Development/Plasticity/Repair

Diphtheria Toxin-Induced Cell Death Triggers Wnt-Dependent Hair Cell Regeneration in Neonatal Mice

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Cochlear hair cells (HCs), the sensory cells that respond to sound, do not regenerate after damage in adult mammals, and their loss is a major cause of deafness. Here we show that HC regeneration in newborn mouse ears occurred spontaneously when the original cells were ablated by treatment with diphtheria toxin (DT) in ears that had been engineered to overexpress the DT receptor, but was not detectable when HCs were ablated *in vivo* by the aminoglycoside antibiotic neomycin. A variety of Wnts (Wnt1, Wnt2, Wnt2b, Wnt4, Wnt5a, Wnt7b, Wnt9a, Wnt9b, and Wnt11) and Wnt pathway component Krm2 were upregulated after DT damage. Nuclear β-catenin was upregulated in HCs and supporting cells of the DT-damaged cochlea. Pharmacological inhibition of Wnt decreased spontaneous regeneration, confirming a role of Wnt signaling in HC regeneration. Inhibition of Notch signaling further potentiated supporting cell proliferation and HC differentiation that occurred spontaneously. The absence of new HCs in the neomycin ears was correlated to less robust Wnt pathway activation, but the ears subjected to neomycin treatment nonetheless showed increased cell division and HC differentiation after subsequent forced upregulation of β-catenin. These studies suggest, first, that Wnt signaling plays a key role in regeneration, and, second, that the outcome of a regenerative response to damage in the newborn cochlea is determined by reaching a threshold level of Wnt signaling rather than its complete absence or presence.

Key words: **apoptosis; hair cell; necrosis; regeneration; Wnt**

Significance Statement

Sensory HCs of the inner ear do not regenerate in the adult, and their loss is a major cause of deafness. We found that HCs regenerated spontaneously in the newborn mouse after diphtheria toxin (DT)-induced, but not neomycin-induced, HC death. Regeneration depended on activation of Wnt signaling, and regeneration in DT-treated ears correlated to a higher level of Wnt activation than occurred in nonregenerating neomycin-treated ears. This is significant because insufficient regeneration caused by a failure to reach a threshold level of signaling, if true in the adult, has the potential to be exploited for development of clinical approaches for the treatment of deafness caused by HC loss.

Introduction

The sensory HCs that detect sound and transmit their signal to the brain via the auditory nerve are susceptible to damage. The sensory nervous system evolved with high degree of specialization but with a decreased ability to regenerate compared to lower species, and loss of sensory HCs is a major cause of deafness. HC damage is typically caused by noise exposure, ototoxic drugs, infections, and aging, and the lack of a regenerative response to replace the cells in the cochlea, the mammalian hearing organ, leads to a high prevalence of acquired forms of deafness, particularly in older adults, with an incidence above age 65 greater then 50%.

In the newborn mouse cochlea, supporting cells have the capacity to generate significant numbers of new HCs with inhibition of Notch after damage *in vitro* (Doetzlhofer et al., 2009; Korrapati et al., 2013; Bramhall et al., 2014; Cox et al., 2014). We

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and others have observed spontaneous regeneration of HCs in models of HC loss in the newborn mouse (Bramhall et al., 2014; Cox et al., 2014). The yield of HCs increased after Notch inhibition (Bramhall et al., 2014), suggesting that pathways of regeneration may be activated, but not sufficient to fully regenerate the organ. The increase in HC number was muted when Wnt signaling was inhibited (Bramhall et al., 2014). Wnts are released after damage in invertebrates and lower vertebrates as a crucial part of the damage response (Kawakami et al., 2006; Chai et al., 2012; Sun and Irvine, 2014). Mechanisms of regeneration in the adult often involve the use of pathways that served to generate the cells in the embryo. In the chick ear, loss of HCs is followed immediately by supporting cell division and transdifferentiation (Bermingham-McDonogh and Rubel, 2003; Cafaro et al., 2007; Daudet et al., 2009). In the present study, we find differences in the newborn mammalian cochlea in the extent of release of Wnts in response to damage induced by diphtheria toxin (DT) versus neomycin, suggesting a conservation of pathways used to drive regeneration. Moreover, the extent of Wnt activation correlates with the regenerative response seen after DT- but not neomycin-induced HC death.

Materials and Methods

Animals. Induced-DTR (*iDTR*) mice, which contain a *loxP*-flanked stop sequence upstream of the DT receptor (DTR) at the *Rosa26* locus, were obtained from The Jackson Laboratory (Stock 007900; Buch et al., 2005). *β-catenin$^{flox(exon3)}$* mice (Harada et al., 1999) were generously provided by M. Taketo (Kyoto University, Kyoto, Japan), *Sox2-Cre-ER* mice (Arnold et al., 2011) by K. Hochedlinger (Harvard Medical School, Boston, MA), and *Gfi1-Cre* mice (Yang et al., 2010) by L. Gan (University of Rochester, Rochester, NY). *Rosa26* reporter mice, containing a *loxP*-flanked stop cassette upstream of a red fluorescent protein variant (Stock 007914; Madisen et al., 2010), were obtained from The Jackson Laboratory. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary.

HC death in iDTR mice. iDTR mice crossed with *Gfi1-Cre* mice received 100 ng of DT at postnatal day 1 (P1), P4, or P6, once a day for 3 d via intraperitoneal injection. Mice of either sex were used for all experiments. Mouse pups were killed 4 d later. *iDTR, Gfi1-Cre*, and wild-type littermates were used as controls for *iDTR;Gfi1-Cre* mice. No differences were seen among the controls, and representative data with iDTR are therefore shown.

Intracochlear delivery of neomycin or Wnt inhibitor. P1 mice were anesthetized by lowering body temperature for the surgical procedure. A postauricular incision was made on the left side, and the bulla was lifted to expose the cochlea. Neomycin (200 nl of a 50 mM solution) or Wnt inhibitor, IWP-2 (10 μM, 200 nl), was injected through the cochlear capsule into scala media at the cochear basal turn with a glass pipette (end diameter, 5 μm) attached to a nanoliter micropump (WPI, UMP3 + Micro4 + NanoFil) at 60 nl/min. After injection, the incision was sutured and the mice were brought to a heating pad to recover. Tissue was analyzed after 4 d.

Constitutive expression of β-catenin in vivo. *Sox2-Cre-ER* mice were mated with *β-catenin$^{flox(exon3)}$* mice. After intracochlear injection of neomycin at P1, tamoxifen dissolved in corn oil (100 μl at 50 mg/ml) was given to the mothers of the compound transgenic mice and passed to the pups via the milk to generate the *β-catenin$^{\Delta exon3}$* mice. Pups were killed at P5. Pups were genotyped for both *Cre* and *β-catenin$^{flox(exon3)}$*.

In vivo *labeling of HCs with FM1-43.* One day before being killed, mice were injected on the back, subcutaneously (3 mg/kg body weight), with FM1-43 (Meyers et al., 2003). After fixing with 4% paraformaldehyde (PFA) for 20 min, FM1-43 uptake in HCs was examined by confocal microscopy.

Labeling necrotic cells with propidium iodide. Organs of Corti, freshly dissected from neonatal mice with fixation, were incubated with propidium iodide at 1.5 μM for 5 min at room temperature. Organs of Corti were washed three times with PBS buffer, fixed, and immunostained.

Labeling apoptotic cells with annexin V. The organ of Corti was stained with annexin V (PromoKine, catalog #PK-CA707-30017) 30 min after neomycin injection, 1 d after DT damage, or without treatment. In brief, the organ of Corti was incubated with FITC-annexin V and Hoechst solution for 15 min in the dark, and then immediately scanned with a confocal microscope.

Cell proliferation. To label dividing cells *in vivo*, 10 μl 5-ethynyl-2′-deoxyuridine (EdU, 10 mg/ml) was given to the pups by subcutaneous injection, twice a day for 4 continuous days. Incorporated EdU was detected with Alexa Fluor azide using Click-iT EdU Image kits (Invitrogen) according to the manufacturer's protocol, before immunostaining.

RT-PCR. Total RNA was extracted from isolated organs of Corti using the RNeasy Mini kit (Qiagen); cDNA was synthesized from RNA (1 μg) with SuperTranscipt III (New England Biolabs). Quantitative PCR was performed for 45 cycles, and GAPDH was used as an internal control in a PerkinElmer ABI PRISM 7700 Sequence Detector (Applied Biosystems). Wnt ligands were analyzed using SYBER Green (ThermoFisher). All other analysis was performed with Taqman probes (Applied Biosystems). Genes with signals occurring later than 45 cycles were considered undetectable. The organs of Corti were harvested and analyzed 1 and 4 d after neomycin or DT injection.

Notch inhibition. The organs of Corti from *iDTR;Gfi1-Cre* mice that had received two doses of DT were dissected and cultured for 12 h before treatment. Organs of Corti were treated with 5 μM dibenzazepine (desphydroxy; LY411575; Santa Cruz Biotechnology) for 3 d and compared to controls given 0.1% dimethyl sulfoxide.

Histology and immunostaining. Cochleae were dissected and immediately fixed for 20 min with 4% PFA (Electron Microscopy Sciences). The organ of Corti was separated from the stria vascularis and fixed further in 4% PFA for 30 min.

Antibodies used in this study were against myosin VIIa (1:800; Proteus), Sox2 (1:500; Santa Cruz Biotechnology), Ki67 (1:100; ThermoFisher), cleaved caspase-3 (1:200; Cell Signaling Technology), and transcriptionally active β-catenin (PY489 β-catenin; Developmental Studies Hybridoma Bank). Species-specific Alexa Fluor-conjugated secondary antibodies were used for detection (1:500; Invitrogen).

Whole-mount preparations of the organ of Corti were analyzed using a Leica TCS SP5 confocal microscopy. The organ of Corti was scanned in the *Z* plane followed by the *Y* plane at the indicated *XZ* line.

Cell counting. Confocal Z stacks of the entire cochlear whole mount were projected into a single image to capture all cells from different planes of focus for counting. This method allowed us to distinguish between inner HCs (IHCs) and outer HCs (OHCs). Atoh1- or myosin VIIa-positive HCs in the pillar cell region were manually counted with Image J software (NIH) from the entire cochlear whole mount. Cell counts for the β-catenin-expressing group were compared to those for littermates without β-catenin overexpression and were analyzed by Student's *t* test.

Results

Death of HCs induced by DT

Mice with the DTR inserted after a loxP-flanked stop sequence become susceptible to cell ablation after Cre recombination and exposure to DT and are thus termed induced-DTR mice (Buch et al., 2005). Gfi1 is expressed in auditory HCs from E15.5 through adulthood (Yang et al., 2010). DT was given at P1, and the cochlea was harvested at P4 from *iDTR;Gfi1-Cre* mice, in which *Cre* is driven by *Gfi1*. No HC damage was observed after DT treatment of iDTR mice without *Gfi1-Cre* and their wild-type littermates (Fig. 1*A*,*B*). Examination of the organ of Corti showed successful HC targeting in *iDTR;Gfi1-Cre* mice without gross structural changes to the organ of Corti (myosin VIIa-positive cells, Fig. 1*B*,*C*). HC death was most evident in the apical region, and the remaining HCs, although myosin VIIa positive, had lost the ability to take up HC transduction channel dye, FM1-43 (Fig. 1*C*). Loss of uptake of FM1-43 was more pronounced than outright cell death and occurred in an apex-to-base gradient (Fig. 1*D*,*E*).

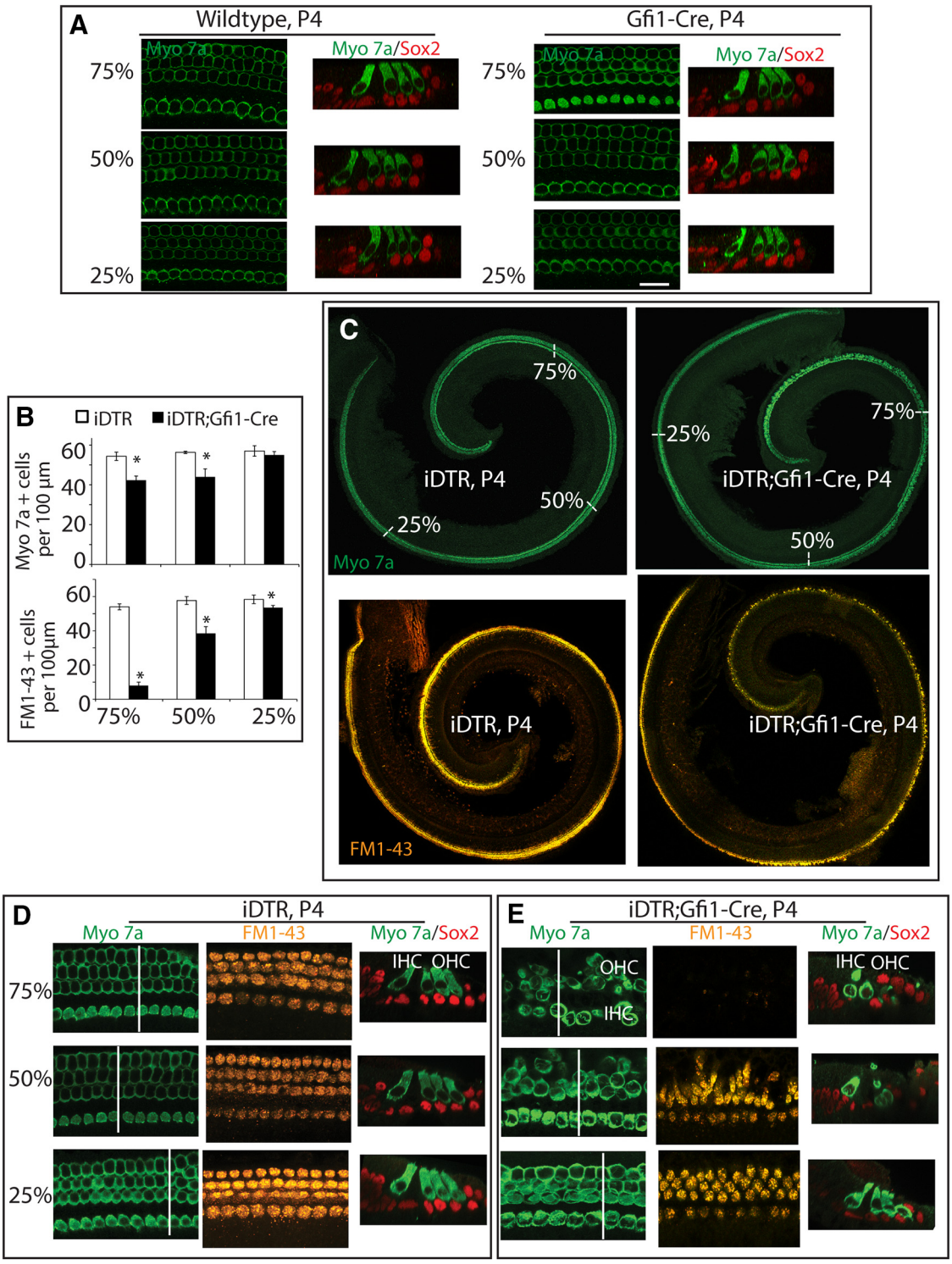

Figure 1. Loss of HCs in different areas of the cochlea (shown as the distance from the hook region of the base to the apex) after DT treatment of transgenic mice. ***A***, HCs were intact in *Gfi1-Cre* mice and their wild-type littermates after DT treatment. ***B***, A decrease in HC number and FM1-43 uptake was seen in the DT-treated $DTR^{flox/+}$;*Gfi1-Cre* (iDTR;Gfi1-Cre) mice. Loss of FM1-43 uptake was more pronounced than outright loss of myosin VIIa-positive cells and was most pronounced in the apical region. The control ear is from a $DTR^{flox/+}$;*Gfi1-Cre*-negative mouse (*iDTR*) that received DT. The cochlea was examined at P4. $^*p < 0.05$. $n = 4$. Error bars indicate SEM. ***C***, There were no gross structural changes in the organ of Corti from *iDTR;Gfi1-Cre* or control mice following systemic DT administration. ***D***, HCs in the control ear were positive for myosin VIIa and FM1-43; the white line indicates an *x–z* scan. ***E***, *iDTR;Gfi1-Cre* mice had lost HCs in an apex-to-base gradient; the disorganized rows of HCs did not contain active transduction channels (FM1-43). Supporting cells had moved apically (*x–z* scan at the white line) after HC loss. Scale bar, 20 μm.

Regeneration of HCs after DT-induced cell death

Sensory epithelial cells in the organ of Corti of control (*iDTR* without *Gfi1-Cre*) mice at P4 did not undergo mitosis (Fig. 2*A*, *C*), as shown by the lack of incorporation of EdU, a marker of DNA replication, and by Ki67, a marker of active phases of the cell cycle. This is consistent with the postmitotic status of auditory HCs and supporting cells in the postnatal sensory epithelium of mammals and the lack of regeneration. Proliferation was only

seen in the mesenchymal cells beneath the basilar membrane (Fig. 2*A*). EdU/myosin VIIa double-positive cells, however, were evident at P4 in organ of Corti in *iDTR; Gfi1-Cre* mice receiving DT at P1 (Fig. 2*B*). Some myosin VIIa-expressing cells in the DT-treated ears also expressed Ki67 (Fig. 2*D*). Sox2 expression was not detected in postnatal HCs of control (*iDTR* without *Gfi1-Cre*) mice at P4 (Fig. 2*E*), but all ectopic myosin VIIa-positive cells showed strong expression of Sox2 after DT treatment (Fig. 2*F*), consistent with our previous observation of Sox2 in new HCs (Bramhall et al., 2014; Kempfle et al., 2016). Sox2-positive HCs were negative for p27Kip1, which was seen in Sox2-positive supporting cells (Fig. 2*G*). A small percentage of myosin VIIa/Sox2 double-positive cells incorporated EdU, suggesting that new HCs were generated both by mitosis and direct transdifferentiation from supporting cells (Fig. 2*H*). When DT was given at later time points, the number of new myosin VIIa/Sox2 double-positive HCs was significantly decreased (Fig. 2*I*).

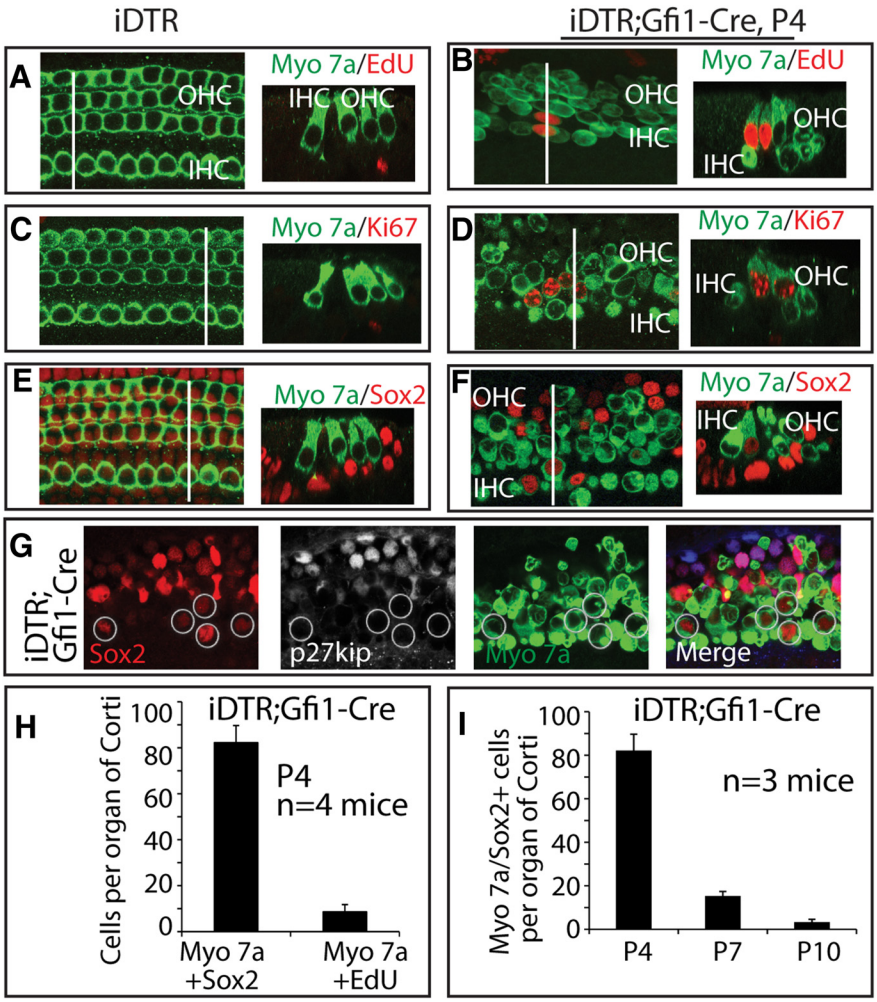

Figure 2. Spontaneous HC regeneration in *iDTR;Gfi1-Cre* mice. ***A***, Lack of EdU incorporation in a control cochlea showed that HCs and supporting cells were postmitotic at P4. HCs were stained with myosin VIIa antibody. The position of an *x–z* scan is indicated by the white line. ***B***, A small number of myosin VIIa-positive cells were EdU positive, along with damaged HCs at P4 in *iDTR;Gfi1-Cre* mice, in which HC death was induced at P1 by DT. Two myosin VIIa-positive cells with EdU are seen in an *x–z* scan (white line). ***C***, HCs in the control ear were negative for Ki67, a protein marker for proliferation. An *x–z* scan was taken at the white line. ***D***, Some of the myosin VIIa-positive cells were colabeled for Ki67. The double-positive cells can be seen in an *x–z* scan (white line). ***E***, At P4, HCs in the control cochlea were negative for Sox2, a marker for supporting cells. An *x–z* scan was taken at the white line. ***F***, Many of the myosin VIIa cells were positive for Sox2 in the DT-treated organ of Corti. An *x–z* scan at the white line shows two myosin VIIa-positive cells in the pillar cell region expressed Sox2. ***G***, Supporting cells were p27Kip positive, and HCs were p27Kip negative. ***H***, Quantification of myosin VIIa/Sox2-positive cells and myosin VIIa/EdU-positive cells in P4 *iDTR;Gfi1-Cre* mice, in which HC death was induced at P1 with DT. $n = 4$ animals per group. ***I***, The number of myosin VIIa/Sox2-positive cells decreased with age, and few of these cells were visible in P10 organ of Corti. $n = 3$ animals at each age. Error bars indicate SEM.

Decreased HC regeneration after inhibition of Wnt signaling

Since Wnt/β-catenin signaling is known to lead to supporting cell proliferation and to generate new HCs when induced in the newborn cochlea *in vivo* (Chai et al., 2012; Shi et al., 2013), we tested for a role of Wnt signaling in the spontaneous regeneration in the *iDTR;Gfi1-Cre* mice. A single dose of IWP-2, an inactivator of porcupine function and inhibitor of Wnt production, directly injected into the DT-treated cochlea *in vivo* decreased the number of new HCs and proliferation (Fig. 3*A*). IWP-2 had no apparent structural effects on the normal or DT-treated ears (Fig. 3*B*). No labeling of supporting or HCs with EdU or Sox2 was seen after treatment of a normal ear with the drug (Fig. 3*C*), but IWP-2 application to DT-treated ears decreased the number of myosin VIIa/Sox2, as well as myosin VIIa/EdU double-positive cells, compared to the (contralateral) ear without IWP-2 treatment (Fig. 3*D*,*E*).

Increased regeneration of HCs by Notch inhibition after DT-induced HC death

Notch inhibition and Wnt/β-catenin signaling manipulation induced new HCs in postnatal organ of Corti and utricle (Doetzlhofer et al., 2009; Lin et al., 2011; Bramhall et al., 2014). We tested whether inactivation of Notch signaling would further augment the regeneration of HCs seen in the DT-treated ears. Treatment of cultured organ of Corti from *iDTR;Gfi1-Cre* mice with LY411575, a γ-secretase inhibitor, significantly increased the number of new HCs (Fig. 4*A*). Based on supporting cell incorporation of BrdU, the differentiation to HCs occurred both by a direct mechanism, without supporting cell mitosis, and by supporting cell division followed by HC differentiation (Fig. 4*B*,*C*). Notch inhibition thus enhanced spontaneous HC regeneration after DT-induced death of HCs.

No regeneration after neomycin-induced HC death

We previously found a low level of spontaneous HC replacement in cultured gentamycin-treated organ of Corti (Bramhall et al., 2014), but did not examine whether HC replacement occurred *in vivo*. To gain access to perilymph, we administered aminoglycosides directly into the scala media of the cochlea. The injection site (Fig. 5*A*) was 15% of the distance from the hook region to the apex (equivalent to 55 kHz after hearing onset). Intracochlear application of 200 nl of neomycin at P1 resulted in dose-dependent (up to 90% at 50 mM) OHC and IHC loss at P4, with increasing IHC damage at high concentrations (Fig. 5*A*,*B*). In

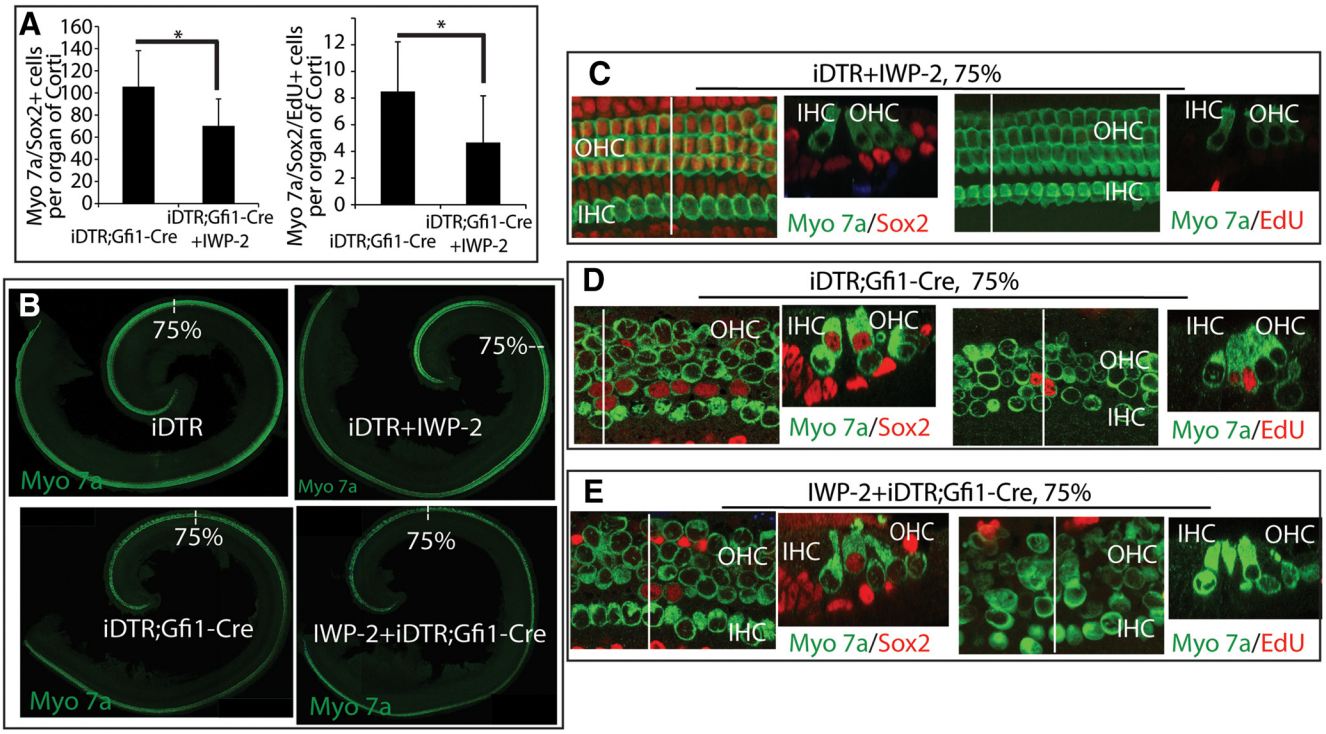

Figure 3. Wnt inhibition decreased spontaneous HC regeneration. ***A***, IWP-2, a Wnt inhibitor, applied directly to the cochlea, significantly decreased the number of myosin VIIa/Sox2 double-positive cells and the number of myosin VIIa/EdU double-positive cells in the organs of Corti upon DT treatment. Error bars indicate SEM. $*p < 0.05$. $n = 4$. ***B***, IWP-2 applied to the normal ear had no effect on gross organ of Corti morphology in the control or *iDTR;Gfi1-Cre* cochleas. ***C***, IWP-2 had no effect on HCs or supporting cells in the control mouse, which was negative for EdU. An *x–z* scan, shown in ***E***, was taken at the white line. ***D***, In the organ of Corti of *iDTR;Gfi1-Cre* mice after DT treatment, some myosin VIIa-positive cells were also positive for Sox2 and incorporated EdU. In an *x–z* scan at the white line, myosin VIIa-positive cells that expressed Sox2 and incorporated EdU were in the pillar cell region. ***E***, IWP-2-treated *iDTR;Gfi1-Cre* ears had fewer myosin VIIa/Sox2 and myosin VIIa/EdU double-positive cells. *x–z* scans were taken at the white lines.

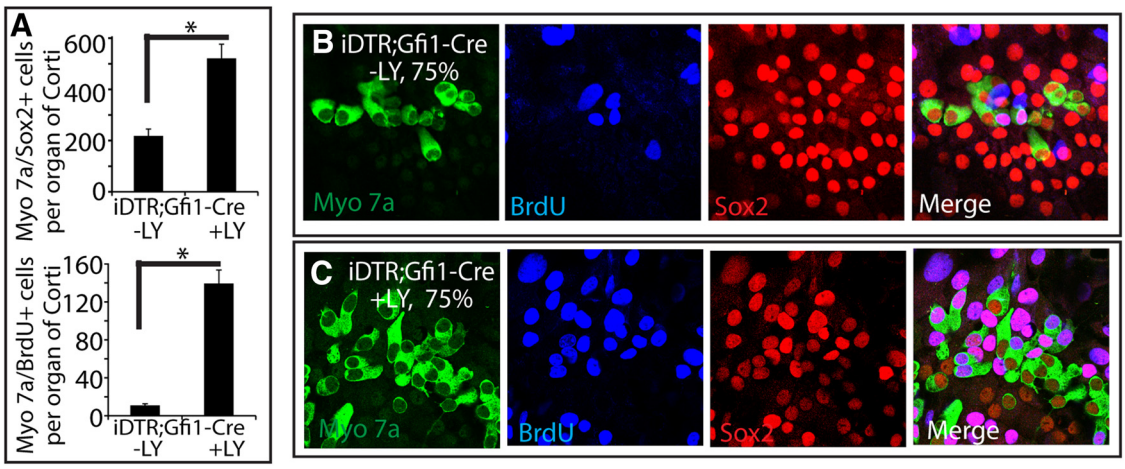

Figure 4. Notch inhibition increased the number of HCs in the DT-treated organ of Corti. ***A***, Treatment of cultured organ of Corti from an *iDTR;Gfi1-Cre* mouse with γ-secretase inhibitor, LY411575 (LY), increased the number of new HCs compared to an *iDTR;Gfi1-Cre* organ of Corti without LY treatment. Error bars indicate SEM. $*p < 0.05$. $n = 3$. ***B***, An *iDTR;Gfi1-Cre* mouse had myosin VIIa/Sox2 double-positive cells, some of which were BrdU positive. ***C***, An organ of Corti isolated from an *iDTR;Gfi1-Cre* mouse and treated with LY411575 gave rise to an increased number of myosin VIIa/Sox2 and myosin VIIa/BrdU double-positive cells.

contrast to the effects of DTR, no myosin VIIa/Sox2 double-positive cells or EdU incorporation were seen after neomycin treatment (Fig. 5*C*,*D*).

Changes in gene expression in DT vs neomycin-treated inner ear

Since previous studies have shown involvement of both Notch and Wnt/β-catenin signaling in HC generation in the newborn ear (Shi et al., 2013), we assessed activation of Wnt and Notch pathways in the DT- and neomycin-treated organ of Corti. No change was seen in Wnts or Wnt pathway molecules 1 d after DT or neomycin injection (Fig. 6*A*,*B*), whereas increased expression of Wnt1, Wnt2, Wnt2b, Wnt4, Wnt5a, Wnt7b, Wnt9a, Wnt9b, and Wnt11, as well as Dickkopf (Dkk) receptor Krm2, were apparent 3 d after DT treatment in the *iDTR;Gfi1-Cre* cochlea, and other Wnt molecules remained either unchanged or undetectable (Fig. 6*C*). In the neomycin-treated ears, however, all Wnt molecules remained unchanged or undetectable, except Wnt16 and Wnt11 (Fig. 6*D*).

We further analyzed the neomycin-induced HC death. Annexin V has a high affinity to phosphatidylserine, which is normally hidden within the cell membrane but appears in the outer leaflet of the cell membrane during apoptosis. Annexin V-positive HCs were observed 30 min after neomycin injection (Fig. 7*A*), but not in the control or DT-treated ears. Cleaved caspase-3-positive cells were also seen in the neomycin-injected ears (Fig. 7*C*), and the number of cleaved caspase-3-positive HCs was significantly increased in the neomycin-injected cochlea (Fig. 7*F*). HCs in *iDTR;Gfi1-Cre* mice showed increased permeability to propidium iodide (Fig. 7*B*). In agreement with differential expression of Wnt ligands, active β-catenin was seen in HCs and supporting cells in the *iDTR;Gfi1-Cre* ear following DT injection, whereas the control and neomycin injected ears did not accumulate active β-catenin (Fig. 7*D*). Cells that had undergone division in the *iDTR;Gfi1-Cre* ear also contained active β-catenin (Fig. 7*E*), while there was a lack of such cells in the neomycin-injected cochlea (Fig. 7*F*).

Regeneration of HCs by forced activation of β-catenin after neomycin-induced HC death

We next examined whether forced activation of Wnt/β-catenin signaling could induce new HC generation following neomycin-induced HC loss. We used *Sox2-Cre-ER* mice to activate β-catenin constitutively in all supporting cells by crossing to a $\beta\text{-}catenin^{flox(exon3)}$ mouse, in which conditional deletion of exon3 ($\beta\text{-}catenin^{\Delta exon3}$) blocks β-catenin degradation and induces accumulation of β-catenin. We administered neomycin to one ear to induce HC death at P1 and tamoxifen systemically to activate β-catenin in both ears at P2. At P5, β-catenin overexpression induced myosin VIIa-positive cells 30% from the base, where there was a complete absence of myosin VIIa cells in control ears (Fig. 8*A*,*B*). No EdU-positive cells were observed in the β-catenin-activated cochlea without HC damage by neomycin (Fig. 8*C*). Myosin VIIa/EdU double-positive cells in the neomycin-treated ear after β-catenin overexpression were seen at the apex in the pillar cell region, along with remaining HCs (Fig. 8*D*), consistent with our previous finding (Shi et al., 2013). The EdU incorporation is likely in supporting cells, since β-catenin upregulation drives supporting cell proliferation and transdifferentiation in the newborn cochlea (Shi et al., 2013), but does not affect hair cells (Shi et al., 2013). The number of new HCs in the neomycin treated, β-catenin-stabilized ears was higher than in ears where β-catenin was upregulated in the absence of damage induced by neomycin (Fig. 8*E*). Lethality of β-catenin overexpression precluded examination at a later age. Thus, forced activation of β-catenin regenerated HCs in neomycin-treated organ of Corti, and more HCs were generated in the neomycin-treated than in the intact organ of Corti.

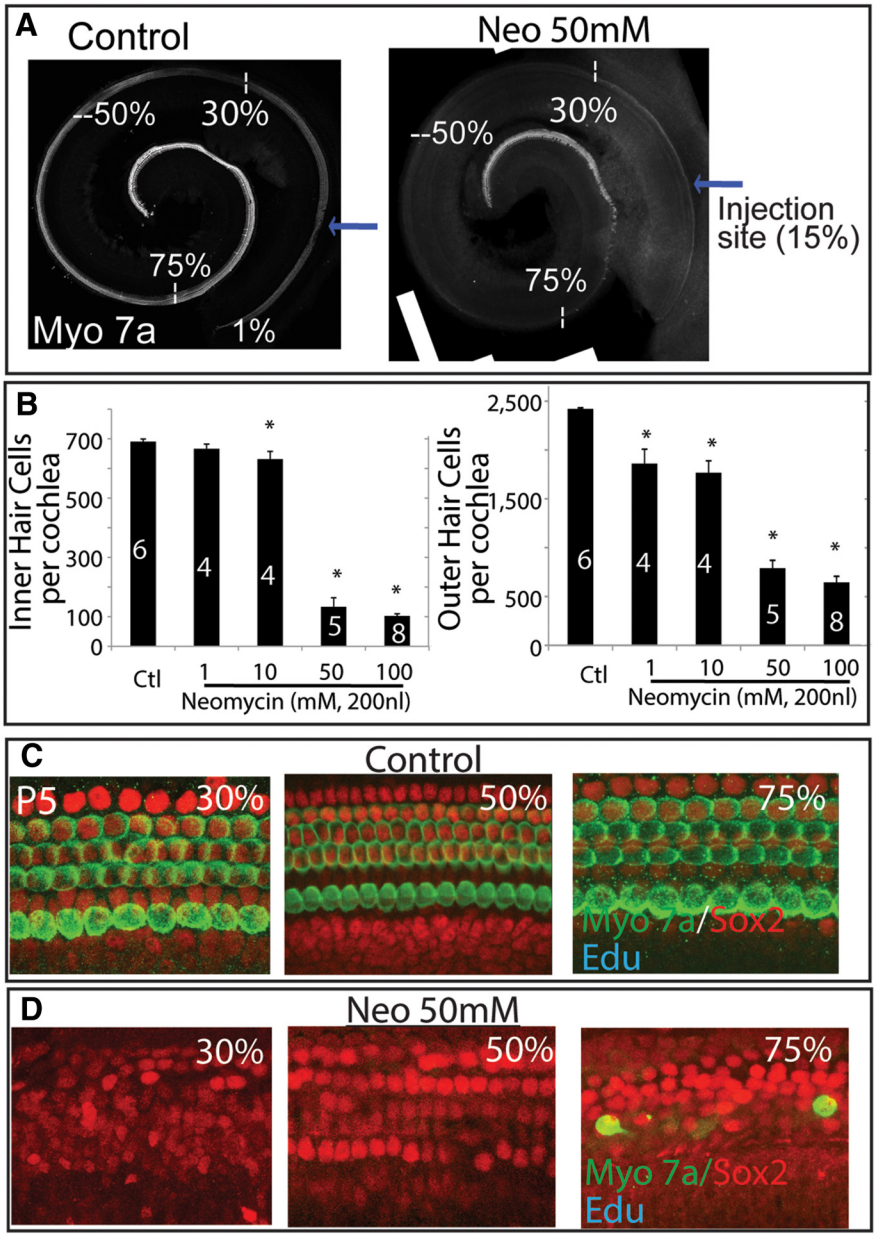

Figure 5. Lack of HC regeneration in the neomycin treated cochlea. ***A***, A single dose (200 nl) of 50 mM neomycin into the P2 cochlea resulted in a complete loss of HCs from the injection site which was 15% from the base (indicated by the arrows). No HC loss was seen in control cochlea with carrier injection between the injection site and the midapex (75% from the base). HCs were stained with myosin VIIa. ***B***, A dose-dependent HC loss was observed with intracochlear injection of neomycin. More IHC than OHC loss was observed at the higher concentrations of neomycin. The numbers of animals per group are indicated in the graph. Error bars indicate SEM. *$p < 0.05$. ***C***, The contralateral ear without neomycin had an overall normal structure, with myosin VIIa-positive HCs, Sox2-positive supporting cells, and no EdU incorporation. ***D***, A complete lack of myosin VIIa-positive cells in the 30 and 50% regions and a nearly complete loss at the 75% region were seen in the neomycin-treated ear. No EdU-positive cells were observed.

Discussion

We show here that significant regeneration of HCs can occur in the newborn mouse cochlea depending on the mechanism of cell death. We find that supporting cells reenter the cell cycle after *in vivo* HC death induced by DT but not neomycin in the newborn cochlea. Mechanisms of cell death induced by ligation of the DTR or by aminoglycosides, after their entry into HCs through trans-

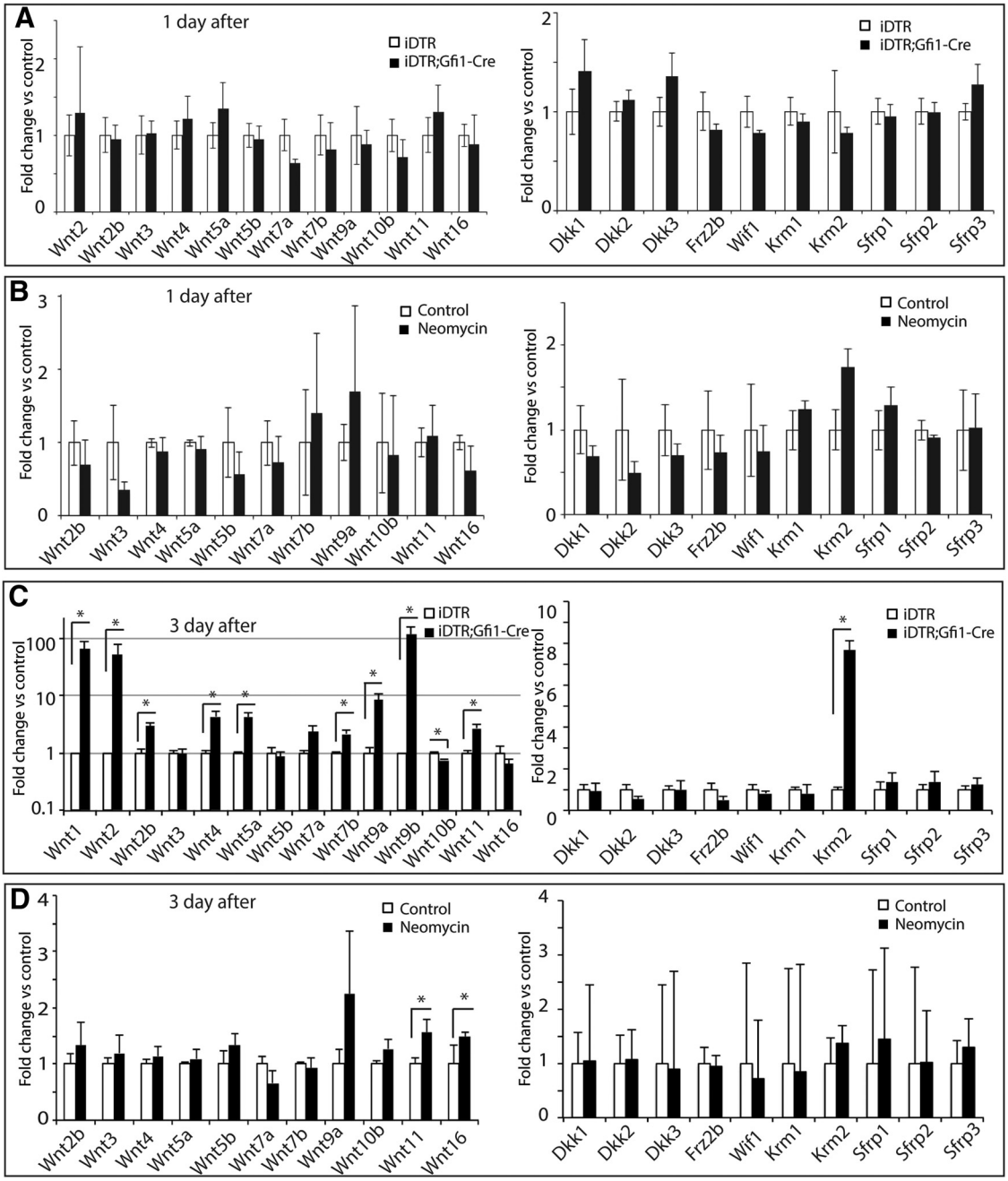

Figure 6. Wnt expression is upregulated in ears with spontaneous HC regeneration. ***A***, Expression of Wnts and Wnt antagonists by quantitative PCR showed no changes 1 d after treatment with DT in *iDTR;Gfi1-Cre* mice. ***B***, No changes in expression of Wnts and Wnt antagonists were observed 1 d after treatment neomycin-induced HC loss. ***C***, Expression of several Wnts showed significant increases 3 d after DT treatment in *iDTR;Gfi1-Cre* mice. Krm2 also increased in *iDTR;Gfi1-Cre* mice. ***D***, Wnt16 and Wnt11 showed small but significant increases 3 d after neomycin treatment. Other Wnt-related molecules showed no change or were undetectable after neomycin treatment. Error bars indicate SEM. $*p < 0.05$. $n = 4$.

duction channels, are distinct. DT binds its receptor, eventually leading to cell membrane rupture and release of cellular components (Collier and Kandel, 1971), whereas gentamicin disturbs mitochondrial function and activates apoptosis cascades (Matsui et al., 2003; Momiyama et al., 2006; Huth et al., 2011) without disrupting the cell membrane (Warchol, 2010). HC loss after neomycin treatment in our experiments occurred within a day, and membrane lipid changes were seen by 30 min. In contrast, DT treatment decreased cell membrane integrity and permeability to propidium iodide, resulting in damage to the bundle (reflected in a loss of mechanotransduction channel function); HC loss was slower and was not preceded by reorganization of membrane lipids. Although cell death after neomycin or DTR was not exclusively apoptotic or necrotic, neomycin, here as in previous studies (Forge and Li, 2000; Warchol, 2010), induced a largely apoptotic mechanism of cell death, whereas DT induced a mixture of apoptotic and necrotic mechanisms.

Wnts were upregulated and β-catenin became transcriptionally active after DT-induced damage, but not after neomycin treatment. Inhibition of regeneration by a Wnt inhibitor suggested a correlation of regeneration with Wnt/β-catenin signaling after DT treatment. A number of Wnts (Wnt1, Wnt2, Wnt2b, Wnt4, Wnt5a, Wnt7b, Wnt9a, Wnt9b, Wnt11) were upregulated after DT-induced cell death. In conjunction with the upregula-

tion of Wnts, an increase in an active form of β-catenin in HCs and supporting cells was consistent with a mechanism involving autocrine and paracrine Wnt signaling. Upregulation of Krm2, a receptor for Wnt inhibitor, Dickkopf, may have been part of a negative feedback loop that would decrease Wnt activity, although Krm2 can also act as an activator of Wnt signaling (Hassler et al., 2007) and could thus increase the response. In the neonatal mouse utricle, *ex vivo* ablation of HCs with neomycin activated the Wnt target gene, *Lgr5*, in the striolar region and led to HC regeneration through mitotic and direct differentiation mechanisms (Wang et al., 2015). Upregulation of these genes is consistent with a recent comprehensive study of the expression of Wnt pathway genes in the cochlea (Geng et al., 2016). Many Wnt ligands, Frizzled receptors, and Wnt antagonists, such as Dkk3, Sfrp1, and Sfrp3, were dynamically expressed both prenatally and postnatally. The level of β-catenin in cells of the sensory epithelium could thus be a result of activity of both Wnts and Wnt antagonists acting in complimentary locations.

Apoptotic cells are extruded by the concerted contraction of their neighbors and formation of a ring of actin and myosin in surrounding cells (Rosenblatt et al., 2001). The supporting cells that surround HCs send out extensions and close off the epithelial surface as a part of the damage response (Meiteles and Raphael, 1994; Anttonen et al., 2012). Actin filaments grow into the space left after loss of the stereociliary bundles of the HCs, resulting in an area that is intact, but without HCs. Actin rings at the surface of the epithelium have been postulated to play a role in preventing regeneration in the mouse (Collado et al., 2011). Cadherins inhibited by Wnt signaling can also act as repressors of cell division (Shi et al., 2014). The integrity of the membrane in cells undergoing apoptosis reduces the inflammatory response, and strand breaks in DNA between nucleosomes in apoptotic cell death are less disruptive to the cell than the random cleavage of necrosis (Günther et al., 2013; Galluzzi et al., 2014). Apoptosis induces less inflammation but can release TNF, leading to recruitment of inflammatory cytokines, and, indeed, developmental apoptosis in the nervous system is a requisite step in the subsequent differentiation of neurons (Hyman and Yuan, 2012) and a general developmental mechanism for tissue morphogenesis (Hernández-Martínez and Covarrubias, 2011; Suzanne and Steller, 2013).

Wnts are released by many other tissues in response to damage and, along with inflammatory cytokines and FGFs, form one arm of the damage response (Hassler et al., 2007; Whyte et al., 2012). Wnt signaling is important for the repair of damage in muscle, intestine, and hair follicles in mammals (Ireland et al., 2004; Silva-Vargas et al., 2005; Snyder et al., 2013) and is critical for blastema formation to initiate limb regeneration in amphibians (Kawakami et al., 2006; Yokoyama et al., 2007; Sun and Irvine, 2014). Signaling leading to apoptotic cell death is crucial to the formation of blastemas and regrowth of amphibian limbs. Cell division in the developing blastema occurs in response to cell death. An essential role for Wnt signaling has been established in regeneration in amphibians and lower vertebrates, although the

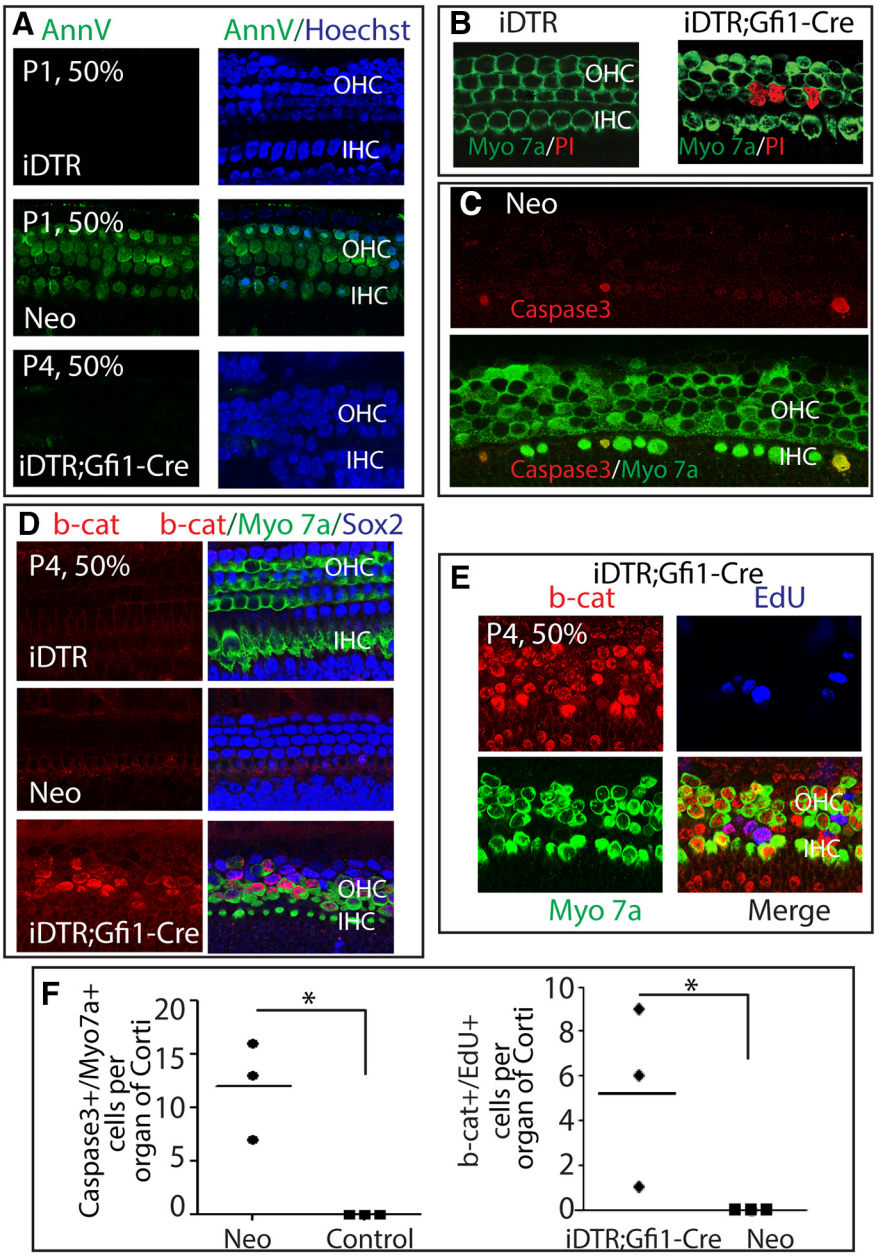

Figure 7. DT-induced HC loss leads to activation of the Wnt pathway. ***A***, Annexin V (AnnV)-positive HCs were observed 30 min after neomycin injection (Neo) into the cochlea at P1. Annexin V-positive cells were not present in the contralateral ear (Control) or in the DT-injected *iDTR;Gfi1-Cre* ear. Hoechst was used to stain the nuclei and locate HCs in the live tissue. ***B***, HCs in *iDTR;Gfi1-Cre* mice, but not control mice, stained with propidium iodide (PI), while supporting cells in control and iDTR ears remained PI negative. ***C***, Cleaved caspase-positive cells were seen in the neomycin-injected ear. ***D***, Transcriptionally active Y498 β-catenin (b-cat) was observed in HCs and supporting cells of a DT-injected *iDTR;Gfi1-Cre* ear, whereas control and neomycin-injected ears did not show accumulation of active β-catenin. ***E***, Active β-catenin was observed in EdU-positive cells in the *iDTR;Gfi1-Cre* ear. ***F***, Caspase-3/myosin VIIa double-positive cells were significantly increased after neomycin treatment. β-catenin/EdU double-positive cells were only observed in *iDTR;Gfi1-Cre* mice after DT treatment. $n = 3$ animals in each group. Error bars indicate SEM. $^*p < 0.05$.

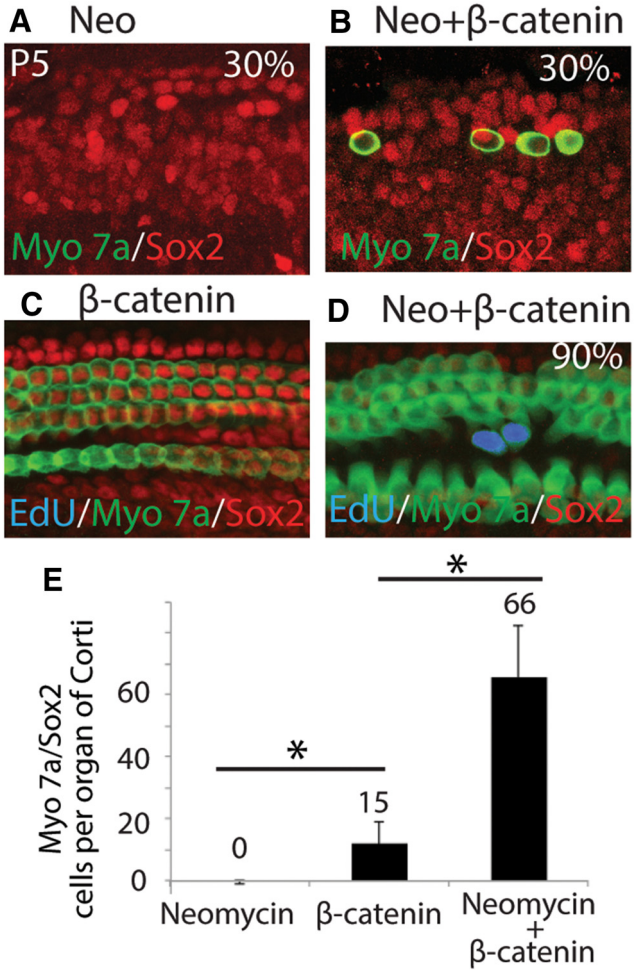

Figure 8. Wnt/β-catenin induced HC regeneration in the neomycin-treated ear. ***A***, A complete loss of HCs was seen in the basal half of the neomycin-treated cochlea. HCs were stained with myosin VIIa and supporting cells with Sox2. ***B***, Constitutive activation of β-catenin in a neomycin-treated ear had myosin VIIa-positive HCs in the region 30% from the base. ***C***, No EdU/myosin VIIa double-positive cells were observed in β-catenin activated cochlea without HC damage by neomycin. ***D***, In the apical region, β-catenin activation induced ectopic myosin VIIa/EdU-positive cells. ***E***, No new HCs were generated in the ears receiving only neomycin. β-catenin activation generated more new HCs in neomycin-injected than intact ears. $n = 4$ animals in each group. $^*p < 0.05$.

exact signal that stimulates the release of Wnts has not been completely characterized. Wnt also organizes the stem cell niche in mammals: Wnt3a released from Paneth cells acts on the adjacent stem cells, constituting a stem cell niche at the base of the crypts in the mammalian gut in which one cell relays a signal to the next (Sato et al., 2011).

The correlation of Wnt activation and regeneration in our studies sheds new light on the role for Wnt, although, as in the studies on limb and fin regeneration, the precise role of cell death in the activation of Wnt signaling remains to be determined. The difference seen between DT- and neomycin-induced HC death was presumably due to the upregulation of Wnt signaling in the DT-treated ear. We cannot exclude the possibility that neomycin upregulates Wnt at later time points than we studied. In the organ of Corti, the Wnt dependence of proliferation was not unexpected, as Wnt upregulation via stabilized β-catenin also leads to proliferation of organ of Corti cells *in vivo* (Chai et al., 2012; Shi et al., 2012, 2013; Kuo et al., 2015). Our previous work also demonstrated that Wnt/β-catenin regulates expression of Atoh1, a key HC gene, leading to HC generation in postnatal organ of Corti (Shi et al., 2010).

In vitro experiments showed detectable regeneration without intervention after treatment of the cochlea dissected from newborn mice with gentamycin (Bramhall et al., 2014). However, supporting cells did not incorporate BrdU and were only generated in significant numbers after inhibition of Notch. A regenerative response to aminoglycoside-induced HC loss *in vitro* could correspond to a higher percentage of necrotic cell death due to the removal of the cochlea from the animal into an artificial and potentially oxidative environment. The lack of a regenerative response to neomycin in vivo could be due to the low Wnt secretion falling below a threshold required for proliferation. Notch inhibition increased proliferation after DT-induced death of HCs, which we believe is due to removing inhibitory effects of Notch on Wnt signaling. Upregulation of Wnt activity can increase Notch signaling and may prevent overexuberant HC proliferation and differentiation, acting as a brake on the regenerating cells. Notch signaling is under the control of Wnt signaling in somitogenesis, through its influence on Dll1 (Galceran et al., 2004; Chesebro et al., 2013; Bone et al., 2014). In an *in vitro* organ of Corti, Notch signaling was maintained in supporting cells, and, indeed, the supporting cells transdifferentiated into HCs when Notch signaling was blocked by inhibition of γ-secretase (Korrapati et al., 2013). In our *in vivo* experiments on Notch inhibition (Mizutari et al., 2013), we found that Notch signaling was upregulated by exposure to a level of noise sufficient to kill HCs and that inhibition of Notch signaling with a γ-secretase inhibitor resulted in differentiation of new HCs from supporting cells even in the adult. Notch inhibition was only effective in increasing the yield of new HCs after damage in other studies when Wnt was active (Bramhall et al., 2014). In the experiments described here, inhibition of Wnt blocked regeneration resulting from damage.

The spontaneous regeneration of HCs in the cochlea of chicks after damage is preceded by upregulation of Wnt signals (Ku et al., 2014), and Wnt signaling increases supporting cell proliferation in newly formed lateral line neuromasts (Ma et al., 2008; Head et al., 2013). Moreover, secretion of Dkk by HCs in the zebrafish, by blocking the effect of Wnt on supporting cell proliferation, imposes a limit on the number of HCs produced, and, thus, Wnt plays an important role in the regeneration of sensory epithelia in lower vertebrates (Wada et al., 2013). Despite the evolutionary loss of regenerative capacity in mammals, some of the same pathways are involved, and chick and fish inner ears may not have fundamentally different responses from the mammal.

Supporting cells and HCs in the organ of Corti exit the cell cycle several days before birth in the mouse and are not replaced after damage in the adult. Although our study did not present direct evidence on supporting cell conversion to HCs, spontaneous conversion of supporting cells to HCs has been demonstrated previously in mouse after damage to the postnatal organ of Corti (Bramhall et al., 2014; Cox et al., 2014). The question of whether potential stem cells in the cochlea are activated by cell death is important because of the role of stem cells in the repair of damage. The regenerative response differed after DT- and neomycin-induced cell death. The regeneration of HCs in the newborn and its correlation with a difference in activation of the Wnt pathway suggests that the ability to regenerate is retained in mammals, and the loss of regenerative capacity as mammals mature may reflect a higher threshold and not an absolute block. A similar threshold must be overcome during limb regeneration in amphibians,

where the ability to regenerate also becomes less pronounced at adult ages and can be restored by Wnt augmentation (Kawakami et al., 2006). Understanding the triggers that initiate regeneration in the newborn mouse model will enable future approaches to regeneration in the cochlea.

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