Regeneration of mammalian cochlear and vestibular hair cells through Hes1/Hes5 modulation with siRNA

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Abstract

The Notch pathway is a cell signaling pathway determining initial specification and subsequent cell fate in the inner ear. Previous studies have suggested that new hair cells (HCs) can be regenerated in the inner ear by manipulating the Notch pathway. In the present study, delivery of siRNA to Hes1 and Hes5 using a transfection reagent or siRNA to Hes1 encapsulated within poly(lactide-co-glycolide acid) (PLGA) nanoparticles increased HC numbers in non-toxin treated organotypic cultures of cochlea and maculae of postnatal day 3 mouse pups. An increase in HCs was also observed in cultured cochlea and maculae of mouse pups pre-conditioned with a HC toxin (4-hydroxy-2-nonenal or neomycin) and then treated with the various siRNA formulations. Treating cochlea with siRNA to Hes1 associated with a transfection reagent or siRNA to Hes1 delivered by PLGA nanoparticles decreased Hes1 mRNA and up-regulated Atoh1 mRNA expression allowing supporting cells (SCs) to acquire a HC fate. Experiments using cochlea and maculae of p27kip1-/-GFP transgenic mouse pups demonstrated that newly generated HCs transdifferentiated from SCs. Furthermore, PLGA nanoparticles are non-toxic to inner ear tissue, readily taken up by cells within the tissue of interest, and present a synthetic delivery system that is a safe alternative to viral vectors. These results indicate that when delivered using a suitable vehicle, Hes siRNAs are potential therapeutic molecules that may have the capacity to regenerate new HCs in the inner ear and possibly restore human hearing and balance function.

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1. Introduction

Hair cells (HCs) and supporting cells (SCs) in the sensory epithelium of the inner ear arise from a common progenitor (Fekete et al., 1998; Lanford et al., 1999; Kiernan et al., 2005; Raft et al., 2007; Riley et al., 1999). Recent evidence suggests that Notch pathway proteins play a role in keeping SCs in their phenotypic state in mammals and prevent them from becoming HCs via a process of lateral inhibition (Landford et al., 1999; Zheng et al., 2000; Zine et al., 2001).

The Notch pathway determines initial specification and subsequent cell fate of sensory progenitors (Kelley, 2006; Murata et al., 2006). Embryonic Notch signaling directs the formation of the intricate SC and HC mosaic of inner ear epithelia. This mosaic pattern is accomplished through lateral inhibition, involving the cell surface receptor Notch and its interactions with ligands, such as Jagged and Delta. Ligand-Notch binding induces the γ-secretase-mediated cleavage of the intracellular domain of Notch, which, in turn, translocates to the nucleus to form a transcription complex that activates the expression of Hes1 and Hes5 (Hair and enhancer of split 1 and 5), inducing a SC fate (Landford et al., 1999; Murata et al., 2006). Hes1 and Hes5 encode two inhibitory basic helix-loop-helix proteins, which down regulate the expression of Atoh1 and other prosensory genes in differentiating SCs (Kelley, 2006). Expression of Atoh1 is critical for the formation of HCs in the normal sensory epithelium (Bermingham et al., 1999). However, in
mammals, Atoh1 expression is attenuated at early postnatal stages and remains low throughout adulthood, while expression of Hes1 becomes elevated at late embryonic and early postnatal stages and is maintained at a relatively high level throughout adulthood, which may be one of the mechanisms that maintains the appropriate complement of HCs and SCs in the inner ear (Zheng et al., 2000). In support of this model, reduction of Hes transcripts in presumptive HCs promotes expression of Atoh1 and leads to terminal differentiation into the HC phenotype (Zheng and Gao, 2000; Zine et al., 2001; Zine and de Ribeaupierre, 2002).

It appears that the Notch pathway is altered shortly after vestibular epithelial injury, allowing HC regeneration in mammals (Batts et al., 2009; Wang et al., 2010). Moreover, regeneration can be enhanced in mammalian cochlear and vestibular epithelia by ectopic Atoh1 expression, using an adenoviral vector (Izumikawa et al., 2005; Shou et al., 2003; Staecker et al., 2007). Decreasing Notch signaling with \( \gamma \)-secretase inhibitors results in ectopic HC replacement in the mammalian cochlea (Hori et al., 2007). Decreasing Notch pathway activity in mouse utricular epithelia in vitro by inhibiting \( \gamma \)-secretase or another enzyme required for Notch activity resulted in a non-mitotic increase in HCs limited to the striolar/juxtastriolar region (Collado et al., 2011; Lin et al., 2011).

Utricular tissue in mice exposed to an ototoxic exhibited a reduction in Hes5 transcript levels that was correlated with an increase in Atoh1 mRNA and the appearance of embryonic-like HCs (Wang et al., 2010). Importantly, a recent report noted that toxin-induced damage to adult guinea pig cochlea resulted in increased Hes1 and Notch1 protein expression and increased levels of the Notch intracellular domain, suggesting active inhibition of a regenerative response under these conditions (Batts et al., 2009). Another recent study reported that siRNA to Hes5 enhanced HC regeneration in mouse utricles (Jung et al., 2013). Therefore, it may be possible to overcome the inhibition imposed on the regenerative process by knocking down mRNA levels for Hes1 and Hes5 and potentially driving SCs toward the formation of new HCs in the injured cochlear and vestibular sensory epithelia.

In the present study, we show that knock down of Hes1 and Hes5 mRNA levels, using small interfering RNAs (siRNAs), leads to an increase in Atoh1 transcript levels, presumably inducing HC differentiation. We used siRNA targeting Hes1 and Hes5 complexed with a transfection reagent or Hes1 siRNA delivered using biodegradable poly(lactide-co-glycolide acid) (PLGA) nanoparticles (NPs) without transfection reagent to inhibit Hes genes in the organ of Corti (OC) and in maculae (utricles and saccules) from neonatal mouse pups pre-treated with toxins to eliminate HCs. An increased number of HCs was observed in toxin-treated cochlear and maculae that had been subsequently treated with siRNA against Hes1 or Hes5. This work presents two main findings: (1) siRNA targeting of Hes genes increased the appearance of HCs in mouse pup OCs and vestibular maculae, and (2) some of the new HCs appeared to have arisen as a result of transdifferentiation of SCs. The results presented herein provide a foundation for a potential therapeutic strategy to regenerate HCs in vivo in mammals. Inner ear delivery of siRNA using PLGA NPs may be a potential approach to treat inner ear diseases.

2. Materials and methods

2.1. CD-1 Mouse pup cochlea collection and culturing conditions

The experimental procedures described herein were approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation. Three-day-old (P3) CD-1 mouse pups (Charles River Laboratories International, Inc., Wilmington, MA) were euthanized, and inner ear capsules were dissected out. The cochlear capsule was carefully opened, and the membranous OC with the stria vascularis was removed. After stripping off the stria vascularis, the basilar membrane with the OC was placed on a collagen gel drop in a 35 mm culture dish. The cochleae were cultured in DMEM with N2 supplements (Invitrogen, Carlsbad, CA) at 37 °C with 5% CO2 and 95% air.

After an initial incubation of 24 h, the cochleae were treated with or without 4-hydroxy-2-nonenal (4-HNE, Cayman Chemical, Ann Arbor, MI) for an additional 24 h. The cultured OCs were then incubated in the absence of 4-HNE in fresh media containing siRNA delivered via lipofection (LF) with JetSTM 10 mM transfection reagent (BIOPARC, Illkirch, France, siRNALF) or Hes1 siRNANPs (Hes1 siRNANP, detailed below). Control cultures consisted of cochleae that were left untreated or were treated with lipofected scrambled siRNA (scRNALF) or scRNA—containing NPs (scRNANP). 4-HNE was prepared as a 60 mM stock solution in DMSO and used at a final concentration of 100–475 \( \mu \)M (Guzman et al., 2006; Ruiz et al., 2006).

2.2. CD-1 Mouse pup maculae collection and culturing conditions

After removing the cochlea, the vestibular capsule was opened and maculae (utricles and saccules) were harvested with the otoconial membrane in place. After opening the membranous sac and stripping off the otoconial membrane, the maculae were cultured on a collagen gel drop in a 35 mm culture dish in drug-free medium for 24 h and then incubated in fresh medium alone (untreated cultures), or in medium containing 4 mM neomycin (Neo, Sigma–Aldrich, St. Louis, MO), or in medium with neomycin plus either scrNANP, Hes1 siRNALF, Hes1 siRNANP or Hes5 siRNALF. The results presented herein provide a foundation for a potential therapeutic strategy to regenerate HCs in vivo in mammals. Inner ear delivery of siRNA using PLGA NPs may be a potential approach to treat inner ear diseases.

2.3. siRNA delivery via lipofection

Twenty four h after incubation with or without 4-HNE, siRNA was added into the dishes, scRNA (at a final concentration of 20 nM, Applied Biosystems/Ambion, Austin, TX), Hes1 siRNA (20 nM), or a combination of Hes1 and Hes5 siRNA (mouse, each at 20 nM; Santa Cruz Biotechnology, Inc. Santa Cruz, CA) were transfected via LF using JetSTM 10 mM transfection reagent (25 nM, BIOPARC, Illkirch, France), and transfectants were cultured for an additional 48 h prior to transitioning to drug-free medium with exchanges of fresh medium every other day (exposed to siRNA for a total of 48 h). The cultures were maintained in vitro for a total of eight days. The Hes1 siRNA and Hes5 siRNA pools each represented a total of three target-specific, 20–25 nucleotide double-stranded RNA molecules designed to knock down either Hes1 or Hes5 expression, respectively (Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

2.4. siRNA delivery via NPs

All NP formulations were fabricated and characterized using a double emulsion technique that was modified for high-density oligonucleotide loading (Cheng and Saltzman, 2011; Cu et al., 2010; Cu and Saltzman, 2009; Woodrow et al., 2009). The amount of siRNA loaded into the PLGA NPs was 100–500 pmol per mg of NPs. Twenty four h after incubation with or without 4-HNE, Hes1 siRNANP (1–800 \( \mu \)g/ml) or scRNANP (negative control, 1–800 \( \mu \)g/ml) were added along with fresh medium into the culture dishes. Media with NPs was exchanged every other day. The cultures were exposed to the NPs for a total of six days (3 doses of NPs, a total of eight days in vitro).
2.5. NP or siRNA uptake in OC cultured tissues

Our experiment was guided by previous studies suggesting that PLGA NPs are taken up over a predictable time interval in cultured cells (Cartier et al., 2009; Davda and Labhasetwar, 2002). To estimate numbers of OC cells and of Jagged1 (Jag1) positive Scs that had taken up NPs or lipofected siRNA, cultured OCs were incubated with either 200 ng/ml of NPs loaded with the lipophilic fluorescent dye, coumarin 6, for 12–48 h or with FAM (a fluorescent dye)-labeled scRNA (Silencer® FAM Labeled Negative Control No. 1, siRNA, 20 nM, Ambion, Austin, TX) coupled with the Jetsi™ transfection reagent for 48 h. NPs containing coumarin 6 were synthesized using a modification of a single emulsion technique (Cu et al., 2010; Cu and Saltzman, 2009).

Six OCs were used for each experimental condition and for each time point (12, 24 or 48 h after NP incubation). Following incubation with the fluorescently-labeled reporter constructs, the OCs were harvested, and the constituent cells were enzymatically-dissociated according to a technique described previously by White and colleagues (White et al., 2006). The dissociated cells were stained with goat anti-Jagged1 antibody (a SC marker, 1:100, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and then Alexa Fluor® 594 donkey anti-goat IgG (1:1000, Invitrogen, Carlsbad, CA). DAPI was used to stain nuclei. After staining, the cells were spread across the surface of a slide and air-dried. Coverslips were applied with antifade medium, and slides were randomly photographed (Leica SP2 confocal microscope, Heidenberg, Germany). Numbers of Jag1 positive or negative cells that had taken up coumarin 6 NPs or FAM labeled scRNA were quantified. One thousand to two thousand cells were counted for each condition. Percentages of cells with coumarin 6 NPs or FAM scRNA were calculated and statistically analyzed (Fig. 1).

To identify the location of transfected cells in cultured OCs, tissues were harvested 48 h after exposure to FAM-labeled scRNA, fixed in 4% formaldehyde diluted in phosphate buffered saline (PBS, pH 7.4) and serial vertical cryosections (10 μm of thickness) were obtained from the middle turns. Cryosections were then stained with goat anti-Jagged1 antibody as described below. Images were acquired on a Leica SP2 confocal microscope (Heidenberg, Germany).

2.6. Hes and Atoh1 mRNA expression in OCs after siRNA treatment

Inhibition of Notch signaling has been shown to up-regulate Atoh1 expression in cultured neonatal mouse utricles (Collado et al., 2011). To study the effects and efficiency of siRNA inhibition on the Notch-responsive effectors Hes1 and Hes5, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to assess changes in the levels of Hes1, Hes5, and Atoh1 among mRNA pools isolated from cultured OCs that had been treated with either 20 nM scRNAFL, Hes1 siRNAFL, or Hes5 siRNAFL or 200–600 μg/ml of empty NPs or Hes1 siRNANP or 475 μg/ml 4-HNE according to the minimum information for publication of quantitative RT-PCR experiments (MIQE) guidelines (Taylor et al., 2010). siRNA-treated cultures were not exposed to 4-HNE.

To this end, cochlear tissue was collected two days after siRNAFL or 4-HNE treatment, or 2, 4 and 7 days after Hes1 siRINANP treatment. At least four cultured OCs in each condition were used for the analysis. Three parallel RNA extracts were prepared for each treatment. Total RNA was isolated using TRizol® (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Invitrogen, Carlsbad, CA). The purity and amount of RNA was determined via spectrophotometry (Shimadzu UV1700 Spectrophotometer, Kyoto, Japan) and gel electrophoresis.

qRT-PCR was performed on an Eppendorf realplex4 Real-Time PCR System (Eppendorf, Hauppauge, NY) with SYBRGreen Gene Expression Assays (Invitrogen, Carlsbad, CA), using Realplex software V2.0 (Eppendorf, Hauppauge, NY). The primers for Hes1 (sc-270146-PR) and Hes5 (sc-72197-PR) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The primer sequences for Atoh1 were as follows: forward, 5′-AGA TCT ACA TCA ACC CTC TGC C-3; reverse, 5′-ACT GCC CTC ATG AGC GTC ACT G-3. Primers specific to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a reference control. For each gene, triplicate qRT-PCR reactions were assayed. To estimate changes in mRNA expression levels after siRNA treatment or ototoxic trauma, the fold change for each gene was determined by the 2-ΔΔCt method (Livak and Schmittgen, 2001). Data from cultures that had been incubated with either 4-HNE or siRNA were normalized to untreated controls or scRNA-treated controls, respectively (Fig. 2).

2.7. Fixation, staining and HC counting in cochlea and maculae

For HC counting, organotypic cultures were fixed in 4% formaldehyde diluted in PBS for 2 h at room temperature. After rinsing in PBS, the tissues were incubated with 0.01% TRITC-labeled phalloidin (Sigma–Aldrich, St. Louis, MO) in PBS for 30 min in the dark. washed three times with PBS, and flat mounted in anti-fade medium on glass slides.

The stereocilia and the cuticular plates of HCs in the cochlea and maculae were examined by fluorescence microscopy as described previously (Ylikoski et al., 1992). To estimate HC numbers, we counted hair bundles and/or cuticular plates under 40× magnification (Olympus BX51, Melville, NY). In the cochlea, two regions from each turn (basal and middle turns, 100 μm in each region = 1.67% of basilar membrane length) were randomly chosen, and the number of inner and outer HCs (IHCs and OHCs, respectively) within each region was estimated and statistically analyzed (Guzman et al., 2006; Ruiz et al., 2006; Takebayashi et al., 2007). In the maculae, four to five areas (50 × 50 μm² per area) were randomly chosen from the striolar and edge regions, and the number of HCs within each area was estimated and statistically analyzed.

siRNA treatment was initiated 24 h after 4-HNE exposure. To estimate HC numbers prior to siRNA treatment, OC cultures were collected 24 h after exposure to 450 μM 4-HNE. The cultures were fixed and stained with TRITC-labeled phalloidin, and HC numbers were estimated as described above.

2.8. HC marker immuno staining in p27kip1-GFP transgenic mouse pup inner ear

HC recovery in the cultured OC after siRNA treatment could be the result of direct transdifferentiation from SCs (Izumikawa et al., 2005, 2008; Lin et al., 2011). To test this hypothesis, we used mouse pups from p27kip1-GFP transgenic line (kindly supplied by the Segil lab at the House Ear Institute, Los Angeles, CA). In this transgenic mouse, GFP expression is observed in all types of SCs but not in HCs. Indeed, more than 95% of Hoechst-stained nuclei in OC constituent cells were GFP positive (White et al., 2006; Doetzhof er et al., 2006). In these SCs, GFP is observed in both the nucleus and cytoplasm, owing to the fact that GFP can passively diffuse across nuclear pore complexes (Wei et al., 2003; Seibel et al., 2007).

We recovered and subsequently culturing of cochlear and macular tissues from p27kip1-GFP transgenic mouse pups were conducted using the same procedures as detailed above. A group of cochlea and maculae were exposed to toxin (4-HNE or neomycin) or siRNA (scRNAFL, Hes1 siRNAFL or Hes1 siRINANP) alone or toxin (4-HNE or neomycin) and siRNA (scRNAFL, Hes1 siRNAFL or Hes1 siRINANP). Cochlea and maculae that were cultured in the absence of toxin and siRNA served as untreated controls. A HC-specific marker,
myosin VIIa, was used to identify HCs in the organotypic cultures. New HCs that transdifferentiated from pre-existing SCs would be predicted to have GFP in their nuclei and exhibit myosin VIIa-positive staining in their cytoplasm (Doetzlhofer et al., 2006).

Cultured cochleae and maculae were fixed in 4% formaldehyde in PBS at day 5–7 in vitro. After washing with PBS, the tissues were blocked in 1% BSA in 1% PBS/T (1% Tween in PBS) and then incubated with rabbit anti-myosin VIIa (1:12.5, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) overnight at 4 °C. After washing with PBS, the tissues were incubated with Alexa Fluor® 594 donkey anti-rabbit IgG (1:1000, Invitrogen, Carlsbad, CA) overnight at 4 °C. The tissues were flat mounted in anti-fade medium on glass slides. Stacks of optical sections (z-series) obtained by confocal microscopy (Leica SP2 confocal microscope, Heidenberg, Germany) were used for evaluating co-expression of the two fluorescent markers in these tissues. Myosin VIIa positive HCs (red) co-expressing nuclear GFP (blue) were identified in the cytoplasm of HCs in the organotypic cultures.
A significant increase of Jag1 negative cells was observed at day 7 (day 8 ± 0.05) after 200 μg/ml BrdU was added to the organotypic cultures for 2 h at 37 °C, and the tissues were then fixed in 4% formaldehyde in PBS. For BrdU staining, tissues were incubated for 2 h at 37 °C with 70% methanol in PBS. The tissues were then washed three times in PBS/T (0.2% Tween-20 in PBS) and incubated with Alexa Fluor 488 donkey anti-rabbit antibody (1:200 dilution) and Alexa Fluor 594 mouse anti-BrdU antibody (1:200 dilution) in the blocking buffer overnight at 4 °C. The tissues were then examined by confocal microscopy as described above.

2.11. Statistical analysis

One way ANOVA (SPSS 14.0 for windows) and a post hoc test (Tukey HSD) were used to determine if there were statistically significant differences among groups and between each of the groups in the HC quantification analyses. A p value of less than 0.05 was considered to be significant in the statistical analyses.

3. Results

3.1. NPs or siRNA are readily taken up in cultured OCs in vitro

As shown in Fig. 1A, different sized clusters of PLGA coumarin NPs (green) were detectable by confocal microscopy within both Jag1 positive (arrows in Fig. 1A) and negative (arrowheads in Fig. 1A) dissociated OC cells after NP exposure. NPs were detected in 13.01–14.29% of Jag1 positive and 13.01–13.43% of Jag1 negative cells at 12 or 24 h after NP exposure (p < 0.05). A greater number of Jag1 positive cells (23.08%) contained NPs at 48 h than at 24 h after NP exposure (p < 0.05), indicative of a pattern of progressive NP uptake by SCs. A modest increase (17.46%) of Jag1 negative cells contained NPs at 48 h after NP exposure, yet the perceived increase was not statistically significant compared to 12 or 24 h (all p > 0.05, Fig. 1D).

However, the true extent of OC cells taking up NPs may be higher...
than we observed due to the fact that the visualization of single, unclusted NPs are not within the resolution limits of the microscope. No NP clusters were observed in untreated cells (data not shown).

FAM labeled scRNA (FAM scRNA) was detected in 44.61% of OC cells after an incubation period of 48 h (Fig. 1B and D). At this time point, more Jag1 positive cells (55.35%, arrows in Fig. 1B) contained siRNA than Jag1 negative cells (38.77%, arrowhead in Fig. 1B, p < 0.05). The location of transfected cells, including both Jag1 positive and negative cells, in cross sections of OCs is shown in Fig. 1C. These data demonstrate that both PLGA-formulated nanoparticles and lipofected double-stranded RNA molecules are readily taken up in the targeted tissue of interest.

3.2. siRNA to Hes1 and Hes5 or Hes1 siRNANP down-regulate targeted transcripts and up-regulate Atoh1 mRNA in cultured cochlear

In comparison to scRNALF controls, Hes1 mRNA expression was inhibited by 62% after Hes1 siRNALF treatment (p < 0.001), Hes5 mRNA expression was inhibited by 66% after Hes5 siRNALF treatment (p < 0.01) based on qRT-PCR data (Fig. 2A). Under these conditions, no cross-inhibition of the two Hes genes was observed (all p > 0.05). Hes1 siRNALF induced marked up-regulation of Atoh1 mRNA (~4-fold, p < 0.001). Hes5 siRNALF also induced significant up-regulation of Atoh1 mRNA (~2-fold, p < 0.01). 4-HNE treatment alone did not induce significant changes in Hes1, Hes5, or Atoh1 mRNA expression compared to untreated cultures (all p > 0.05, Fig. 2A).

Hes1 siRNA encapsulated within PLGA NPs also resulted in significant down-regulation of Hes1 mRNA and up-regulation of Atoh1 mRNA levels, although the effects were not clearly discernible until seven days after a low dose (200 µg/ml) of Hes1 siRNANP treatment in OC tissues (all p < 0.05), suggesting slow release of siRNA from NPs. A high dose (600 µg/ml) Hes1 siRNANP treatment induced significant down-regulation of Hes1 mRNA (0.38 fold) and up-regulation of Atoh1 mRNA (1.63 fold) levels 4 days after NP treatment compared to scRNALF controls, respectively (all p < 0.05). Such significant changes in the mRNA levels were not observed in the OCs treated with 200–600 µg/ml NPs for 2 days or 200–400 µg/ml NPs for 4 days (all p > 0.05). Throughout the analytical time-course, Hes1 siRNANP did not alter expression pattern of Hes5 (all p > 0.05, Fig. 2B). These results are consistent with the observation that blockage of Notch signaling is accompanied with up-regulation of Atoh1 transcription (Collado et al., 2011).

3.3. siRNA to Hes1 or Hes1/5 results in supernumerary HCs in non-toxin treated OC cultures

Quantification of HCs in the middle turn of cochlear cultures from P3 mice treated with Hes1 or Hes1/5 siRNALF consistently demonstrated atypically high IHC numbers (arrowheads in Figs. 3G and 4). We consistently observed supernumerary IHCs which produced about a 53% (Hes1 siRNALF) or 57% (Hes1/5 siRNALF) increase in total IHC numbers per unit of basilar membrane in the middle turns (all p < 0.001, Fig. 4). In addition, Hes1 siRNALF and Hes1/5 siRNALF induced approximately 29% and 20% increases in OHC numbers, respectively (all p < 0.001, Fig. 4), as well as multiple areas where more than three rows of OHCs were seen in the middle turn (arrows in Fig. 3G). There were also areas outside of the OC where there appeared to be a small number of ectopic HCs, as determined by the presence of a cuticular plate and stereociliary bundles (data not shown). An increase in HC numbers was not observed in the basal turns (all p > 0.05, Fig. 4). These results were obtained using the standard transfection reagent JetST™ 10 mM. scRNALF control experiments using the same transfection reagent were also performed, and no increases in IHC or OHC numbers were observed under these conditions (Figs. 3B and 5).

3.4. siRNA to Hes1 increases HC numbers in OC cultures exposed to 4-HNE

We estimated the number of OHCs eliminated by the toxin before the siRNA treatment was initiated. Twenty-four hours after 4-HNE (450 µM) exposure and prior to siRNA treatment, an approximately 70% reduction in the number of OHCs was observed in the middle turns, and an approximately 76% reduction in the number of the OHCs was observed in the basal turns. After eight days in culture, 4-HNE (100–475 µM) consistently eliminated 80–89% of the OHCs in the middle turns of OCs, while most of the IHCs remained intact (Fig. 3D). We repeated the toxin exposure experiments three times and always observed an approximately 80±5% reduction in the number of OHCs. Significant reduction in the number of IHCs was only observed after exposure to a high dose of 4-HNE (475 µM, p < 0.05, 0.01 or 0.001, Figs. 5 and 6).

However, when 4-HNE-ablated OCs were subsequently cultured with Hes1 siRNA, using a standard transfection reagent (JetST™ 10 mM), we consistently observed a marked increase in the number of OHCs in the middle turn compared to ototoxin-treated cultures that had been subsequently treated with scRNA (Figs. 3E and H, 5 and 6). In general, the OHC population in the middle turn that was observed following this experimental treatment was organized into three parallel rows and had cuticular plates in conjunction with orderly stereociliary bundles (Fig. 3H). This was in marked contrast to controls treated with 4-HNE alone (Fig. 3D) or 4-HNE followed by scRNALF (Fig. 3E), conditions which exhibited an 80–89% reduction in OHCs in the middle turn (Figs. 5 and 6). In addition, we observed a reproducibly higher number of IHCs in the middle turn after 4-HNE and Hes1 or Hes1/5 siRNALF treatment compared to 4-HNE alone or 4-HNE plus scRNA controls (arrowheads in Figs. 3H, 5 and 6). siRNA-induced increases in middle turn HC numbers were also seen at different doses of toxin exposure (100–475 µM 4-HNE, Fig. 6). Unexpectedly, treatment with 4-HNE plus Hes1/5 siRNALF did not increase OHCs in the middle turn to the same extent as treatment with 4-HNE plus Hes1 siRNALF (29% vs. 76–83%, p < 0.001, Fig. 6). This result suggests competitive uptake or antagonistic effects between Hes1 siRNA and Hes5 siRNA. In contrast, siRNA-induced increases in OC and IHC numbers were not seen in the basal turn under the same experimental conditions (7–17% of OHCs observed in 4-HNE plus siRNA treatment groups, all p > 0.05 compared to groups exposed to 4-HNE alone, Fig. 6).

3.5. Hes1 siRNANPs increase HC numbers in OC cultures

Treatment of non-toxin-damaged OC cultures with Hes1 siRNANP (50 µg/ml) significantly increased OHC numbers in the middle turns compared to untreated cultures (p < 0.05, Fig. 7), indicating Hes1 siRNANP can also promote de novo HC generation in a non-toxin-damaged culture condition. However, these effects were not seen in the basal turns (p > 0.05, Fig. 7). No significant change in HC number was found at low doses (1 or 10 µg/ml) of Hes1 siRNANP treatment in toxin-damaged OC cultures (200 µM 4-HNE) compared to cultures exposed to 4-HNE alone (p > 0.05, Fig. 7). Treatment of toxin-damaged (200 µM 4-HNE) OC cultures with 50 µg/ml Hes1 siRNANP significantly increased OHC and IHC numbers in the middle turn and IHC number in the basal turn in comparison to 4-HNE alone or to 4-HNE plus the lower dose of NPs (1 or 10 µg/ml, p < 0.01 or 0.001, Fig. 7), consistent with siRNA-specific increases in HC numbers at higher doses of Hes1 siRNANP treatment (Figs. 3I and 8). However, increased HC numbers were
not seen in OCs exposed to a higher dose of 4-HNE (475 mM) and subsequently treated with 100 or 200 mg/ml of Hes1 siRNANP (data not shown). This result suggested that higher doses of NPs may be needed when higher doses of toxin are applied (see dose response study below). As expected, scRNANP (50–200 μg/ml) had no impact on HC numbers in untreated OCs or in OCs exposed to 4-HNE (all \( p > 0.05 \), Figs. 3C and F, 5).

### 3.6. A dose response effect was found in the Hes1 siRNANP treatment in OC cultures

qRT-PCR analyses indicated that Hes1 siRNA encapsulated within PLGA NPs exhibits a prolonged release profile (Fig. 2B). Compared to about an 80% HC population in toxin damaged OC cultures treated with siRNA plus JetSI™ 10 nM transfection reagent, only about a 50% HC population was observed following 50 μg/ml NP treatment (\( p < 0.01 \), Figs. 6 and 7), suggesting that a higher concentration of NPs is needed to match the results of Hes1 siRNALF treatment. To identify a NP dose that phenocopied the 20 nM Hes1 siRNALF-induced response in HC numbers over the same timecourse, cultured OCs were exposed to a high dose of 4-HNE (450 mM) and subsequently treated with increasingly higher doses (400, 600 or 800 μg/ml) of NPs. As shown in Fig. 8, a dose response effect was observed in the Hes1 siRNANP treatment strategy, such that an increased number of HCs were observed as the dose of NPs increased. A high dose (600 μg/ml) Hes1 siRNANP treatment was capable of promoting similar HC numbers in the middle turn as was observed previously for 20 nM Hes1 siRNALF in 4-HNE-ablated OCs (\( p > 0.05 \), Figs. 6 and 8). Treatment with a high dose of NPs (>400 or 600 μg/ml) also resulted in a significant
increase in OHC numbers in the basal turn and IHC numbers in the middle turn following exposure to 4-HNE (\( P < 0.05 \) or \( 0.01 \), Fig. 8).

3.7. siRNA to Hes1 increases HC numbers in macular cultures exposed to neomycin

The vestibular sensory epithelia in mammals have a limited regenerative capacity (Forge et al., 1993; Kawamoto et al., 2009; Kopke et al., 2001; Lopez et al., 1997; Stone et al., 1998). siRNA to Hes1 may increase this capacity. We tested this hypothesis in neonatal mouse macular cultures exposed to neomycin, a toxin that kills vestibular HCs.

Cell density measurements were first conducted for six utricles and six saccules from mouse pups that had been cultured without either toxin or siRNA treatment and stained with phalloidin. No significant differences in cell densities were found between the utricles and the saccules in the striola or in the non-striolar (edge) regions (all \( p > 0.05 \)). Therefore, cell quantification results from each of these regions in mouse utricles and saccules were combined in each experimental group and statistically analyzed (Fig. 9E).

Compared to untreated cultures (Fig. 9A), HC numbers were markedly reduced in maculae after either exposure to neomycin alone (Fig. 9B) or after neomycin exposure followed by scRNALF treatment (Fig. 9C). In comparison to maculae treated with neomycin alone or neomycin plus scRNALF, more HCs were found in the maculae treated with neomycin plus Hes1 siRNALF in both striolar and edge regions (Fig. 9E, all \( p < 0.001 \)). However, more HCs observed under these conditions exhibited shorter stereocilia (arrow in Fig. 9D), perhaps indicative of an immature phenotype. The cuticular plates of the immature HCs were also stained by phalloidin (arrow in Fig. 9D).

3.8. Transdifferentiation of new HCs from SCs

To ascertain whether Hes1 siRNA treatment induces the transdifferentiation of SCs into HCs, we examined siRNA-treated OC

![Graph showing HC numbers in OCs from neonatal mice.](image)

**Fig. 4.** Hes1 siRNALF significantly increased HC numbers in OCs from neonatal mice. Compared to untreated cultures, treatment of OC cultures with Hes1 siRNALF or Hes1/5 siRNALF resulted in a significant increase in the number of IHCs and OHCs in the middle turn (***, all \( p < 0.001 \)) but not in the basal turn (all \( p > 0.05 \)). There is a significant difference in OHC numbers between the Hes1 siRNALF and Hes1/5 siRNALF groups (#, \( p = 0.05 \)) in the middle turn. ** indicates \( p < 0.001 \) compared to the untreated cultures. # indicates \( p < 0.05 \) when the Hes1 siRNALF group is compared to the Hes1/5 siRNALF group. Error bars represent standard error of the means (numbers in the brackets indicate the number of OCs in which HCs were counted). These data were obtained from OCs that had been cultured eight days in vitro.

![Graph showing HC numbers in OCs treated with scRNA.](image)

**Fig. 5.** scRNA had no significant impact on HC numbers in untreated or 4-HNE (475 \( \mu M \)) treated OC cultures. scRNA treatment did not change HC numbers in the absence of 4-HNE (scRNALF or scRNANP alone) or in 4-HNE-exposed cultures (4-HNE plus scRNALF or scRNANP) compared to the untreated cultures or the cultures expose to 4-HNE alone, respectively (all \( p > 0.05 \)). There was a significant reduction in the number of OHCs and IHCs after 4-HNE exposure compared to the untreated cultures (** and * indicate \( p < 0.001 \) and \( 0.05 \), respectively). Error bars represent standard error of the means (numbers in the brackets indicate the number of OCs in which HCs were counted). These data were obtained from OCs that had been cultured eight days in vitro.
cultures for the presence of nascent HCs coexpressing the HC marker, myosin VIIa and the SC marker, p27kip1/GFP. p27kip1 protein is typically expressed only in SCs of the embryonic and postnatal inner ear sensory epithelia (Chen and Segil, 1999; Löwenheim et al., 1999; Minoda et al., 2007). However, some utricular HCs have been shown to weakly express p27kip1 protein at P7 (Laine et al., 2010). In the inner ear of the p27kip1/GFP transgenic mice, GFP expression is regulated by the p27kip1 promoter and is thus expressed specifically in SCs (Doetzlhofer et al., 2006; White et al., 2006). To discern whether HCs in the p27kip1/GFP transgenic mice exhibit GFP expression, we first examined the expression pattern of GFP in uncultured OCs at an equivalent developmental stage (P9) to when Hes1 siRNA-induced transdifferentiation of supernumerary HCs was observed in vitro. In uncultured P9 OCs, the pattern of GFP expression was restricted to the SCs that surrounded Myosin VIIa-positive IHCs and OHCs, and no signal overlap was observed between these two

Fig. 6. Hes1 siRNALF treatment significantly increased HC numbers in 4-HNE (100–475 μM) exposed OC cultures. 4-HNE treatment significantly decreased OHC numbers in the middle and basal turns (** or ***, p < 0.01), while only high doses of 4-HNE (475 μM) significantly decreased IHC numbers in the middle and the basal turns compared to the untreated cultures (*** or ****, p < 0.01 or 0.001). Treatment of 4-HNE damaged OC cultures with Hes1 siRNALF (20 nM) resulted in a significant increase in IHCs and OHCs in the middle turn (# or ###, p < 0.05 or 0.001) at all doses of 4-HNE tested. Treatment of 4-HNE damaged OC cultures with Hes1/S siRNALF resulted in a significant increase in IHCs in the middle turn (p < 0.001) but not in OHCs (p > 0.05) compared to cultures treated with 4-HNE alone. Increased OHC and IHC numbers were not observed in the basal turn after 4-HNE plus Hes1 or Hes1/S siRNALF treatment (** and *** indicate p < 0.001 and 0.01, respectively, compared to untreated cultures. # and ## indicate p < 0.001 and 0.05, respectively, compared to each dose of 4-HNE only treated cultures. Error bars represent standard error of the means (numbers in the brackets indicate the number of cochleae in which HCs were counted). These data were obtained from OCs that had been cultured eight days in vitro.

Fig. 7. Hes1 siRNA delivered by nanoparticles significantly increased HC numbers with low dose 4-HNE (200 μM) damaged OC cultures. Treatment of non-toxin damaged OC cultures with Hes1 siRNANP (50 μg/ml) increased OHC numbers compared to untreated cultures in the middle turn (*, p < 0.05). Low doses of NP treatment (1–10 μg/ml) in 4-HNE damaged OCs did not change HC number compared to 4-HNE only exposed (all p > 0.05). Treatment of 4-HNE-damaged OC cultures with 50 μg/ml Hes1 siRNANP significantly increased IHC and OHC numbers in the middle turn and IHC number in the basal turn compared to cultures exposed to 4-HNE alone (## or ###, p < 0.01 or 0.001). No increase in OHC numbers was observed in the basal turn under these treatment conditions (all p > 0.05). ***, ** and * indicate p < 0.001, 0.01 and 0.05, respectively, compared to untreated cultures. ### and ## indicate p < 0.001 and 0.01, respectively, compared to each dose of 4-HNE only treated cultures. Error bars represent standard error of the means (numbers in the brackets indicate the number of cochleae in which HCs were counted). These data were obtained from OCs that had been cultured eight days in vitro.
untreated cultures. ###, ## and # indicate found in the maculae under all experimental conditions (Fig. 12). Myosin VIIa positive HCs with non-green nuclei were also (numbers in the brackets indicate the number of OC in which HCs were counted). These data were obtained from OCs that had been cultured eight days in vitro.

A dose-dependent response was observed in the number of HCs in OCs treated with 450 μM 4-HNE and Hes1 siRNANP. Following 4-HNE exposure, more HCs were observed in cultured OCs as the dose of NPs was increased. High dose (600 μg/ml) Hes1 siRNANP treatment in 4-HNE damaged OCs resulted in a similar HC increase in the middle turn compared to cultures treated with 20 nM Hes1 siRNALF following 4-HNE (475 μM) exposure (p < 0.05, Fig. 7). Treatment with a high dose of NPs (>400 or 600 μg/ml) also resulted in significant OHC increase in the basal turn and IHC increase in the middle turn (# or ##, p < 0.05 or 0.01). *** and * indicate p < 0.001 and 0.05, respectively, compared to untreated cultures. ###, ## and # indicate p < 0.001, 0.01 and 0.05, respectively, compared to the 4-HNE only exposed group. Error bars represent standard error of the means (numbers in the brackets indicate the number of OC in which HCs were counted). These data were obtained from OCs that had been cultured eight days in vitro.

Fig. 8. A dose-dependent response was observed in the number of HCs in OCs treated with 450 μM 4-HNE and Hes1 siRNANP. Following 4-HNE exposure, more HCs were observed in cultured OCs as the dose of NPs was increased. High dose (600 μg/ml) Hes1 siRNANP treatment in 4-HNE damaged OCs resulted in a similar HC increase in the middle turn compared to cultures treated with 20 nM Hes1 siRNALF following 4-HNE (475 μM) exposure (p < 0.05, Fig. 7). Treatment with a high dose of NPs (>400 or 600 μg/ml) also resulted in significant OHC increase in the basal turn and IHC increase in the middle turn (# or ##, p < 0.05 or 0.01). *** and * indicate p < 0.001 and 0.05, respectively, compared to untreated cultures. ###, ## and # indicate p < 0.001, 0.01 and 0.05, respectively, compared to the 4-HNE only exposed group. Error bars represent standard error of the means (numbers in the brackets indicate the number of OC in which HCs were counted). These data were obtained from OCs that had been cultured eight days in vitro.

Decreased numbers of SCs have previously been observed in OCs or utricles following manipulation of the Notch pathway (Collado et al., 2011; Kiernan et al., 2005; Takebayashi et al., 2007). Consistent with these previous studies, decreased numbers of Jag1-positive SCs were observed in undamaged or toxin-damaged OCs treated with Hes1 siRNALF (Fig. 13B, D and E), suggesting that the increased numbers of HCs that arise following depletion of Hes1 do so at the expense of SCs.

Very few BrdU positive cells were found in the OCs under all treatment conditions (Fig. 14 and data not shown), suggesting that non-mitotic HC regeneration is the major mechanism underlying the increased numbers of HCs observed following treatment with Hes1 siRNA. This is one of the two mechanisms of HC replacement described in the avian auditory organ (Duncan et al., 2006). No myosin VIIa positive HCs had BrdU positive staining (Fig. 14A–D). These results suggest that most of the new HCs observed upon knock down of Hes1 mRNA transdifferentiate from SCs in the inner ear through a non-mitotic process.

4. Discussion

Deafness and loss of balance are commonly caused by a loss of sensory HCs due to toxins, infection, trauma, aging, and other factors (Brigande and Heller, 2009; Cotanche, 2008). Once lost, cochlear HCs in mammals do not spontaneously regenerate, resulting in permanent hearing impairments (Bermingham-McDonogh and Rubel, 2003; Johnsson and Hawkins, 1976). In contrast to mammals, the avian auditory organ readily regenerates lost HCs and recovers hearing function (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). The SCs or a subset of SCs in birds and fish have, indeed, been shown to act as HC precursors (Adler and Raphael, 1996; Ma et al., 2008). The vestibular sensory epithelia in mammals have been shown to exhibit a limited regenerative capacity (Forge et al., 1993; Kopke et al., 2001; Lopez et al., 1997; Stone et al., 1998), but the mammalian auditory sensory epithelium does not regenerate, as the cochlear SCs seemingly lose the capacity for proliferation shortly after birth (White et al., 1988; Ryals and Rubel, 1988). The SCs or a subset of SCs in birds and fish have, indeed, been shown to act as HC precursors (Adler and Raphael, 1996; Ma et al., 2008). The vestibular sensory epithelia in mammals have been shown to exhibit a limited regenerative capacity (Forge et al., 1993; Kopke et al., 2001; Lopez et al., 1997; Stone et al., 1998), but the mammalian auditory sensory epithelium does not regenerate, as the cochlear SCs seemingly lose the capacity for proliferation shortly after birth (White et al., 1988; Ryals and Rubel, 1988).
The present study demonstrated increased HC numbers in cochlear and macular explants cultured from neonatal mice that had been exposed to ototoxins and subsequently treated with siRNA against either Hes1 or Hes1/Hes5. Treatment with Hes1 or Hes5 siRNALF effectively reduced the targeted transcripts in neonatal mouse cochlear tissues and reciprocally increased the expression levels of Atoh1. The downregulation of Hes messages was accompanied by a significant increase in the number of both IHCs and OHCs under these conditions. After HC lesioning with a toxin, there was a significant increase in OHC numbers in tissues exposed to siRNA against Hes1 alone or Hes1 and Hes5 together. These HCs appeared in orderly rows in anatomically correct regions and demonstrated cuticular plates and stereociliary bundles. The siRNA-mediated response was robust, with an approximately 5-fold increase in OHC number noted in the middle turn of toxin-damaged cultured OCs compared to toxin-ablated controls treated with scRNA (73.49% vs. 12.91%, \( p < 0.001 \), Figs. 5 and 6). After toxin-induced elimination of sensory HCs from neonatal

**Fig. 9.** siRNA treatment significantly increased HC numbers in macular cultures following neomycin exposure. Examples of fluorescence microscopy images from mouse macular (utricle and sacule) cultures treated with neomycin only (Neo, B), neomycin plus scRNALF (Neo/Sc, C) or neomycin plus Hes1 siRNALF (Neo/Si, D). An image from an untreated macular culture is shown in A. Few HCs survived in the maculae after neomycin only (B), neomycin plus scRNALF (C) treatment. However, more HCs with short stereocilia were found in the maculae treated with Hes1 siRNALF (arrow in D). The cuticular plates (ring like staining) of the immature HCs were also stained by phalloidin (D). All images were taken from maculae that had been cultured eight days in vitro. HC numbers from each condition were counted and statistically analyzed (E). Significantly decreased HC numbers (averaged or in edge and striola areas) were found in the neomycin only treated compared to the untreated cultures (all \( p < 0.001 \)). scRNALF treatment did not change HC number in neomycin damaged maculae in all areas compared to neomycin plus scRNALF in all areas (all \( p > 0.05 \)). More HCs were found in the neomycin plus Hes1 siRNALF treated maculae compared to neomycin plus scRNALF in all areas (all \( p < 0.001 \)). *** indicates \( p < 0.001 \) compared to the untreated cultures. ### indicates \( p < 0.001 \) compared to the neomycin plus scRNALF group. Error bars represent standard error of the means (the number of maculae counted was 6 in each group). Scale bar = 10 \( \mu \)m in D for A–D.
Fig. 10. Comparison of p27kip1/-GFP expression in uncultured and cultured OCs from p27kip1/-GFP transgenic mice. Examples of confocal images from untreated and uncultured OCs from p27kip1/-GFP neonatal (P9) mice (A–C), untreated OC cultures (D–F), OC cultures treated with scRNALF alone (G–I), and OC cultures treated with 4-HNE alone (J–L). HCs were stained with anti-myosin VIIa (red, column 1 at left) while SCs have GFP positive nuclei and cytoplasm (green, column 2 at middle). Images in column 3 (right) are the merged images from column 1 and column 2. All HCs have myosin VIIa positive cytoplasm with non-green nuclei in OC cultures in all conditions. Few OHCs survived in the OC treated with 4-HNE (arrows in J and L, note that most IHCs are not shown in J and L). Scale bar = 10 μm in L. Images in D–L were taken from OCs that had been cultured six days in vitro.
Hes1 siRNA treatment induced transdifferentiation of SCs into new HCs in 4-HNE damaged OCs of p27kip1/GFP transgenic neonatal mouse pups. OC cultures were treated with Hes1 siRNA only (A–C) or exposed to 4-HNE and subsequently treated with either Hes1 siRNA (D–I) or Hes1 siRNA NP (J–L). HCs were stained with anti-myosin VIIa (red, column 1 at left) while SCs have GFP positive nuclei and cytoplasm (green, column 2 at middle). Images in column 3 (right) are the merged images from column 1 and column 2. OHCs with red cytoplasm (myosin VIIa) and green nuclei (p27kip1/GFP) were found in OCs treated with Hes1 siRNA alone (arrow in C), 4-HNE plus Hes1 siRNA (arrows in F and I), or 4-HNE plus Hes1 siRNA NP (arrow in L), indicating that these HCs transdifferentiated from SCs. Myosin VIIa positive OHCs and IHCs with non-green nuclei were found in the OCs in all conditions (A–L). Scale bar = 10 μm in L for A–L. All images were taken from OCs that had been cultured six days in vitro.

Fig. 11. Hes1 siRNA treatment induced transdifferentiation of SCs into new HCs in 4-HNE damaged OCs of p27kip1/GFP transgenic neonatal mouse pups. OC cultures were treated with Hes1 siRNA only (A–C) or exposed to 4-HNE and subsequently treated with either Hes1 siRNA (D–I) or Hes1 siRNA NP (J–L). HCs were stained with anti-myosin VIIa (red, column 1 at left) while SCs have GFP positive nuclei and cytoplasm (green, column 2 at middle). Images in column 3 (right) are the merged images from column 1 and column 2. OHCs with red cytoplasm (myosin VIIa) and green nuclei (p27kip1/GFP) were found in OCs treated with Hes1 siRNA alone (arrow in C), 4-HNE plus Hes1 siRNA (arrows in F and I), or 4-HNE plus Hes1 siRNA NP (arrow in L), indicating that these HCs transdifferentiated from SCs. Myosin VIIa positive OHCs and IHCs with non-green nuclei were found in the OCs in all conditions (A–L). Scale bar = 10 μm in L for A–L. All images were taken from OCs that had been cultured six days in vitro.
Fig. 12. Hes1 siRNA treatment induced transdifferentiation of SCs to new HCs in neomycin (Neo) damaged maculae of p27kip1/GFP transgenic neonatal mouse pups. Examples of confocal images from maculae without treatment (untreated cultures, A–C), treated with neomycin (D–F), or exposed to neomycin and then treated with either Hes1 siRNALF (G–I) or Hes1 siRNANP (J–L) are shown. HCs were stained with anti-myosin VIIa (red, column 1 at left) while SCs are GFP positive (green, column 2 at middle). Images in column 3 (right) are the merged images from column 1 and column 2. HCs with red cytoplasm (myosin VIIa) and green nuclei (p27kip1/-GFP) were found in maculae treated with Hes1 siRNALF (arrows in I) or Hes1 siRNANP (arrows in L), indicating that these HCs transdifferentiated from SCs. Myosin VIIa positive HCs with non-green nuclei were observed in maculae under all conditions (A–L). Fewer HCs were observed in maculae treated with neomycin alone (D–F) compared to untreated cultures (A–C). More HCs were observed in the maculae exposed to neomycin and treated with either Hes1 siRNALF (G–I) or Hes1 siRNANP (J–L) compared to neomycin only treated controls (C–F). Scale bar = 10 μm in L for A–L. All images were taken from maculae that had been cultured six days in vitro.
mouse maculae, Hes1 siRNA treatment was associated with a significant increase in the appearance of apparently immature HCs bearing short stereociliary bundles. scRNA experiments suggest that these responses were specific to the siRNA targets under investigation.

4.1. HC regeneration or protection?

Limited autonomous HC regeneration has been reported in mammalian utricles, and treatment with siRNA to Hes5 has been shown to enhance utricular HC regeneration (Forge et al., 1993, 1998; Jung et al., 2013; Walsh et al., 2000). Consistent with previous reports, immature HCs were found in all cultured maculae in the present study. However, a significant increase in the number of HCs was only seen in maculae treated with Hes1 siRNALF (Fig. 9), suggesting siRNA to Hes1 can promote new HC formation in maculae cultured in vitro. No autonomous cochlear HC regeneration has been reported in mammals. However, new cochlear and vestibular HCs have been generated when Atoh1 was overexpressed in vitro (Zheng and Gao, 2000) and in vivo (Kawamoto et al., 2003; Staecker et al., 2007), suggesting that cochlear SCs possess an intrinsic potential to transdifferentiate into HCs through molecular manipulation of the Atoh1 pathway or the Notch pathway (Doetzlhofer et al., 2006; Hori et al., 2007; Sinkkonen et al., 2011; White et al., 2006). Consistent with these previous studies, we observed de novo HC formation induced by Hes1 and Hes5 siRNA in undamaged organotypic OC cultures (Figs. 3G and 4). However, the increased numbers of HCs induced by Hes1 siRNA following
exposure of cultured OCs to the ototoxin 4-HNE could be a result of protection or regeneration. The results of the present study support the latter of these two possibilities, as evidenced by the siRNA-specific occurrence of HCs that co-expressed myosin VIIA and p27kip1/-GFP, following 4-HNE exposure and subsequent down-regulation of Hes1 expression (Fig. 11). Moreover, there were significantly more HCs observed after siRNA treatment than were observed to have survived at the 24 h time point post-toxin. We initiated siRNA treatment 24 h after toxin exposure, at a time point when approximately 70% of OHCs were observed to be ablated by the toxin in the cultures. Thus the presence of ~70% of OHCs would suggest addition of new HCs, since only ~30% of OHCs survived the toxin. Our NP treatment regimen also supports HC regeneration. Hes1 mRNA inhibition and Atoh1 mRNA up-regulation occurred

Fig. 14. Few BrdU positive cells were found in the OCs after toxin and Hes1 siRNA application. Examples of BrdU labeling in untreated OC cultures (A and A’) and in OC cultures treated with either Hes1 siRNA LiF (B and B’), 4-HNE alone (C and C’) or 4-HNE plus Hes1 siRNA LiF (D and D’). Images in the left column (A–D) were from the middle turns of flat mounted OCs, and images in the right column (A’–D’) were from cross-sections of the middle turns of OCs. Brackets indicate the area where HCs were located (HCs were labeled with myosin VIIa in A’–D’). Similar numbers of BrdU positive cells were found in the OCs under all conditions. The majority of BrdU positive cells were outside of the area where HCs were located (arrows in A–D and B’–D’) with only a few observed in areas where HCs were located (arrowheads in C and D). No myosin VIIa positive HCs exhibited positive BrdU staining under all conditions (A’–D’). Scale bar = 200 μm in A–D, 10 μm in D’ for A’–D’. All images were taken from OCs that had been cultured four days in vitro.
between day four and day seven of Hes1 siRNANP treatment, over a period when more than 86% of OHCs were observed to be lost in the middle turn following toxin exposure.

The HC regeneration observed in the cultured OC may be directly attributable to stimulation of Atoh1-responsive gene expression pathways, as expression of Atoh1 significantly increased after Hes1 siRNALF or Hes1 siRNANP treatment. Others have reported that increasing Atoh1 expression by using a γ-secretase inhibitor can result in the formation of new HCs in the cochlea in vivo (Yamamoto et al., 2006). We have observed that Hes1 siRNALF treatment induced ~four-fold increase in Atoh1 mRNA levels, supporting new HC generation through up-regulation of Atoh1. Hes5 siRNALF treatment induced only ~2 fold upregulation of Atoh1. This result may explain why adding Hes5 siRNA did not combinatorially induce significantly more HC production (Fig. 8).

Low expression of Hes1 in the inner ear after birth may also explain this phenomenon (Hartman et al., 2008).

Significant HC regeneration after exposure to ototoxic drugs and siRNA treatment was only observed in the middle turn in the OC, not in the basal turn, when 20 nM of Hes1 siRNANP or < 200 μg/ml of Hes1 siRNANP was applied. OHC regeneration in basal turns of OC explants was only observed when higher doses of Hes1 siRNANP were administered (Fig. 8). These results suggest a regional specificity for HC regeneration. This may be due to the fact that neonatal OC tissues are not fully developed at birth and that SCs in the basal turn of cochlear explants lose their capacity to respond to Hes1 down-regulation before the middle turn does (Shnerson et al., 1981; Tang et al., 2006). Alternatively, the basal turn of the cochlea is known to be more susceptible to ototoxins (Rybak and Ramkumar, 2007). As such, it is possible that the degree of toxicity to the SC population at the basal turn may have rendered it less responsive to Hes1 siRNA treatment, requiring significantly higher doses to elicit a transdifferentiation phenotype. However, we cannot formally rule out that our siRNA treatment regimen supports a degree of HC protection, because slightly more OHCs survived (~30% of OHC survival in the middle turn) when siRNA was administered (24 h after toxin exposure) than the day when the final analyses were conducted (day 8 in vitro, ~13% of OHC survival in the middle turn, p < 0.05) in the present study. There is also a possibility that HCs were damaged to a point of not being recognizable as HCs, but did not die and then were repaired rather than regenerated. While our findings for the HES1 siRNA treatment (i.e. predominant OHC regeneration) in OC organotypic cultures differ from those reported for Hes1 knockout mice, in which increases in IHCs were the predominant feature (Zine et al., 2001; Tateya et al., 2011), the increased complexity of the lateral inhibition mosaic that exists within the neurosensory network of the postnatal OC may elicit a unique transdifferentiation response relative to those observed during embryonic development. This may be due, at least in part, to functional redundancies imposed by the Hey family of genes within the SCs surrounding postnatal IHCs (Tateya et al., 2011). Therefore, it may be possible to induce a more robust HC regenerative response by employing siRNA therapeutic strategies that also incorporate siRNAs targeted against the Hey family of genes.

4.2. Regeneration through transdifferentiation and cell proliferation

In the mammalian vestibular system, one explanation for the disappearance of HC bundles after toxin exposure with their later reappearance is that the HCs are first injured causing a loss of the stereocilia followed by HC repair with the reconstitution of new HC bundles (Forge et al., 1993, 1998). However, the regenerative capability of the hair bundle is lost in mammalian cochlear HCs (Jia et al., 2009). Furthermore, a degree of post-traumatic HC recovery has been documented in surviving HCs beneath the reticular lamar surface following scar formation (Sokownik et al., 1996). To explore these possibilities, we utilized a transgenic mouse model that specifically expresses a p27kip1−/−-GFP reporter construct only in cochlear and macular SCs (White et al., 2006). Based on our co-expression analyses, it is probable that some new HCs regenerate, at least in part, from p27kip1−/−-GFP positive SCs in both OC and macular cultures pre-treated with ototoxins. Supporting our observation, a previous study demonstrated that mammalian cochlear SCs have the capacity to divide and transdifferentiate into new HCs in vitro (White et al., 2006). A recent in vivo study also demonstrated that manipulation of Notch pathway by a γ-secretase inhibitor can induce new HC regeneration from transdifferentiation of SCs in young mice (Mizutari et al., 2013). It has been proposed that the presence of Hes1 after a lesion may prohibit the occurrence of transdifferentiation in the surviving SCs in aminoglycoside-damaged guinea pig OCs (Batts et al., 2009). In our model, we found myosin VIIa positive cells with GFP positive nuclei, which suggests that new HCs arose from transdifferentiation of SCs. The myosin VIIa positive cells with non-GFP-labeled nuclei could represent surviving HCs or transdifferentiated HCs that have matured and no longer express the p27kip1−/−-GFP transgene, as differentiation of HCs has been shown to correspond with downregulation of this reporter (Chen and Segil, 1999). Consistent with our results, only a few myosin positive new HCs had GFP positive nuclei when p27kip1−/−-GFP positive SCs differentiated in vitro (Doetzlhofer et al., 2006). The fact that very few HCs survived in tissues exposed to toxin alone (Figs. 10–L and 12D–F) also supports the conclusion that these new HCs are likely to represent HCs that have transdifferentiated from SCs. Therefore, we contend that siRNA-mediated reduction of Hes1 protein, in turn, allowed for enhanced Atoh1 expression, promoting a subpopulation of SCs to adopt a HC fate. However, cytoplasmic translocation of p27kip1−/− was found in gentamicin damaged HCs in the avian inner ear (Torchinsky et al., 1999). Therefore, there is a possibility that expression of p27kip1−/−-GFP in the myosin VIIa positive cells may indicate degeneration of HCs. However, we never observed any GFP positive HCs in 4–HNE alone treated cultures. A cell lineage trace study is needed to rule out this possibility in the future.

Very few BrdU positive cells were found in the HC region of cochlear after toxin and siRNA application, suggesting a nonmitotic replacement process, which is one of the two regenerative mechanisms described in the avian auditory organ (Duncan et al., 2006). This result is confirmed by data from OC cultures of p27kip1−/−-GFP transgenic mouse pups. Normally p27kip1−/−-GFP is only expressed in SCs (White et al., 2006). However, myosin VIIa labeled, GFP-positive cells were observed in OCs and maculae treated with Hes1 siRNA (Figs. 11 and 12), while a decreased number of Jag1 positive SCs were observed in OCs under the same conditions (Fig. 13). All these results support a process of HC transdifferentiation from existing SCs in the absence of widespread proliferation. Consistent with our results, a previous study demonstrated that inhibition of the Notch pathway evoked a proliferation-independent increase in HC number in the OC (Takebayashi et al., 2007). While these results do not formally rule out the possibility that proliferation contributes to the regenerative process, they do indicate that, under the conditions tested, proliferation would have played a minor role.

4.3. Advantages of using siRNA and NPs

Using siRNA has several advantages. siRNA works at the RNA level, and no foreign DNA or viral vector are integrated into the host genome. Higher levels of siRNA molecules can be incorporated into polymer delivery carriers, and enhanced transfection can occur when compared to plasmid DNA for example (Salcher and Wagner, 2010). Silencing RNA technology is rapidly advancing to the clinic (Davis et al., 2010) and is being applied to the ear (Alvarado et al., 2010).
2011; Maeda et al., 2005; Mukherjea et al., 2008). In the auditory system, siRNA has previously been used to more closely study the proliferative mechanism of a cochlear cell line (Ozeki et al., 2007). According to data generated from ongoing clinical trials, the use of siRNA to catalyze mRNA degradation may be a promising approach to prevent or treat human diseases (Castanotto and Rossi, 2009; Whitehead et al., 2009). However, delivery of siRNA is one of the most challenging problems in modern medicine. One aspect of this challenge is protecting and transfecting the siRNA into the desired target tissues. We have developed a promising approach using a nontoxic, biodegradable PLGA polymer-based NP delivery platform (Barnes et al., 2007; Dormer et al., 2008; Du et al., 2013; Gao et al., 2010; Ge et al., 2007; Kopke et al., 2006; Mondalek et al., 2006; Wang et al., 2011; Wassel et al., 2007). Saltzman et al. discovered a methodology that significantly enhances the loading of siRNA into PLGA NPs, which are readily taken up into epithelial cells without the cytotoxicity seen with other methods (Cartiera et al., 2009; Cu and Saltzman, 2009; Woodrow et al., 2009). PLGA is a nontoxic and FDA-approved polymer for other applications. Using these PLGA NPs, Woodrow and colleagues reported substantial silencing of target transcripts in vivo with low toxicity after topical delivery to a sensitive epithelial tissue (Woodrow et al., 2009), providing excellent intracellular delivery and transfection efficiency. In the present study, we used lipofected siRNA targeting Hes1 and Hes5 or Hes1 siRNANP (without transcription reagent) to inhibit Hes genes in toxin-exposed OCs and maculae (utricles and saccules) from neonatal mouse pups. An increased number of HCs was found in siRNA treated cochleae and maculae after toxin damage compared to those exposed to toxin alone. Therefore, siRNA targeting of Hes genes may be an effective strategy for the post-traumatic regeneration of mammalian HCs in vivo in the future. PLGA NPs can cross the round window membrane of the inner ear in vitro and in vivo (Du et al., 2013; Gao et al., 2010; Ge et al., 2007), suggesting that PLGA NP-mediated inner ear delivery of siRNAs against these target genes may be a viable clinical approach to accomplish this aim.

This approach may have advantages over other regenerative approaches in terms of translation to the clinic. The approach described in our study avoids the use of viral vectors, which could pose a safety hazard (Bergmans et al., 2008). Encapsulating siRNA into NPs likely enhances siRNA transfection through cell uptake of the NPs and protection of the siRNA from extracellular and internal degradation, which ultimately allows for a degree of sustained siRNA release from the NPs by diffusion and hydrolysis of the polymer. The NP formulation used in the experiments described herein is nontoxic to delicate epithelial tissues (Cu et al., 2011; Woodrow et al., 2009). Consistent with these prior findings, scRNANP did not exhibit any discernible signs of intrinsic toxicity to inner ear tissues cultured in vitro (Figs. 3 and 5).

The results presented in this study indicate that Hes1 siRNAs are potential therapeutic molecules that could possibly regenerate new HCs in the inner ear. Whether these new HCs are functional remains to be tested. However, new HCs regenerated from manipulation of the Notch pathway has previously been shown to correlate with partial hearing recovery in mouse ears damaged by noise exposure (Mizutari et al., 2013). Nonetheless, additional experimentation needs to be performed to confirm that this regenerative phenotype can occur in vivo in adult tissues with an accompanying recovery of function.

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