Published in final edited form as:

Hear Res. 2013 October ; 304: 91–110. doi:10.1016/j.heares.2013.06.011.

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 cochleae and maculae of postnatal day 3 mouse pups. An increase in HCs was also observed in cultured cochleae 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 cochleae 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 cochleae and maculae of p27^{kip1}/GFP transgenic mouse pups demonstrated that newly generated HCs trans-differentiated 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.

Keywords

hair cell regeneration; inner ear; Notch pathway; siRNA; nanoparticle; mouse

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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; Kiernen 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 (Lanford 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* (Hairy and enhancer of split 1 and 5), inducing a SC fate (Lanford 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 Ribaupierre, 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 γ -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 γ -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 ototoxin, 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 cochleae 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 (utricle and saccule) from neonatal mouse pups pre-treated with toxins to eliminate HCs. An increased number of HCs was observed in toxin-treated cochleae 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.

MATERIALS AND METHODS

CD-1 Mouse pup cochleae 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 hours, the cochleae were treated with or without 4-hydroxy-2-nonenal (4-HNE, Cayman Chemical, Ann Arbor, MI) for an additional 24 hours. The cultured OCs were then incubated in the absence of 4-HNE in fresh media containing siRNA delivered via lipofection (LF) with JetSI™ 10 mM transfection reagent (BIOPARC, Illkirch, France, siRNALF) or *Hes1* siRNA NPs (*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 μM (Guzman et al., 2006; Ruiz et al., 2006).

CD-1 Mouse pup maculae collection and culturing conditions

After removing the cochleae, the vestibular capsule was opened and maculae (utricle and saccule) 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 hours 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 scRNALF, *Hes1* siRNALF, *Hes1* siRNANP or scRNANP. We only used *Hes1* siRNA in macular studies due to the fact that we did not see any synergistic effects in OCs when *Hes1* and *Hes5* siRNA (*Hes1/5* siRNALF) were used in combination (detailed in the Results section below).

siRNA delivery via lipofection

Twenty four hours 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 JetSI™ 10 mM transfection reagent (25 nM, BIOPARC, Illkirch, France), and transfectants were cultured for an additional 48 hours prior to transitioning to drug-free medium with exchanges of fresh medium every other day (exposed to siRNA for a total of 48 hours). 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).

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 hours after incubation with or without 4-HNE, *Hes1* siRNANP (1-800 µg/ml) or scRNANP (negative control, 1-800 µg/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*).

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 (Cartiera 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 µg/ml of NPs loaded with the lipophilic fluorescent dye, coumarin 6, for 12-48 hours or with FAM (a fluorescent dye)-labeled scRNA (*Silencer*® FAM Labeled Negative Control No. 1 siRNA, 20 nM, Ambion) coupled with the JetSI™ transfection reagent for 48 hours. 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 hours 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 NPs or FAM labeled scRNA were quantified. One thousand to two thousand cells were counted for each condition. Percentages of cells with coumarin 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 hours 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).

***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 20nM scRNALF, *Hes1* siRNALF, or *Hes5* siRNALF or 200-600 µg/ml of empty NPs or *Hes1* siRNANPs or 475 µM 4-HNE according to the minimum information for publication of quantitative rtPCR experiments (MIQE) guidelines (Taylor et al., 2010).

To this end, cochlear tissue was collected two days after siRNALF or 4-HNE treatment, or 2, 4 and 7 days after *Hes1* siRNANP treatment. siRNA-treated cultures were not exposed to 4-HNE. 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 ACG CTC TGT C-3'; reverse, 5'-ACT GGC CTC ATC AGA 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 ototoxic trauma, the fold change for each gene was determined by the 2^{-CT} 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).

Fixation, staining and HC counting in cochleae and maculae

For HC counting, organotypic cultures were fixed in 4% formaldehyde diluted in PBS for two hours 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 40X magnification (Olympus BX51, Melville, NY). In the cochleae, two regions from each turn (basal and middle turns, 100 μm in each region $\approx$ 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 $\times$ 50 μm^2 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 hours after 4-HNE exposure. To estimate HC numbers prior to siRNA treatment, OC cultures were collected 24 hours after exposure to 450 μM 4-HNE exposure. The cultures were fixed and stained with TRITC-labeled phalloidin, and HC numbers were estimated as detailed above.

HC marker immunostaining in p27^{kip1}/GFP transgenic mouse pup inner ears

HC recovery in the cultured OC after siRNA treatment could be the result of direct transdifferentiation from SCs (Izumikawa et al., 2005 and 2008; Lin et al., 2011). To test this hypothesis, we used mouse pups from p27^{kip1}/green fluorescent protein (GFP) transgenic mice (kindly supplied by the Segal 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 and Doetzlhofer 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).

Harvesting and subsequent culturing of cochlear and macular tissues from p27^{kip1}/GFP transgenic mouse pups were conducted using the same procedures as detailed above. A group of cochleae and maculae were exposed to toxin (4-HNE or neomycin) or siRNA (scRNA, *Hes1* siRNALF or *Hes1* siRNANP) alone or toxin (4-HNE or neomycin) and siRNA (scRNA, *Hes1* siRNALF or *Hes1* siRNANP). Cochleae 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 trans-differentiated 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 to 7 *in vitro*. After washing with PBS, the tissues were blocked in 1% BSA in 1% PBS/T 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 p27^{kip1}/GFP (*green*) were considered to be HCs trans-differentiated from SCs (Doetzlhofer et al., 2006).

Although low levels of p27^{kip1} mRNA have been detected by rtPCR, no detectable protein levels of p27^{kip1} have been observed in auditory HCs at postnatal day 7 (P7). However, some utricular HCs have been shown to weakly express p27^{kip1} protein at this developmental stage (Laine et al., 2010). To determine the p27^{kip1}/GFP expression patterns in p27^{kip1}/GFP transgenic mice, fresh-fixed OCs and maculae from p27^{kip1}/GFP transgenic mice were examined at P9, the developmental stage at which the *in vitro* transdifferentiation of SCs was observed (detailed in Results). Inner ears were harvested from p27^{kip1}/GFP transgenic mouse pups and fixed immediately in 4% formaldehyde in PBS. Post-fixation, OCs were dissected out in PBS and incubated with rabbit anti-myosin VIIa IgG to label HCs. Expression patterns of p27^{kip1}/GFP and myosin VIIa were characterized using confocal microscopy as described above.

SC labeling with anti-Jagged1 antibody

Previous studies have shown that the increases in HC numbers within inner ear tissues following manipulation of the Notch pathway arise at the expense of pre-existing SCs (Collado et al., 2011; Kiernan et al., 2005; Takebayashi et al., 2007). In the present study, a significant increase of HCs was observed after treatment of siRNA. If this was caused by transdifferentiation of SCs into HCs, a significant decrease in the number of SCs should occur. To address this issue, at least six cultured OCs were harvested from each condition (untreated cultures, scRNALF, *Hes1* siRNALF, 4-HNE and scRNALF, 4-HNE and *Hes1* siRNALF, as detailed above) at day 8 *in vitro*, and serial vertical cryosections (10 µm of thickness) were obtained from the middle turns for immunostaining with the SC marker, Jagged1. The sections were immunolabeled with goat anti-Jagged1 antibody (1:250, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and rabbit anti-myosin VIIa (1:12.5, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) followed by incubation with Alexa Fluor® 594 donkey anti-rabbit IgG and Alexa Fluor® 488 donkey anti-goat IgG (each at 1:1000, Invitrogen, Carlsbad, CA). DAPI was used to stain nuclei. Images were acquired from serial sections on a Leica SP2 confocal microscope (Heidenberg, Germany). Jag1 positive cells in OCs on each cross-section were counted and statistically analyzed as described previously (Takebayashi et al., 2007).

BrdU labeling in the cultured OC

Previous studies demonstrated increased SC proliferation following Notch pathway manipulation in either mouse cochleae (Kiernan et al., 2005; Takebayashi et al., 2007) or

within the lateral line of zebrafish (Ma et al., 2008). BrdU labeling was conducted to study cell proliferation in the cultured cochlear tissue after exposure to 4-HNE (400 μ M) and/or treatment with siRNA. After a 4-day incubation period, 10 μ g/ml BrdU was added to the organotypic cultures for 2 hours at 37 °C, and the tissues were then fixed in 4% formaldehyde in PBS. For BrdU staining, tissues were incubated in 2 M HCl for 30 minutes at 37 °C. The tissues were then blocked with 1% BSA + 5% normal serum for 1 hour and incubated with Alexa Fluor® 488 mouse anti-BrdU antibody (1:200 dilution, Invitrogen, Carlsbad, CA) in the blocking buffer overnight at 4 °C. Cultures in which no BrdU was added were used as negative controls. The tissues were washed three times in PBST (0.2% Tween in PBS) and mounted onto slides for fluorescence microscopy.

To identify the types of cells that exhibited positive BrdU staining, fixed OC cultures were immersed in 30% sucrose in PBS overnight, and middle turns of OCs were cryosected at 10 μ m. After incubation with anti-BrdU antibody, the cryosections were then incubated with rabbit anti-myosin VIIa antibody (1:50, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) followed by incubation with Alexa Fluor® 594 donkey anti-rabbit IgG (1:1000, Invitrogen, Carlsbad, CA) overnight at 4 °C. The tissues were then examined by confocal microscopy as described above.

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.

RESULTS

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% Jag1 negative cells at 12 or 24 hours after NP exposure (*p* > 0.05). A greater number of Jag1 positive cells (23.08%) contained NPs at 48 hours than at 24 hours 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 hours after NP exposure, yet the perceived increase was not statistically significant compared to 12 or 24 hours (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, unclustered 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 hours (Fig. 1B and D). At this time, 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. 2C. These data demonstrate that both PLGA-formulated nanoparticles and lipofected double-stranded RNA molecules are readily taken up in the targeted tissue of interest.

siRNA to *Hes1* and *Hes5* or *Hes1* siRNANP down-regulate targeted transcripts and up-regulate *Atoh1* mRNA in cultured cochleae

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 low dose of (200 µg/ml) *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 scRNANP 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 timecourse, *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).

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 JetSI™ 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).

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 μ M) 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 μ M) 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 $80 \pm 5\%$ 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 μ M, $p < 0.05$, 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 (JetSI™ 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, F 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 (Fig. 3H) with orderly stereociliary bundles. 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 μ M 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 OHC 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).

Hes1 siRNANPs increase HC numbers in OC cultures

Treatment of non toxin-damaged OC cultures with *Hes1* siRNANP (50 μ g/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 μ g/ml) of *Hes1* siRNANP treatment in toxin-damaged OC cultures (200 μ M 4-HNE) compared to 4-HNE only exposed cultures ($p > 0.05$, Fig. 7). Treatment of toxin-damaged (200 μ M 4-HNE) OC cultures with 50 μ g/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 μ g/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 μ M) and subsequently treated with 100 or 200 μ g/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 μ g/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).

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 OC cultures treated with siRNA plus JetSI™ 10 mM transfection reagent, only about a 50% HC population was found in 50 μ g/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 μ M) and subsequently treated with increasingly higher doses (400, 600 or 800 μ g/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 μ g/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 μ g/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).

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 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 only or neomycin plus scRNALF, more HCs were found in the maculae treated with neomycin plus *Hes1* siRNALF in both striola 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).

Transdifferentiation of new HCs from SCs

To ascertain whether *Hes1* siRNA treatment induces the transdifferentiation of SCs into HCs, we examined siRNA-treated OC cultures for the presence of nascent HCs coexpressing the HC marker, myosin VIIa and the SC marker, p27^{kip1}/GFP. p27^{kip1} 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 p27^{kip1} protein at P7 (Laine et al., 2010). In the inner ear of the p27^{kip1}/GFP transgenic mice, GFP expression is regulated by the p27^{kip1} promoter and is thus expressed specifically in SCs (Doetzlhofer et al., 2006; White et al., 2006). To discern whether HCs in the p27^{kip1}/GFP transgenic mice exhibit GFP expression, we first examined the expression pattern of GFP in un-cultured 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 cell populations (Fig. 10A-C). The GFP expressed in the uncultured SCs was distributed throughout the cell, exhibiting diffuse signal intensity in both the nucleus and cytoplasm, consistent with previous reports on the distribution pattern of eGFP (Chalfier et al., 1994; Kain et al., 1995). Although a similar GFP expression pattern was observed in cultured OC controls (untreated and scRNALF treated OCs, Fig. 10D-I), the uncultured P9 OCs exhibited higher intensity GFP expression in SCs that surrounded IHCs and a lower GFP signal intensity in SCs that surrounded OHCs (Fig. 10A-C). The few HCs that survived exposure to either 4-HNE (arrows in Fig. 10J-L) or neomycin (Fig. 12D-F) in the organotypic cultures did not exhibit any discernible changes in the pattern of GFP expression compared to untreated (Figs. 10D-F and 12A-C) and scRNALF treated (Fig. 10G-I) controls as evidenced by fluorescence microscopy.

We exploited the fact that GFP-positive SCs exhibited a diffuse intracellular localization pattern, and surveyed the organotypic cultures for HCs that unambiguously exhibited both GFP positive nuclei and myosin VIIa positive cytoplasm. HCs with this co-expression pattern were found only in cultured OCs treated with *Hes1* siRNALF alone (arrow in Fig. 11C) or in cultures exposed to 4-HNE and subsequently treated with either *Hes1* siRNALF (arrows in Fig. 11F and I) or *Hes1* siRNANP (arrow in Fig. 11L). These double-labeled HCs were often found in the middle turn of OC tissues at day six *in vitro*. These new HCs were typically located along the outside edges of OHC rows (arrows in Fig. 11C, F and L) or in the region where OHCs were normally located (arrow in Fig. 11I). HCs with GFP positive nuclei and myosin VIIa positive cytoplasm were also found in maculae treated with neomycin and *Hes1* siRNALF (arrows in Fig. 12I) or neomycin and *Hes1* siRNANP (arrows in Fig. 12L). Myosin VIIa positive HCs with non-green nuclei were also found in the maculae under all experimental conditions (Fig. 12) with fewer HCs surviving in maculae treated with neomycin alone (Fig. 12D-F). These findings strongly suggest that the new HCs arise from SCs (GFP positive cytoplasm and nuclei) that transdifferentiate into HCs (myosin VIIa positive cytoplasm with GFP positive nuclei) rather than from repair of injured HCs, which would not demonstrate GFP expression.

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 knockdown of *Hes1* mRNA trans-differentiate from SCs in the inner ear through a non-mitotic process.

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., 2006). 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 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.

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 and 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 p27^{kip1}/GFP, following 4-HNE exposure and subsequent downregulation of *Hes1* expression (Fig. 11). Moreover, there were significantly more HCs observed after siRNA treatment than were observed to have survived at the 24 hour time point post-toxin. We started siRNA treatment 24 hours 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 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 (data not shown).

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 a ~four-fold increase in *Atoh1* mRNA levels, supporting new HC generation through up-regulation of *Atoh1*. *Hes5* siRNALF treatment induced only a ~2 fold upregulation of *Atoh1*. This result may explain why adding *Hes5* siRNA did not combinatorily induce significantly more HC production (Fig. 8). Low expression of *Hes5* in the inner ear after birth may also explain this phenomenon (Hartman et al., 2009).

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* siRNALF or < 200 μ g/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 regime supports a degree of HC protection, because slightly more OHCs survived (~30% of OHC survival in the middle) when siRNA was administered (24 hours 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 and 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.

Regeneration through trans-differentiation and cell proliferation

In the mammalian vestibular system, one explanation for the disappearance of HC bundles after toxic 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 and 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 laminar surface following scar formation (Sobkowicz et al., 1996). To explore these possibilities, we utilized a transgenic mouse model that specifically expresses a p27^{kip1}/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 p27^{kip1}/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 (Mizutani et al., 2013). It has been proposed that the presence of *Hes1* after a lesion may prohibit the occurrence of trans-differentiation 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 p27^{Kip1}/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 p27^{Kip1}/GFP positive SCs differentiated *in vitro* (Doetzlhofer, 2006). The fact that very few HCs survived in tissues exposed to toxin alone (Figs. 10J-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 p27^{Kip1} was found in gentamicin damaged HCs in the avian inner ear (Torchinsky et al., 1999). Therefore, there is a possibility that expression of p27^{Kip1}/GFP in the myosin VIIa positive cells may indicate degeneration of HCs. However, we never saw 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 cochleae 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 p27^{Kip1}/GFP transgenic mouse pups. Normally p27^{Kip1}/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.

Advantages of using siRNA and NPs

Using siRNA has several advantages: firstly, 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, 2010) and is being applied to the ear (Alvarado et al., 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 transfection reagent) to inhibit *Hes* genes in toxin-exposed OCs and maculae (utricle and saccule) 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 intracellular 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, siRNANP 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 (Mizutani 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.

Acknowledgments

This study was supported by grants from Hough Ear Institute and Integris Health, Oklahoma City, Oklahoma (RDK). The authors would like to thank Dr. Neil Segil at House Ear Institute for providing p27^{kip1}/GFP transgenic mice and Jim Henthorn at the University of Oklahoma Health Science Center for assistance with confocal microscopy. We are also very grateful to Dr. Douglas Cotanche at Boston University School of Medicine for his thoughtful review of and suggestions for this manuscript.

ABBREVIATIONS

GFP	green fluorescent protein
HC	hair cell
<i>Hes1</i> and <i>Hes5</i>	hairy and enhancer of split 1 and 5
4-HNE	4-hydroxy-2-nonenal
NP	nanoparticle
OC	the organ of Corti
PLGA	poly(lactide- <i>co</i> -glycolide acid)
qRT-PCR	quantitative real-time polymerase chain reaction
SC	supporting cell
scRNA	scrambled siRNA
siRNA	small interfering RNA
LF	lipofection

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>New hair cells can be regenerated in the inner ear by manipulating the Notch pathway.
>siRNA to *Hes* genes decreased *Hes1* mRNA and up-regulated *Atoh1* expression. >Hair cells recovered in cultured inner ears treated with toxins/siRNA formulations. >*Hes* siRNAs delivered using a suitable vehicle are potential therapeutic molecules.

Figure 1.

NPs (A) or siRNA (B, C) were readily taken up by cultured OC cells from mouse pups. The OC cells were stained with anti-Jag1 IgG (red) and DAPI (blue) and examined by confocal microscopy (A-C). Examples of clusters of Coumarin NPs (green) were found in Jag1 positive (arrows in A) and negative (arrowheads in A) OC cells 48 hours after NP exposure. FAM-labeled scRNA (FAM scRNA, green) was also found in Jag1 positive (arrows in B and C) and negative cells (arrowheads in B and C) arising from dissociated OC explants (B) and in cross sectional views of intact OCs (C). The number of Jagged1 positive or negative cells that had taken up coumarin NPs or FAM scRNA was counted and statistically analyzed (D). Equal numbers of Jag1 positive (13.01-14.29%) and Jag1 negative (13.01-13.43%) cells had NPs at 12 or 24 hours after NP exposure ($p > 0.05$). More Jag1 positive cells (23.08%) contained NPs 48 hours compared to 24 hours after NP exposure ($p < 0.05$). More Jag1 positive cells (55.35%) contained scRNA than Jag1 negative cells (38.77%) 48 hours after scRNA exposure ($p < 0.05$). Scale bar = 10 μ m in B (applies to images shown in A, B) and C.

Figure 2.

Hes1 or *Hes5* siRNA treatment inhibited *Hes1* or *Hes5* mRNA expression, respectively, and up-regulated *Atoh1* mRNA expression in OC cultures as analyzed by qRTPCR. As shown in graph A, the mRNA levels were significantly reduced 2 days after application of the siRNALF ($p < 0.001$ or 0.01). A significant up-regulation of *Atoh1* and down-regulation of *Hes1* mRNA were observed at day 7 (day 8 *in vitro*, all $p < 0.05$) but not at day 2 or day 4 (all $p > 0.05$) after 200 $\mu\text{g/ml}$ *Hes1* siRNANP treatment (B). ***, ** and * indicate $p < 0.001$, 0.01 and 0.05 , respectively. Error bars represent standard error of the means.

Figure 3.

Effects of siRNA treatment on HCs in OC cultures. Examples of phalloidin staining in the middle turn of untreated neonatal mouse OC cultures (A) or in OC cultures treated with scRNALF alone (B), scRNANP alone (C), 4-HNE alone (D), 4-HNE plus scRNALF (E), 4-HNE plus scRNANP (F), *Hes1* siRNALF alone (G), 4-HNE plus *Hes1* siRNALF (H) or 4-HNE plus *Hes1* siRNANP (I). All images were taken from OCs that had been cultured eight days *in vitro*. Three rows of OHCs and one row of IHCs were observed in OCs from the untreated cultures (A) and scRNA-treated cultures (B and C). One row of IHCs and few, dispersed OHCs (open arrowheads in D and E) were observed in 4-HNE treated (D) and 4-HNE plus scRNA treated (E and F) OCs. Extra OHCs (arrows in G-I) and IHCs (arrowheads in G-I) were observed in OCs treated with *Hes1* siRNALF alone (G) or in OCs treated with 4-HNE plus *Hes1* siRNA (H and I) OCs. Scale bar = 10 μ m in F for A-I.

Figure. 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 between OHC numbers in the *Hes1* siRNALF and *Hes1/5* siRNALF groups (#, $p < 0.05$) in the middle turn. *** indicate $p < 0.001$ 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*.

Figure 5.

scRNA had no significant impact on HC numbers in untreated or 4-HNE (475 μ 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 exposed 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 in F indicate the number of OCs in which HCs were counted). These data were obtained from OCs that had been cultured eight days *in vitro*.

Figure 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 (all $p < 0.001$), 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 *Hes1/5* 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 *Hes1/5* siRNALF treatment (all $p > 0.05$). ***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*.

Figure 7.

Hes1 siRNA delivered by nanoparticles significantly increased HC numbers in low dose of 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 cultures exposed to 4-HNE alone. 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*.

Figure 8.

A dose-dependent response was observed in the number of HCs in OCs treated with 450 μ M 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 μ g/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 μ M) exposure ($P > 0.05$, Fig. 7). Treatment with high dose of NPs (> 400 or 600 μ g/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*.

Figure 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 scRNAi (Neo/Sc, C) or neomycin plus *Hes1* siRNA (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 scRNAi (C) treatment. However, more HCs with short stereocilia were found in the maculae treated with *Hes1* siRNA (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 $p < 0.001$). scRNAi treatment did not change HC number in neomycin damaged maculae in all areas compared to neomycin only treated (all $p > 0.05$). More HCs were found in the neomycin plus *Hes1* siRNA treated maculae compared to neomycin plus scRNAi 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 scRNAi group. Error bars represent standard error of the means (the number of maculae counted was 6 in each group). Scale bar = 10 μm in D for A-D.

Figure 10.

Comparison of p27^{kip1}/GFP expression in uncultured and cultured OCs from p27^{kip1}/GFP transgenic mice. Examples of confocal images from untreated and uncultured OCs from p27^{kip1}/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 μ m in L for A-L. Images in D-L were taken from OCs that had been cultured six days *in vitro*.

Figure 11.

Hes1 siRNA treatment induced transdifferentiation of SCs into new HCs in 4-HNE damaged OCs of p27^{kip1}/GFP transgenic neonatal mouse pups. OC cultures were treated with *Hes1* siRNALF only (A-C) or exposed to 4-HNE and subsequently treated with either *Hes1* siRNALF (D-I) or *Hes1* siRNANP (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 (p27^{kip1}/GFP) were found in OCs treated with *Hes1* siRNALF alone (arrow in C), 4-HNE plus *Hes1* siRNALF (arrows in F and I), or 4-HNE plus *Hes1* siRNANP (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 (AL). Scale bar = 10 μ m in L for A-L. All images were taken from OCs that had been cultured six days *in vitro*.

Figure 12.

Hes1 siRNA treatment induced transdifferentiation of SCs to new HCs in neomycin (Neo) damaged maculae of p27^{kip1}/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 (p27^{kip1}/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 μ m in L for A-L. All images were taken from maculae that had been cultured six days *in vitro*.

Figure. 13.

The number of Jag1 positive SCs decreased in OCs following *Hes1* siRNA treatment. Examples of confocal images from untreated OC cultures (A), or OCs treated with *Hes1* siRNALF (B), 4-HNE plus scRNALF (C), or 4-HNE plus *Hes1* siRNALF (D) and labeled with anti-Jagged1 antibody (green, arrowheads), anti-myosin VIIa antibody (red, brackets-OHCs, arrows-IHCs), and DAPI (blue, nuclei) are shown. Note that four rows of OHCs are observed in the OC treated with either *Hes1* siRNALF alone or *Hes1* siRNALF plus 4-HNE (bracket in B and D) and no OHCs were observed in the cross-sectional view of the OC treated with scRNALF and 4-HNE (C). The number of Jag1 positive SCs was counted in the OCs and statistically analyzed (E). Significantly fewer Jag1 positive SCs were observed in the OC cultures treated with either *Hes1* siRNALF alone or *Hes1* siRNALF plus 4-HNE compared to the number of Jag1 positive cells observed in OCs treated with scRNALF alone or scRNALF plus 4-HNE treated cultures (decreased by 23.56% and 25.86%, respectively, all $p < 0.01$). No significant difference in Jag1 positive cell numbers was observed in OCs treated with scRNA compared to untreated cultures ($p > 0.05$). ** indicate $p < 0.01$. Scale bar = 10 μm in D for A-D. All images were taken from OCs that had been cultured eight days *in vitro*.

Figure 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* siRNALF (B and B'), 4-HNE alone (C and C') or 4-HNE plus *Hes1* siRNALF (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 middle turn 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 μ m in A-D, 10 μ m in D' for A'-D'. All images were taken from OCs that had been cultured four days *in vitro*.