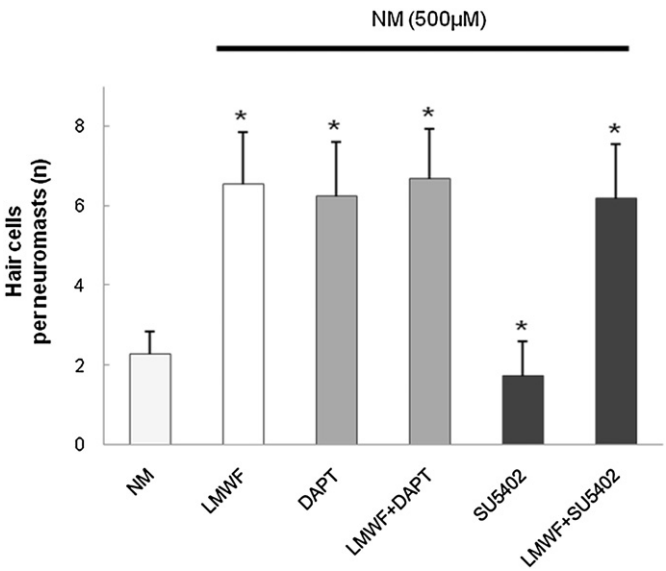

Fig. 7. Hair cells regenerated more rapidly in larvae treated with the Notch singling inhibitor DAPT after neomycin, compared to larvae treated with neomycin only ($p < 0.001$, $n = 10$ respectively). The rate of DAPT-induced hair cell regeneration was similar to that induced by LMWF ($p = 0.382$, $n = 10$) 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).

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 and Raible, 2008; Millimaki et al., 2007; Ma and Raible, 2009). 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). 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 amino glycoside 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 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.

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 (1), 17–20.

Baird, R.A., Torres, M.A., Schuff, N.R., 1993. Hair cell regeneration in the bullfrog vestibular otolith organs following amino glycoside toxicity. Hear Res 65, 164–174.

Behra, M., Bradsher, J., Sougrat, R., Gallardo, V., Allende, M.L., Burgess, S.M., 2009. Phoenix is required for mechanosensory hair cell regeneration in the zebrafish lateral line. PLoS Genet 5 (4), e1000455.

Changotade, S.I., Korb, G., Bassil, J., Barroukh, B., Willig, C., Colliec-Jouault, S., Durand, P., Godeau, G., Senni, K., 2008. Potential effects of a low-molecular-weight fucoidan extracted from brown algae on bone biomaterial osteoconductive properties. J Biomed Mater Res A 87 (3), 666–675.

Chiu, L.L., Cunningham, L.L., Raible, D.W., Rubel, E.W., Ou, H.C., 2008. Using the zebrafish lateral line to screen for ototoxicity. J Assoc Res Otolaryngol 9 (2), 178–190.

Fukuta, K., Nakamura, T., 2008. Induction of hepatocyte growth factor by fucoidan and fucoidan-derived oligosaccharides. J Pharm Pharmacol 60, 499–503.

Harris, J.A., Cheng, A.G., Cunningham, L.L., MacDonald, G., Raible, D.W., Rubel, E.W., 2003. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (Danio rerio). J Assoc Res Otolaryngol 4 (2), 219–234.

Hori, R., Nakagawa, T., Sakamoto, T., et al., 2007. Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea. NeuroReport 18 (18), 1911–1914.

Itoh, M., Chitnis, A.B., 2001 Apr. Expression of proneural and neurogenic genes in the zebrafish lateral line primordium correlates with selection of hair cell fate in neuromasts. Mech Dev 102 (1–2), 263–266.

Jung, Y.M., Yoo, K.M., Park, D.C., Kim, T.K., Lee, H.S., Ku, S.K., 2008. Single dose toxicity study of low molecular fucoidan in mice. Off J Korean Society Toxicology 24 (1), 79–86.

Jung, Y.M., Kim, T.K., Park, D.C., Lee, Y.H., 2007. Bioavailable fucoidan and methods for preparing the same. Korean Patent. (http://www.wipo.int/pctdb/en/wo.jsp?WO=2008041799).

Kang, D.H., Jun, K.Y., Lee, J.P., Pak, C.S., Na, Y., Kwon, Y., 2009. Identification of 3-acetyl-2-aminoquinolin-4-one as a novel, nonpeptidic scaffold for specific calpain inhibitory activity. J Med Chem 52 (9), 3093–3097.

Kim, C.H., Kang, S.U., Pyun, J., Lee, M.H., Hwang, H.S., Lee, H., 2008. Epicatechin protects auditory cells against cisplatin-induced death. Apoptosis 13 (9), 1184–1194.

Kim, K.R., Kwon, J.-L., Kim, J.-S., No, Z., Kim, H.R., Cheon, H.G., 2005. EK-6136 (3-methyl-4-(O-methyl-oximino)-1-phenylpyrazolin-5-one): a novel Cdc25B inhibitor with antiproliferative activity. Eur J Pharmacol 528 (1–3), 37–42.

Li, B., Lu, F., Wei, X., Zhao, R., 2008. Fucoidan: structure and bioactivity. Molecules 13 (8), 1671–1695 (Review).

Lippe, W.R., Westbrook, E.W., Ryals, B.M., 1991. Hair cell regeneration in the chicken cochlea following amino glycoside toxicity. Hear Res 56, 203–210.

Luyt, C.E., Meddahi-Pellé, A., Ho-Tin-Noe, B., Colliec-Jouault, S., Guezennec, J., Louedec, L., et al., 2003. Low-molecular-weight fucoidan promotes therapeutic revascularization in a rat model of critical hindlimb ischemia. J Pharmacol Exp Therapeut 305, 24–30.

Ma, E., Rubel, E., Raible, D., 2008. Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. J Neurosci 28, 2261–2273.

Please cite this article in press as: Moon, I.S., et al., Fucoidan promotes mechanosensory hair cell regeneration following amino glycoside-induced cell death, Hearing Research (2011), doi:10.1016/j.heares.2011.07.007

Ma, E., Raible, D., 2009. Signaling pathway regulating zebrafish lateral line development. Curr Biol 19, R381–R386.
Millimaki, B.B., Sweet, E.M., Dhason, M.S., Riley, B.B., 2007. Zebrafish atoh1 genes: classic proneural activity in the inner ear and regulation by FGF and Notch. Development 134, 295–305.
Nechiporuk, A., Raible, D.W., 2008. FGF-dependent mechanosensory organ patterning in zebrafish. Science 320 (5884), 1774–1777.
Nicolson, T., 2005. The genetics of hearing and balance in zebrafish. Annu Rev Genet 39, 9–22.
Owens, K.N., Santos, F., Roberts, B., Linbo, T., Coffin, A.B., Kinsely, A.J., Simon, J.A., Rubel, E.W., Raible, D.W., 2008. Identification of genetic and chemical modulators of zebrafish mechanosensory hair cell death. PLoS Genet 4 (2), e1000020.
Parinov, S., Kondrichin, I., Korzh, V., Emelyanov, A., 2004. *Tol2* transposon-mediated enhancer Trap to identify developmentally regulated zebrafish genes in vivo. Dev Dyn 231, 449–459.
Rubel, E.W., Dew, L.A., Roberson, D.W., 1995. Mammalian vestibular hair cell regeneration. Science 267, 701–707.
Ruben, R.T., 1967. Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. Acta Otolaryngol 220, 1–44.
Rybak, L., Ramkumar, V., 2007. Ototoxicity. Kidney Int 72, 931–935.
Schuknecht, H.F., Gacek, M.R., 1993. Cochlear pathology in presbycusis. Ann Otol Rhinol Laryngol 102, 1–16.
Stone, J.S., Rubel, E.W., 2000. Cellular studies of auditory hair cell regeneration in birds. Proc Natl Acad Sci USA 97, 11714–11721.
Sweeney, E.A., Lortat-Jacob, H., Priestley, G.V., Nakamoto, B., Papayannopoulou, T., 2002. Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells. Blood 99, 44–51.
Ton, C., Parng, C., 2005 Oct. The use of zebrafish for assessing ototoxic and otoprotective agents. Hear Res 208 (1–2), 79–88.
Wang, J., Zhang, Q., Zhang, Z., Zhang, J., Li, P., 2009. Synthesized phosphorylated and aminated derivatives of fucoidan and their potential antioxidant activity in vitro. Int J Biol Macromol 44, 170–174.
Westerfield, M., 2000. The zebrafish book;a guide ofr the laboratory use of zebrafish(Danio rerio), fourth ed. University of Oregon Press, Eugene, OR.
Whitfield, T.T., 2002. Zebrafish as a model for hearing and deafness. J Neurobiol 53, 157–171.
Xiao, T., Roeser, T., Staub, W., Baier, H., 2005. A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. Dev 132, 2955–2967.

Please cite this article in press as: Moon, I.S., et al., Fucoidan promotes mechanosensory hair cell regeneration following amino glycoside-induced cell death, Hearing Research (2011), doi:10.1016/j.heares.2011.07.007