

In Vivo Proliferative Regeneration of Balance Hair Cells in Newborn Mice

Joseph C. Burns,^{1,2*} Brandon C. Cox,^{4*} Benjamin R. Thiede,¹ Jian Zuo,⁴ and Jeffrey T. Corwin^{1,3}

Departments of ¹Neuroscience, ²Biomedical Engineering, and ³Cell Biology, University of Virginia School of Medicine Charlottesville, Virginia 22908, and ⁴Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

The regeneration of mechanoreceptive hair cells occurs throughout life in non-mammalian vertebrates and allows them to recover from hearing and balance deficits that affect humans and other mammals permanently. The irreversibility of comparable deficits in mammals remains unexplained, but often has been attributed to steep embryonic declines in cellular production. However, recent results suggest that gravity-sensing hair cells in murine utricles may increase in number during neonatal development, raising the possibility that young mice might retain sufficient cellular plasticity for mitotic hair cell regeneration. To test for this we used neomycin to kill hair cells in utricles cultured from mice of different ages and found that proliferation increased tenfold in damaged utricles from the youngest neonates. To kill hair cells *in vivo*, we generated a novel mouse model that uses an inducible, hair cell-specific CreER allele to drive expression of diphtheria toxin fragment A (DTA). In newborns, induction of DTA expression killed hair cells and resulted in significant, mitotic hair cell replacement *in vivo*, which occurred days after the normal cessation of developmental mitoses that produce hair cells. DTA expression induced in 5-d-old mice also caused hair cell loss, but no longer evoked mitotic hair cell replacement. These findings show that regeneration limits arise *in vivo* during the postnatal period when the mammalian balance epithelium's supporting cells differentiate unique cytological characteristics and lose plasticity, and they support the notion that the differentiation of those cells may directly inhibit regeneration or eliminate an essential, but as yet unidentified pool of stem cells.

Introduction

In many non-mammalian vertebrates, continuous proliferation of glial and sustentacular stem cells gives rise to new neurons and sensory cells and is paralleled by life-long regeneration (Zupanc, 2008). Such is the case in fish, amphibians, reptiles, and birds, where mechanoreceptive hair cells are produced throughout life and can be regenerated from supporting cells to restore lost hearing, balance, and lateral line functions (Corwin, 1981, 1985, 1986; Corwin and Cotanche, 1988; Jorgensen and Mathiesen, 1988; Ryals and Rubel, 1988; Popper and Hoxter, 1990; Lanford et al., 1996; Warchol, 2011). In those species, hair cell death leads to robust proliferation of supporting cells, whose progeny give rise to replacement hair cells that become innervated and restore sensory functions within weeks. Also, supporting cells sometimes replace lost hair cells by converting directly into a hair cell phe-

notype without passing through S-phase (Stone and Cotanche, 2007; Collado et al., 2011b; Lin et al., 2011).

Proliferative or “mitotic” hair cell regeneration appears to be the dominant process responsible for the recovery of hearing, balance, and lateral line sensitivity following hair cell losses in many non-mammals. The irreversibility of similar deficits in mammals has been ascribed to sharp, embryonic declines in the cellular production capacity of inner ear sensory epithelia that were measured through pulse labeling (Ruben, 1967). It remains unclear, however, exactly what restricts regeneration in mammalian ears and what relative contributions proliferative and non-proliferative forms of regeneration make to the limited repair processes that have been observed in balance epithelia from humans and other mammals (Forge et al., 1993, 1998; Warchol et al., 1993; Li et al., 1995; Tanyeri et al., 1995; Kuntz and Oesterle, 1998; Ogata et al., 1999; Oesterle et al., 2003; Kawamoto et al., 2009; Lin et al., 2011). In addition, recent estimates based on cell density measures suggest that hair cell numbers increase during neonatal maturation of murine utricles (Kirkegaard and Nyengaard, 2005). These findings point to the possibility that an unrecognized period of neonatal plasticity might allow murine vestibular organs to repair hair cell losses through significant mitotic regeneration.

To investigate this, we used neomycin to kill hair cells in utricles cultured from young mice, and measured large proliferative responses that declined with age. Although substantial numbers of cells were labeled with BrdU in the youngest utricles, none differentiated into new hair cells under our culture conditions. Therefore, to circumvent limitations of *in vitro* experi-

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*J.C.B. and B.C.C. contributed equally to this work.

Correspondence should be addressed to Jeffrey T. Corwin, Department of Neuroscience, University of Virginia Medical School, 409 Lane Road, Charlottesville, VA 22908. E-mail: jtc2k@virginia.edu.

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ments, we generated mice that carried a hair-cell-specific CreER allele that allows inducible expression of diphtheria toxin fragment A (DTA). Inducing DTA expression soon after birth killed hair cells and resulted in significant mitotic hair cell replacement in the utricle *in vivo*, but the response decreased significantly when hair cell death was induced in 5-d-old mice. These findings show that neonatal rodent balance organs possess the machinery for significant mitotic hair cell regeneration *in vivo*, but regeneration becomes impaired or inhibited later during the time when maturing mammalian supporting cells differentiate characteristics not found in their non-mammalian counterparts.

Materials and Methods

Animal models. Animal work was approved by the Animal Care and Use Committees of the University of Virginia and St. Jude Children's Research Hospital. Swiss Webster mice were obtained from Charles River. ROSA26^{DTA} mice (stock #6331) and ROSA26^{eYFP} mice (stock # 6148) were purchased from The Jackson Laboratory. Atoh1-CreERTM mice were provided by S. Baker at St. Jude Children's Research Hospital (Chow et al., 2006). Mice of either sex were injected with tamoxifen (3 mg/40 g, i.p.; Sigma-Aldrich) and 5-ethynyl-2-deoxyuridine (EdU; 10 μg/g, i.p.; Invitrogen) at the ages indicated.

Dissection and culture of utricles. Labyrinths were dissected from temporal bones in ice-cold DMEM/F-12 (Invitrogen), the utricles were isolated, and the roof, otoconia, and nerve were mechanically removed under aseptic conditions. The dissected organs contained the entire sensory epithelium, a small portion of the surrounding nonsensory epithelium, and the underlying connective tissue matrix. For organ culture, dissected utricles were adhered to glass-bottom dishes (Mat-Tek) coated with Cell-Tak (BD Biosciences) as described previously (Meyers and Corwin, 2007) and cultured in DMEM/F12 with 1% FBS (Invitrogen), 3 μg/ml 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich), 0.25 μg/ml Fungizone (Invitrogen), and 10 μg/ml ciprofloxacin (Bayer) at 37°C and 5% CO₂. Utricles were adhered to Cell-Tak to maintain the flat architecture of the sensory epithelium and prevent the tissue from folding. To kill hair cells, 3 mM neomycin sulfate (Sigma-Aldrich) was added to the culture media for 24 h and then washed out with three rinses of media. For preliminary tests of other culture conditions that might affect the capacity for cells to differentiate into hair cells, we cultured limited numbers of utricles on Nucleopore filters (Laine et al., 2010) and cultured some utricles with 0% and 10% FBS.

Quantification of S-phase entry and hair cell numbers. In all applicable experiments, the number of BrdU- or EdU-labeled nuclei was manually counted using the Cell Counter plugin in ImageJ. EdU-positive/myosin VIIA-positive cells were counted separately. To quantify the amount of hair cell death in utricles from Atoh1-CreERTM; ROSA26^{DTA/+} mice, the number of hair cells per 50 μm² was counted at nine different locations spaced along the anterior–posterior axis of the medial, striolar, and lateral regions of each utricle. The same procedure was used to quantify the percentage of eYFP-positive/myosin VIIA-positive hair cells in utricles from Atoh1-CreERTM; ROSA26^{eYFP/+} mice.

Immunocytochemistry. Rabbit anti-myosin VIIA (1:200, Proteus Bio-Sciences) was used to label hair cell somata, and rabbit anti-espina (1:10; generous gift from Dr. James Bartles, Northwestern University, Evanston, IL) was used to label stereocilia. Mouse anti-BrdU (1:50; BD Biosciences) was used to label cells that had incorporated BrdU during S-phase. Rabbit anti-GFP (1:200, Invitrogen) was used to label eYFP.

Vestibular organs were fixed in either fresh 4% paraformaldehyde in PBS for 1 h at room temperature (RT) or in Shandon Glyofixx (Thermo Scientific) overnight at 4°C. After fixation, specimens were washed in PBS then preincubated for 1 h at RT in blocking solution, containing PBS and 0.2% Triton X-100 (PBS-T) as well as 10% NGS (Vector Laboratories). Samples to be labeled with anti-BrdU were incubated with DNase I (0.5 kunitz/μl; Sigma) for 1 h at 37°C before adding the blocking solution. For detection of EdU, utricles were rinsed in PBS after the blocking step, and a copper-catalyzed Click-iT reaction kit was used per the manufacturer's instructions (Invitrogen). Samples were then incubated in the appropriate primary antibodies in PBS-T with 2% NGS overnight, fol-

lowed by 3 rinses in PBS-T and incubation with Alexa-conjugated secondary antibodies (1:200, Invitrogen) and/or phalloidin (5 U/ml, Invitrogen) in PBS-T for 2 h at RT. Utricles were rinsed in PBS 3 times and mounted in SlowFade (Invitrogen). Specimens were imaged using Zeiss LSM 510 and LSM 700 confocal microscopes.

Statistics. For statistical comparisons, GraphPad Prism was used to conduct one-way or two-way ANOVAs followed by a Bonferroni's multiple-comparisons test (α level = 0.05 in all cases). All descriptive statistics are presented as mean \pm SEM.

Results

Damage-induced proliferation declines postnatally in cultured mouse utricles

It has remained unclear whether the limited hair cell regeneration capacity of vestibular organs in adult mammals is the result of an evolutionary loss of essential recovery processes that are found in non-mammals or results from the evolution of mammalian attributes that inhibit regeneration (Burns et al., 2008; Brigande and Heller, 2009). Here, we cultured utricles from P0, P2, P4, and P8 mice in BrdU-containing medium and killed hair cells using a 24 h treatment with 3 mM neomycin (Cunningham et al., 2002) to investigate the hypothesis that mitotic regeneration in adult mammals is restricted by derived characteristics that mammalian ears express during postnatal maturation. By 72 h after neomycin washout, the maculae of damaged P0 utricles contained 10-times more BrdU-positive nuclei than controls (192 ± 19 vs 19 ± 2 ; $p < 0.05$, Two-way ANOVA with Bonferroni's multiple-comparisons test; Fig. 1; $n = 8$), but damage-induced proliferation declined quickly with age (Fig. 1C; $n = 8$). Also, no BrdU-positive/myosin VIIA-positive cells were detected in any of the samples, even when utricles were cultured for 21 d. If hair cells had differentiated from the progeny produced from those damage-induced cell divisions, then BrdU-positive/myosin VIIA-positive cells should have formed. We suspected that the *in vitro* environment we were using provided insufficient support for hair cell differentiation, so we used mouse genetics to circumvent limitations of the culture environment and to directly investigate whether hair cells could be regenerated *in vivo*.

Utricular hair cells can be reproducibly ablated *in vivo* using an inducible mouse model

By crossing Atoh1-CreERTM mice with ROSA26^{DTA/+} mice, we generated offspring in which we could induce hair-cell-specific expression of DTA using the Atoh1 enhancer to drive CreER expression in hair cells (Chow et al., 2006; Weber et al., 2008). Cre-mediated excision of the stop sequence in the ROSA26-loxP-stop-loxP-DTA (ROSA26^{DTA}) allele results in cell-autonomous ablation of Cre-positive cells (Ivanova et al., 2005; Abrahamsen et al., 2008). Utricles from Atoh1-CreERTM; ROSA26^{DTA/+} mice, injected with tamoxifen once each at P0 and P1, exhibited significant, 19% \pm 4%, 41% \pm 6%, and 48% \pm 4% decreases in hair cell density as measured at P2, P5, and P11, respectively ($p < 0.05$; Two-way ANOVA with Bonferroni's multiple-comparisons test; Fig. 2A, C; $n = 4$).

To determine whether the amount of hair cell loss we observed corresponded with the number of hair cells that exhibit Cre activity, we bred Atoh1-CreERTM mice with ROSA26^{eYFP/+} reporter mice and injected them with tamoxifen once a day on P0 and P1. In utricles fixed at P6, we found 50% \pm 5% of myosin VIIA-expressing hair cells also expressed eYFP (Fig. 2B, C; $n = 4$). This level of Cre activity is similar to the percentage decrease in hair cell number we observed in utricles from Atoh1-CreERTM; ROSA26^{DTA/+} mice at P5 and P11, and slightly lower, but still consistent with the 60% recombination reported from Atoh1-

CreERTM; ROSA26^{LacZ/+} mice, where Cre activity was assessed on P6 after tamoxifen injections at P0/P1 (Chow et al., 2006).

In utricles of Atoh1-CreERTM; ROSA26^{DTA/+} mice that were fixed on P2, P5, or P10 following tamoxifen injections on P0/P1, quantification showed that comparable decreases in the spatial density of myosin VIIA-positive hair cells occurred in the medial, striolar, and lateral regions (Fig. 2C, purple bars). The spatial density variations that resulted from DTA-mediated hair cell loss at P5 and P10 resembled spatial variations in the incidence of eYFP-expressing hair cells observed in utricles of Atoh1-CreERTM; ROSA26^{eYFP/+} reporter mice that were fixed at P6 after the same tamoxifen induction regimen (Fig. 2C, green bars).

We injected another group of Atoh1-CreERTM; ROSA26^{eYFP/+} mice with tamoxifen once a day on P4 and P5 and found that 33% ± 4% of their myosin VIIA-positive hair cells also expressed eYFP at P10 (Fig. 2E,F), which suggested that hair cells could be ablated at such older ages. When we gave Atoh1-CreERTM; ROSA26^{DTA/+} mice the same P4/P5 tamoxifen induction regimen and fixed their utricles at P9 we observed a significant 29% ± 5% decrease in utricular hair cell density compared with controls ($p < 0.05$; Two-way ANOVA with Bonferroni's multiple-comparisons test; Fig. 2D,F; $n = 4$). Thus, hair cells in the utricles of mice can be reproducibly ablated *in vivo* using inducible expression of DTA both early and late during the first postnatal week.

In all but one utricle from the Atoh1-CreERTM; ROSA26^{eYFP/+} mice that were induced with tamoxifen at P0/P1 or P4/P5, we detected 1–5 eYFP-positive/myosin VIIA-negative cells that resembled supporting cells and extended down to the basal lamina. The intensity of eYFP in these cells appeared lower than eYFP in neighboring hair cells (data not shown). Since hair cells are being added to the sensory epithelium at these ages (Kirkegaard and Nyengaard, 2005), we suspect that the eYFP-expressing cells may be nascent hair cells in the earliest stages of differentiation. The presence of eYFP-positive/myosin VIIA-negative cells suggests that our method for inducing cell death may ablate some cells that are not fully differentiated hair cells. However, the low numbers of eYFP-positive/myosin VIIA-negative cells suggests that the effects of non-hair-cell-specific cell loss would be minimal compared with the effects of killing over a thousand differentiated, myosin VIIA-positive hair cells.

DTA-induced hair cell death evokes a significant proliferative response in the sensory epithelium of newborn mouse utricles

To determine whether killing hair cells in the utricles of newborn Atoh1-CreERTM; ROSA26^{DTA/+} mice would lead to a damage-induced proliferative response *in vivo*, we injected mice with tamoxifen at P0 and P1, and followed that treatment with a single

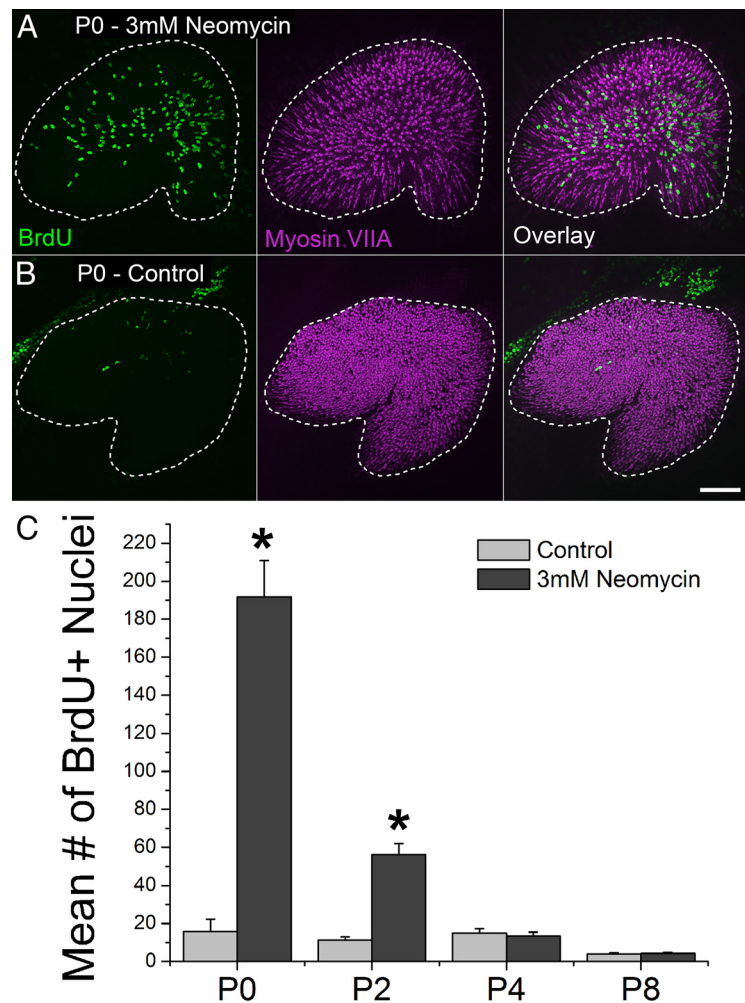


Figure 1. Cells in the utricles of newborn mice respond to hair cell death with robust proliferation *in vitro*. **A, B**, Confocal images of utricles from P0 mice that were treated with 3 mM neomycin or vehicle control for 24 h and cultured for 72 h thereafter (BrdU, green, was included in the media throughout). Hair cells are labeled with an antibody to myosin VIIA (purple). A dashed line marks the macula's edge. Scale bar, 100 μ m. **C**, Graph of BrdU-positive nuclei in the macula of neomycin-damaged and control utricles from mice of different ages, $n = 8$ per age. Asterisks indicate a significant difference between treatment and controls ($p < 0.05$).

injection of EdU given at either P2, P3, P4, or P8. Utricles from these mice were all fixed 24 h after the EdU injection and processed to reveal EdU and myosin VIIA labeling. Utricular maculae in mice that received EdU at P2, P3, and P4 exhibited 1.8-fold, 5.8-fold, and 10.3-fold more EdU-positive nuclei than littermate control mice lacking Cre or DTA ($p < 0.05$, Two-way ANOVA with Bonferroni's multiple-comparisons test; Fig. 3; $n = 4$). Since cell proliferation ceases by P2 in the undamaged mouse utricular sensory epithelia, as shown by the data in Ruben's (1967) investigation of terminal mitoses, the significant increases in proliferation that occurred here in Atoh1-CreERTM; ROSA26^{DTA/+} mice, which were up to 4 d old, appear to be responses evoked by hair cell death. However, EdU labeling did not differ significantly between the groups of experimental and control mice that received EdU at P8 ($p = 0.2426$, Two-way ANOVA with Bonferroni's multiple-comparisons test; Fig. 3C; $n = 4$), leading to the conclusion that the damage-induced proliferative responses subsided 4–8 d after hair cell death was induced through the tamoxifen-mediated expression of DTA on P0/P1.

To determine whether a proliferative response could be extended later in life by inducing the death of hair cells at an older age, we gave mice tamoxifen injections at P4/P5 and a single EdU

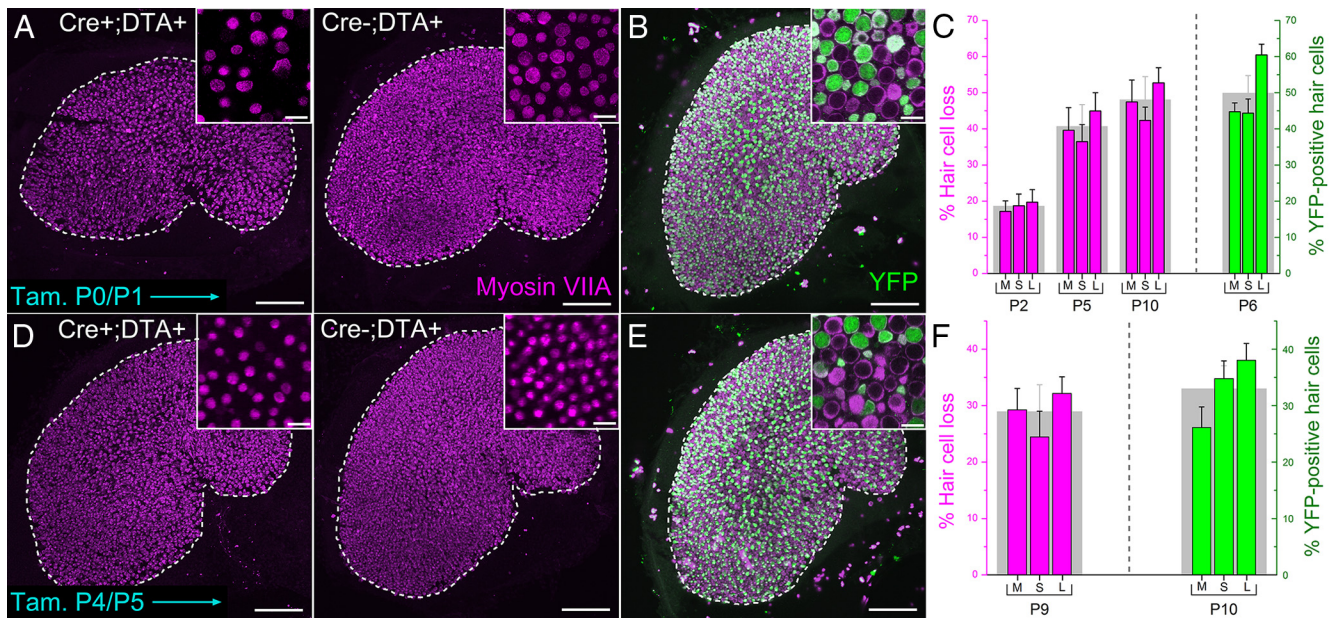


Figure 2. Hair cells in neonates can be ablated *in vivo* using an inducible mouse model. **A**, Confocal images of utricles from an *Atoh1-CreERTm; ROSA26^{DTA/+}* mouse and a *ROSA26^{DTA/+}* control mouse injected with tamoxifen at P0 and P1 and killed at P10. Hair cells are labeled with antibodies to myosin VIIA (purple). Scale bar, 100 μ m. Insets show zoomed regions of the sensory epithelium. Scale bar, 10 μ m. **B**, Confocal image of a utricle from an *Atoh1-CreERTm; ROSA26^{eYFP/+}* mouse induced with tamoxifen at P0/P1 and killed at P6. eYFP is shown in green. Scale bar, 100 μ m. Inset shows zoomed region of the sensory epithelium. Scale bar, 10 μ m. **C**, Graph of the percentage difference in the density of hair cells between utricles from *Atoh1-CreERTm; ROSA26^{DTA/+}* mice and control mice that were induced with tamoxifen at P0/P1 and killed at the ages indicated (bars in purple), $n = 4$. M, medial; S, striolar; L, lateral regions of the utricle, respectively. The gray bar behind the colored columns shows the average for the entire utricle. Also shown is the percentage of eYFP-positive hair cells in utricles from *Atoh1-CreERTm; ROSA26^{eYFP/+}* mice induced with tamoxifen at P0/P1 and killed at P6 (bars in green), $n = 4$. Data expressed as mean \pm SEM. **D–F**, Same as in **A–C**, except that mice were induced with tamoxifen at P4/P5. Utricles in **D** and **E** were fixed from mice killed at P9 and P10, respectively. Data represented by green bars in **F** were taken from utricles fixed at P10. Note different scales for graphs in **C** and **F**.

injection at P6, P7, or P8. In utricles fixed 24 h after the injection of EdU, the mean number of EdU-positive cells detected in the utricular maculae of the *Atoh1-CreERTm; ROSA26^{DTA/+}* mice (P7 = 2 ± 1 , P8 = 5 ± 2 , and P9 = 5 ± 2) and the maculae from the littermate controls lacking Cre or DTA (P7 = 3 ± 1 , P8 = 2 ± 1 , and P9 = 3 ± 1) was low at all ages, and sample differences between the DTA mouse model and controls did not reach significance ($p = 0.3319$, Two-way ANOVA; $n = 3–4$). Thus, the proliferation that is evoked by hair cell damage in mouse utricles *in vivo* declines with postnatal age along an age-related time course that is similar to progressive declines in supporting cell proliferation that have been measured *in vitro* (Gu et al., 1997, 2007; Hume et al., 2003; Davies et al., 2007; Meyers and Corwin, 2007; Burns et al., 2008; Lu and Corwin, 2008).

Mitotic hair cell regeneration occurs in newborn mouse utricles *in vivo*

In utricles from the P0/P1 tamoxifen-induced *Atoh1-CreERTm; ROSA26^{DTA/+}* mice that were fixed between P3 and P9, just 24 h after they had each received an injection of EdU, none of the EdU-positive cells had become myosin VIIA-positive hair cells. However, utricles from other groups of P0/P1 tamoxifen-induced *Atoh1-CreERTm; ROSA26^{DTA/+}* mice that each received a single EdU injection on P4 and were fixed at either P10 or P15 contained substantial fractions of EdU-positive cells that were positive for myosin VIIA (P10 = $23\% \pm 0.8\%$, P15 = $22\% \pm 2\%$; Fig. 4C–F). The somata of these cells had shapes characteristic of hair cells, and hair bundles that labeled with an antibody to espin protruding from their apical surfaces (Fig. 4C–E). Many of these EdU-positive hair cells appeared in close proximity to a myosin VIIA-negative cell that contained an EdU-positive nucleus located in the basal nuclear layer that is comprised of supporting

cell nuclei (Fig. 4D, E, arrowheads). In contrast, no EdU-positive/myosin VIIA-positive cells were detected in utricles from littermate Cre-negative or DTA-negative control mice that were fixed on P10 or P15 after receiving EdU on P4. These results reveal that the vestibular organs of neonatal mice must contain all the elements necessary for responding to hair cell loss with cell proliferation that leads to mitotic hair cell regeneration *in vivo*.

Discussion

A transient regenerative potential in murine balance organs

In showing that newborn mice are capable of significant mitotic regeneration of vestibular hair cells *in vivo*, these results challenge the notion that mammals lack molecular circuitry that is essential to mitotic hair cell regeneration and expressed only in non-mammalian ears. Our results also show that changes in the regenerative capabilities of the vestibular sensory epithelia occur early in the postnatal maturation of rodents, since hair cell death induced by giving tamoxifen to 5-d-old *Atoh1-CreERTm; ROSA26^{DTA/+}* mice resulted in only low numbers of EdU-positive utricular cells, while utricles of mice that were given tamoxifen at P0/P1, followed by a single injection of EdU at P4, and fixation on the next day, contained nearly twice as many proliferating cells as had been reported previously after days to weeks of continuous BrdU labeling of ototoxin-damaged vestibular organs in adult rodents (Tanyeri et al., 1995; Li and Forge, 1997; Kuntz and Oesterle, 1998; Ogata et al., 1999; Oesterle et al., 2003; Kawamoto et al., 2009). While our data fail to add support for the notion that mammalian ears lack essential molecular circuits found in non-mammalian ears, it remains unclear exactly how maturational changes inhibit, disable, or shut down *in vivo* hair cell regeneration in young mammals.

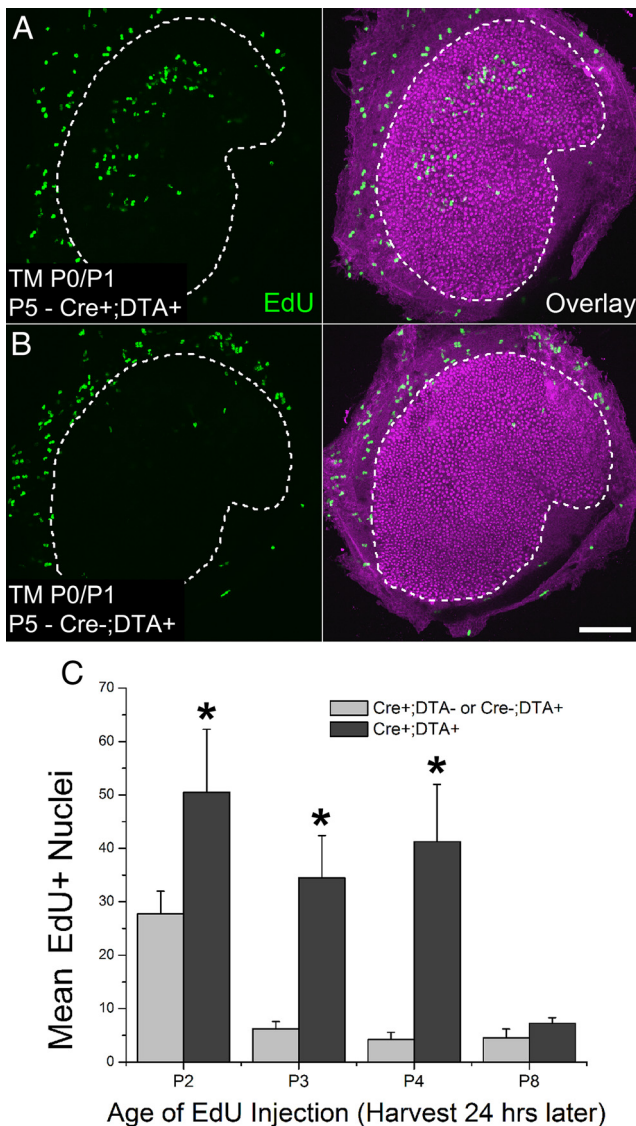


Figure 3. Cells in the utricles of newborn mice respond to hair cell death with robust proliferation *in vivo*. **A, B**, Confocal images of utricles from an Atoh1-CreERTM; ROSA26^{DTA/+} mouse and a ROSA26^{DTA/+} control mouse induced with tamoxifen (TM) at P0/P1. EdU (green) was injected once at P4 and utricles were harvested 24 h later. Hair cells are labeled with an antibody to myosin VIIA (purple). Scale bar, 100 μ m. **C**, Graph of the number of EdU-positive nuclei in maculae of Atoh1-CreERTM; ROSA26^{DTA/+} or control mice of different ages that were induced with tamoxifen at P0/P1 and received EdU 24 h before utricle fixation, $n = 4$ per age. Data expressed as mean \pm SEM. Asterisks indicate significant difference between treatment and controls ($p < 0.05$).

What drives the neonatal decline in mitotic regeneration?

The age-related declines in the early and robust proliferation responses observed here in neomycin-treated utricle cultures and *in vivo* are reminiscent of responses observed in vestibular epithelia that were delaminated from rodent utricles and cultured with Glial Growth Factor 2 (rhGGF2), insulin, or serum. Over 40% of the cells entered S-phase in 72 h when such epithelia were cultured from newborn rodents, but little or no labeling occurred in 72 h in such epithelia from 2- and 3-week-old rodents. However, when adult epithelia were allowed to spread by culturing for 1 week or longer, proliferation increased 20-fold with rhGGF2 over control cultures, even though the overall incidence of proliferating cells remained low (Gu et al., 1996, 1997, 2007; Mont-

couquiou and Corwin, 2001a,b; Davies et al., 2007; Lu and Corwin, 2008).

Since rhGGF2 and insulin signal through different receptors and intracellular pathways, and since rhGGF2 is a ligand for ErbB receptors that appear to be expressed continuously, the decline in proliferative response does not appear to be due to receptor downregulation (Zheng et al., 1999; Hume et al., 2003; Gu et al., 2007). Instead, the decline in proliferative response mirrors an age-related decline in the propensity for rodent supporting cells to change from columnar to spread shapes both in cultured sheets and vestibular epithelia healing *in situ* (Davies et al., 2007; Meyers and Corwin, 2007). Contrasting with this, supporting cells in utricular epithelia from adult birds continue to exhibit rapid spreading and high rates of proliferation that are undiminished from those of hatchlings (Burns et al., 2008; Collado et al., 2011a).

For both humans and mice, the decrease in spreading could stem from postnatal thickening of F-actin belts that bracket the apical junctions between neighboring vestibular supporting cells (Meyers and Corwin, 2007; Burns et al., 2008). In mice, those belts grow 13-fold thicker and come to occupy 89% of the adult supporting cell's area at the level of the adherens junction. There is also a strong inverse correlation ($r = -0.98$) between the belt thickening that appears unique to these cells and proliferative responses that occur in damaged murine balance epithelia at different postnatal ages. In contrast, corresponding perijunctional F-actin belts in the supporting cells of chicken utricles, which exhibit lifelong regeneration of hair cells, remain thin even in adulthood (Burns et al., 2008).

Differences between mammals and the birds, sharks, bony fish, and amphibians that all exhibit rapid mitotic hair cell regeneration also extend to the cadherins at intercellular junctions of supporting cells (Hackett et al., 2002; Warchol, 2007; Burns et al., 2008; Collado et al., 2011b; J. Burns, E. Oliver, and J.T. Corwin, unpublished observations). Chicken vestibular epithelia express little or no E-cadherin, while vestibular supporting cells in mammals express E-cadherin strongly, and at levels that increase six-fold during postnatal maturation. Such correlations led to the hypothesis that the postnatal development of the junctional specializations of mammalian supporting cells contributes to the striking differences between the hair cell regeneration capacities of mammals and non-mammals, and to the development of postnatal limits to the regeneration responses that occur in mammalian ears, but the potential for causality and a defined mechanism remain to be explored (Burns et al., 2008).

Cell cycle regulatory proteins that are expressed in many tissues, such as the retinoblastoma protein (pRb) and the cyclin-dependent kinase inhibitors, p27^{Kip1}, p21^{Cip1}, and p19^{INK4d}, have established influences on the proliferation and postmitotic state of cells during the embryonic development of the mammalian cochlea, so it seems likely that such proteins may have roles in limiting vestibular regeneration (Chen and Segil, 1999; Löwenheim et al., 1999; Mantela et al., 2005; Sage et al., 2005; Weber et al., 2008; Groves, 2010; Laine et al., 2010; Yu et al., 2010; Huang et al., 2011). Although murine vestibular epithelia exhibit weak expression of p27^{Kip1}, cyclin D1 is expressed in numerous vestibular supporting cell nuclei in neonatal mice and in considerably fewer cells within the vestibular epithelia of older mice (Laine et al., 2010).

Recently, when Lopenon et al. (2011) used an adenovirus vector to overexpress cyclin D1 in supporting cells of utricles cultured from adult (P50) mice to test whether its decreased expression limits proliferation in adults, they observed that a

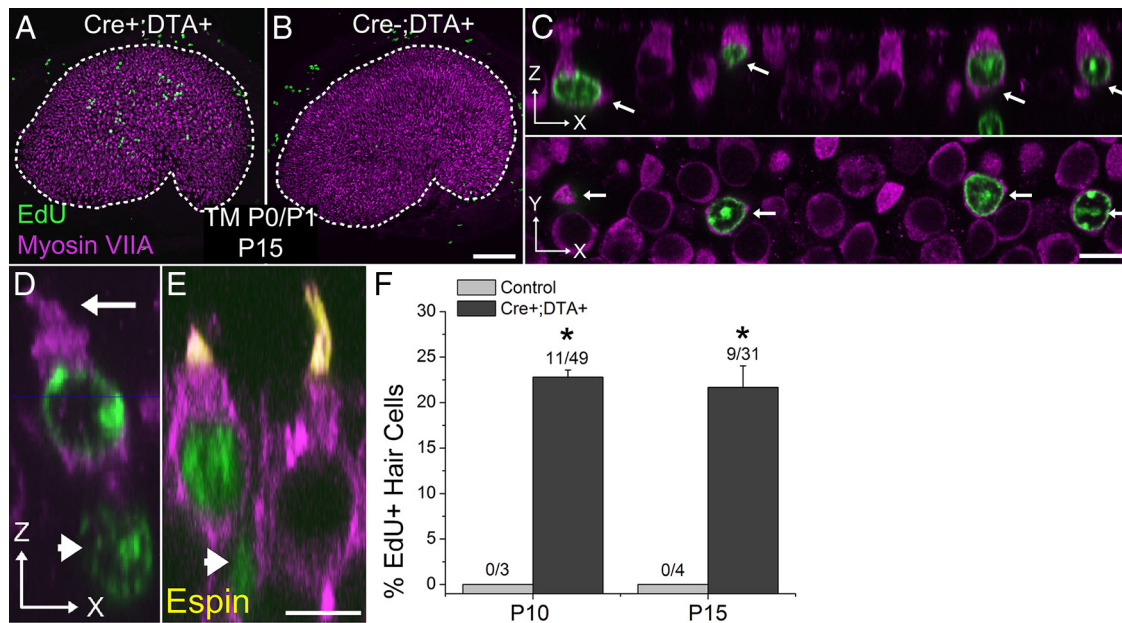


Figure 4. Damage-induced proliferation gives rise to new hair cells *in vivo*. **A, B**, Confocal image of a utricle from a P15 *Atoh1-CreERTM*; *ROSA26^{DTA/+}* mouse and a control mouse lacking Cre that received tamoxifen (TM) at P0/P1 and EdU (green) at P4. Hair cells are labeled with an antibody to myosin VIIA (purple). Scale bar, 100 μ m. **C**, Confocal images of a zoomed region in **A**. Top (*x-z* view), Orthogonal view along the long axis of the hair cells. Arrows indicate four EdU-positive/myosin VIIA-positive hair cells. Bottom (*x-y* view), Cross-sectional view taken at the level of the hair cell nuclei. Scale bar, 10 μ m. **D, E**, High-resolution confocal images of regenerated hair cells in utricles from P15 *Atoh1-CreERTM*; *ROSA26^{DTA/+}* mice that received tamoxifen at P0/P1 and EdU at P4. Image is parallel to the long axis of the hair cells (*x-z* view). Arrow marks a hair bundle, and arrowheads mark EdU-positive hair cell/supporting cell pairs. Regenerated hair cells have shorter hair bundles than preexisting hair cells, as shown by labeling with an espin antibody (**D**). Scale bar, 10 μ m. **F**, Graph of the percentage of EdU-positive cells that were also positive for myosin VIIA in utricles from P10 and P15 *Atoh1-CreERTM*; *ROSA26^{DTA/+}* or control mice that were injected once with EdU at P4 (tamoxifen given at P0/P1), $n = 4$. In the fractions above the bars, the mean number of EdU-positive/myosin VIIA-positive cells is shown in the numerator, and the mean number of EdU-positive cells is shown in the denominator. Data in graphs are expressed as mean \pm SEM. Asterisks indicate significant difference between treatment and controls ($p < 0.05$).

large percentage of the transduced supporting cells passed through the G₁-S restriction point, as shown by BrdU incorporation and Ki-67 immunostaining. However, the number of cells that progressed from S-phase to mitosis was equivalent to just 0.6% of the Ki-67+ cells. To explore age dependence, they over-expressed cyclin D1 in utricles cultured from P9 neonates, finding a slight, but still significant increase (to 2.5% of the amount of Ki-67+ cells) in the cells that progressed through mitosis. Such results are consistent with the potential existence of an intrinsic barrier to cell cycle progression (Loponen et al., 2011). The apparent age dependence of such a barrier also is consistent with the sharp *in vivo* decline in proliferation observed here in the DTA mouse model when tamoxifen injections were given at P4/P5. Postnatal reinforcement of intercellular junctions and cytoskeletons of mammalian supporting cells might contribute to such an intrinsic barrier by restricting dedifferentiation and cell cycle progression (Burns et al., 2008), but it remains to be determined whether those cytological changes have such effects.

What cells give rise to the regenerated hair cells in mice?

During the proliferative regeneration observed here in mice and the well established regeneration that occurs in non-mammals, resident cells divide and produce progeny that can become hair cells and supporting cells, but it is yet to be established whether those progeny arise from undifferentiated reserve stem cells or from more mature supporting cells that dedifferentiate and become multipotent progenitors. Both may occur and are consistent with results obtained when murine vestibular organs are dissociated and placed in suspension culture. Such cultures contain yet-to-be-identified stem cells that can be serially passaged and expanded before giving rise to hair cells, and in addition,

endodermal and mesodermal cell types (Li et al., 2003; Oshima et al., 2007). Similar to the observations from more intact cultures of vestibular epithelia, the yields of dissociation-derived stem cells decrease with increasing rodent age, but it is not yet known whether the decrease occurs through the gradual death or because of the differentiation of cells within the stem cell pool (Ronaghi et al., 2012).

The results obtained in cultures here contrast with the mitotic replacement of hair cells that occurs when vestibular organs from frogs and chickens are treated with aminoglycoside antibiotics and maintained in culture (Baird et al., 1996; Matsui et al., 2000). BrdU labeled 100–200 or more cells in the sensory epithelium of each newborn mouse utricle that we cultured after neomycin killed hair cells, but the progeny of those cells did not differentiate into hair cells under the various conditions we tested. Yet, it seems likely that conditions conducive to hair cell differentiation could be identified through parametric testing, since methods have been described for guiding the progeny of murine embryonic stem cells and induced pluripotent cells to differentiate as hair cells (Oshima et al., 2010).

The results here are consistent with the hypothesis that rapid neonatal maturation of uniquely specialized intercellular junctions contributes to restricting the plasticity that immature mammalian supporting cells appear to exhibit, since those junctions change markedly between P0 and P4 (Burns et al., 2008; Collado et al., 2011a); but causality has not been established. This and alternative hypotheses now may be more readily tested, since the findings here show that when preexisting hair cells are killed at an early stage of neonatal life, mice can mitotically regenerate significant numbers of hair cells *in vivo*. Results here also highlight the potential usefulness of the DTA mouse model for investigating

how limits to regeneration in more mature mammalian ears eventually may be overcome.

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