

The Cyclin Dependent Kinase Inhibitor p27^{Kip1} Maintains Terminal Differentiation in the Mouse Organ of Corti

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Abstract: *Background:* In mammals, sensory hair cells and supporting cells that comprise the auditory epithelium (i.e. organ of Corti) lose the ability to proliferate or regenerate after embryogenesis and are considered terminally differentiated. Previously, it was demonstrated that deletion of p27^{Kip1}, a cyclin dependent kinase inhibitor, extends the period of cellular proliferation beyond embryogenesis in the organ of Corti, resulting in supernumerary supporting cells and hair cells.

Methodology/Principle Findings: We now report that p27 deletion results in an increased number of inner and outer hair cells at one month of age. These hair cells display normal phenotypes including uptake of AM1-43, a mechanotransduction dye. Outer Hair Cells (OHCs) in the p27^{-/-} cochlea appropriately express Prestin, the cellular protein essential for OHC electromotility. This has not been observed in new hair cells induced by deletion of Rb or over expression of Atoh1.

Ototoxic antibiotics (aminoglycosides) can cause hair cell loss resulting in hearing loss. In p27^{Kip1} ^{+/-} and ^{-/-} mice, aminoglycosides induce de novo supporting cell proliferation and hair cell regeneration. Thus, in addition to mediating cell cycle withdrawal during embryogenesis, p27 blocks cycle re-entry after embryogenesis and maintains the terminally differentiated state in the organ of Corti. In p27^{Kip1} ^{+/-} and ^{-/-} mice, Cyclin E expression was observed in supporting cells on postnatal day 0, increased in expression by postnatal day 7 and was maintained into adulthood. Following p27 inhibition, Cyclin E expression in supporting cells is presumed to drive cell cycle re-entry.

Conclusions/Significance: We propose that CyclinE is the dominant cyclin in the supporting cells and release from p27 inhibition allows supporting cell to proliferation and hair cell regeneration.

Keywords: Hair cell, terminal differentiation, p27^{Kip1}, regeneration.

INTRODUCTION

Terminal differentiation is a process whereby cells permanently withdraw from the cell cycle and develop highly specialized characteristics. In mammals, examples of terminally differentiated cells include central nervous system neurons, cardiac and skeletal myocytes, and optic and inner ear sensory cells. Several reports indicate a capacity for the regeneration of some of these cell types from less differentiated cell types. For example, in the ependymal layer and subventricular zone of the brain, populations of “precursor cells” exhibit ongoing proliferation *in vivo* [1]. Some of these proliferating cells are capable of differentiating into glia or neurons as they migrate into regions of the forebrain [2-4] or *in vitro* [5]. This phenomenon can be up-regulated or induced in response to a cytotoxic injury to surrounding areas including the neocortex [6, 7]. In the ciliary margin of the eye, a region directly adjacent to the neural retina and retinal pigmented epithelium lay retinal “stem cells” that retain the capacity to proliferate and differentiate into rod photoreceptors, bipolar neurons and Mueller glia *in vitro* [8].

However, in all of these examples, terminally differentiated cells are regenerated through the proliferation and migration of non-terminally differentiated cells. The molecular signals that prevent terminally differentiated cells from re-entering the cell cycle, proliferating, and regenerating, remain largely unknown.

The auditory sensory epithelium of the mammalian cochlea (i.e. organ of Corti) is a highly specialized, terminally differentiated structure. The organ of Corti is composed of two classes of cells: hair cells that mechanotransduce acoustic energy, and supporting cells of which there are several subclasses. In mammals, hair cells are arranged into one row of inner hair cells and three rows of outer hair cells. Supporting cells provide structural integrity to the mechanics of the system and mediate fluid and electrolyte balance that is critical to auditory function. Supporting cells are classified into five morphological subtypes. Hair cells and supporting cells in the postnatal or adult mammalian cochlea do not proliferate, and they are not regenerated following injury or death [9, 10]. Thus, sensorineural hearing loss, whether from acoustic or ototoxic trauma is considered permanent in mammals.

Because cellular proliferation depends on the enzymatic activity of cyclin complexes with cyclin dependent kinases (CDKs), negative regulators of cyclin/CDK activity may

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prevent cell cycle re-entry in quiescent or terminally differentiated cells. Inhibitors of CDK function include the Ink4 proteins, including the tumor suppressor p16^{Ink4a}, which disrupt binding of the D-type cyclins to CDK4 and CDK6. p27 is a member of a second family of CDK inhibitors, the Cip/Kip family which includes p21^{Cip1/Waf1} and p57^{Kip2}. These proteins are capable of binding multiple G1 phase cyclin-CDK complexes [11] and induce a G1-phase cell cycle arrest when over expressed in cells *in vitro* [12, 13]. In contrast, targeted deletion of one or both copies of p27 leads to mice with increased rates of postnatal growth and multi-organ hyperplasia *in vivo* [14-16].

Previous work demonstrated that p27 expression in the mouse organ of Corti starts between embryonic day 12 to 14 (E12 – E14) [17], at the time when auditory epithelial cells undergo their final or “terminal” mitosis [18]. By E16, expression of p27 protein is localized to the supporting cell population and this specific pattern is maintained into adulthood [17, 19]. After E16, hair cells and supporting cells begin to differentiate, but it is not until postnatal day 8 that hair cells begin to function and hearing onset begins. In contrast, supporting cell proliferation and their subsequent differentiation into hair cells occurs after E16 in p27^{-/-} mice [17], and continues into adulthood, although at reduced levels [19]. These observations indicate that p27 is required for cell cycle withdrawal in the developing organ of Corti during embryogenesis, and that p27 is not essential for the subsequent differentiation of either the hair cell or supporting cell phenotype.

In addition, several *in vitro* studies support the hypothesis that p27 functions as part of the developmental clock which is responsible for cell cycle withdrawal during embryogenesis. p27^{-/-} oligodendrocytes, which are non-terminally differentiated glial cells of the central nervous system, are capable of one to two more rounds of cell division in culture than their wildtype counterparts [20-22]. Another hypothesis is that p27 is required to maintain quiescence in the post-mitotic auditory supporting cells. Although not mutually exclusive this second hypothesis predicts that loss of p27 in these post-mitotic cells would induce cell cycle re-entry. In either situation, cytotoxic stimuli may increase the ongoing level of supporting cell proliferation in p27^{-/-} cochlea. This induction has been observed in supporting cells dissociated from p27^{-/-} cochlea and placed into culture [23] or by targeting p27 using RNAi in neonatal cochlear cultures [24].

RESULTS

To determine the effect of ongoing hair cell production and hair cell loss in p27^{-/-} cochlea, we quantified hair cell density in the organs of Corti of p27^{-/-}, +/- and +/+ mice at P26 (Table 1). p27^{-/-} cochlea had an average increase of 23% ($P < .001$) in the number of inner hair cells (IHCs) when compared with the number of IHCs in p27^{+/-} and +/+ age-matched littermates. In one region, 180 degrees from the apex, p27^{-/-} cochlea had a 13.3% increase ($P < .001$) in the number of OHCs when compared with the same region in p27^{+/+} (45.9 vs 40.5 outer hair cells (OHCs)/100 μ m). However, this difference, was not significant when the number of OHCs was averaged over all regions of the p27^{-/-} cochlea and compared to the number of OHCs observed in

p27^{+/+} cochlea. We then determined whether active cell death was occurring using an *in situ* TUNEL labeling method to identify cells with fragmented DNA. In all cochleae examined, no TUNEL-positive cells were observed in the organ of Corti, although TUNEL-positive cells were observed in regions outside the organ of Corti. For scoring purposes, the apical 180 degrees of the cochlea was quantified for the numbers of TUNEL positive cells. On average, p27^{+/+} cochlea had 7.7 apoptotic cells per cochlea ($n = 6$, SE = 6) while p27^{-/-} cochlea had 8.3 apoptotic cells per cochlea ($n = 6$, SE = 4). In neither case were TUNEL labeled cells co-labeled with the hair cell marker MyoVIIa. The finding that cochlea from 4 week old p27^{-/-} mice do not exhibit an increase in TUNEL labeling relative to their age matched p27^{+/+} littermates, suggests that there no significant difference in hair cell death at this time point.

Table 1. p27^{-/-} Cochlea have Greater Densities of Hair Cells

p27 ^{Kip1}	Location	Cochlea (n)	IHC	OHC
+/+	90	6	13.0 + 0.0	42.8 + 2.5
+/-	90	16	13.1 + 0.7	44.1 + 2.9
-/-	90	11	16.0 + 1.9*	43.5 + 2.3
+/+	180	6	13.3 + 0.8	40.5 + 5.2
+/-	180	16	13.2 + 0.8	43.1 + 2.2
-/-	180	11	17.0 + 1.3*	45.9 + 3.3*
+/+	360	6	13.7 + 0.8	41.7 + 5.1
+/-	360	16	13.4 + 0.5	43.1 + 2.8
-/-	360	11	16.2 + 1.8*	41.0 + 4.6

* $P < 0.001$ by ANOVA test. Density of inner and outer hair cell regions (IHC and OHC, respectively) in p27 littermates at P26. Cell counts from a 100 μ m length of sensory epithelium at three different locations along organ of Corti. Location is listed in degrees from the apical tip of the cochlea. Comparisons were between p27^{+/+}, +/- and -/- within the same location.

The mechanotransduction ability of auditory hair cells in one month old p27Kip1^{-/-} and p27Kip1^{+/+} mice was examined using AM1-43, a fixation stable mechanotransduction dye analogous to FM1-43 [25]. Results indicate that IHCs and OHCs from one month old p27Kip1^{-/-} mice display the same ability to uptake AM1-43 as p27Kip1^{+/+} littermates when evaluated in whole mount (Fig. 1A, B, E, F) and cross section (Fig. 2A, B, E, F). The expression of Prestin (the OHC motor protein responsible for electromotility and cochlear amplification) was examined using a Prestin antibody under immunofluorescence. Whole mount (Fig. 1C-F) and cross sectional (Fig. 2C-F) analyses reveal equivalent Prestin staining in p27Kip1^{-/-} mice when compared to p27Kip1^{+/+} littermates. Furthermore, supernumary OHCs exhibit the ability to uptake AM1-43 and express Prestin along their basolateral membrane (Figs. 1F, 2F). IHCs in both p27Kip1^{-/-} and +/+ littermates, did not exhibit Prestin immunostaining (Figs. 1E, F, 2E, F).

We then investigated whether cytotoxic injury to the organ of Corti could partially relieve the constraint to cell cycle re-entry when administered *in vivo* to p27 knockout

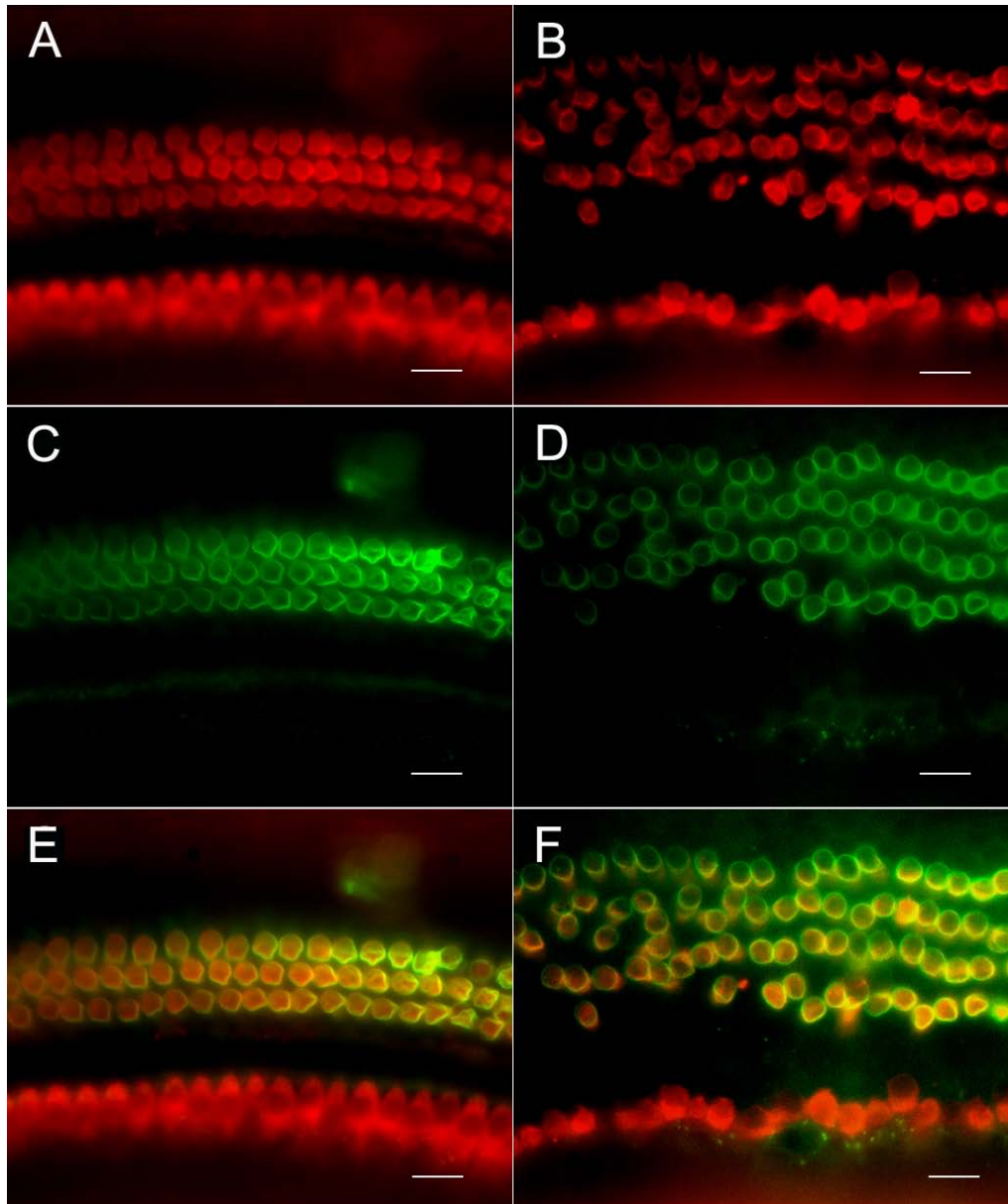


Fig. (1). Mechanotransduction and cell motility in cochlea from p27 $+/+$ and p27 $-/-$ mice. Whole mount mouse cochlea showing normal cellular uptake of the mechanotransduction indicating dye AM1-43 (Red) for (A) p27 $+/+$ and (B) p27 $-/-$ genotypes in both inner and outer hair cells. Immunostaining for the outer hair cell motor protein Prestin (Green) shows normal staining pattern in Outer Hair Cells for (C) p27 $+/+$ and (D) p27 $-/-$ genotypes. Merged images for AM1-43 uptake (Red) and Prestin immunostaining (Green) shows a normal staining pattern for (E) p27 $+/+$ and (F) p27 $-/-$ genotypes. Notably, the super-numary outer hair cells in the p27 $-/-$ genotype have normal uptake of the mechanotransduction dye AM1-43 and a normal pattern of Prestin expression (B, D, F). Scale bar = 25 μ m.

mice. Amikacin sulfate, an aminoglycoside antibiotic that induces hair cell injury and death, was administered to p27 $-/-$, p27 $+/-$ and p27 $+/+$ mice from postnatal days 7 to 12 (P7 – P12). BrdU was injected on P10-P12. Immunocytochemical staining for the presence of BrdU positive nuclei of cells resident within the cochlea was performed at early time points (0 and 2 days post cessation of injections) in order to determine the number and identity of proliferating cells (Fig. 3, Table 2). Because unincorporated BrdU has a short half live *in vivo* (i.e. 2-4 h) analysis of cochleae at later time points allowed us to determine the fate of these newly

proliferating cells. Cochleae were compared from mice that received amikacin/BrdU with mice that received BrdU only, at either 4 h, 2 days, 7 days or 14 days following the last BrdU injection (P12, P14, P19 and P26, respectively n= 4-12 cochlea at each time point) (Fig. 4). In p27 $-/-$ mice injected with amikacin/BrdU, the level of BrdU-positive cells increased by 26.1 % at P12 ($P > 0.05$), increased by 17.3% ($P > .05$) at P14, increased by 32.7% ($P < .001$) at P19 and increased by 67.2% ($P < .001$) at P26 when compared to p27 $-/-$ mice injected with BrdU only. These data indicate that ototoxic injury to the organ of Corti further stimulates supporting cell proliferation in dependent of p27.

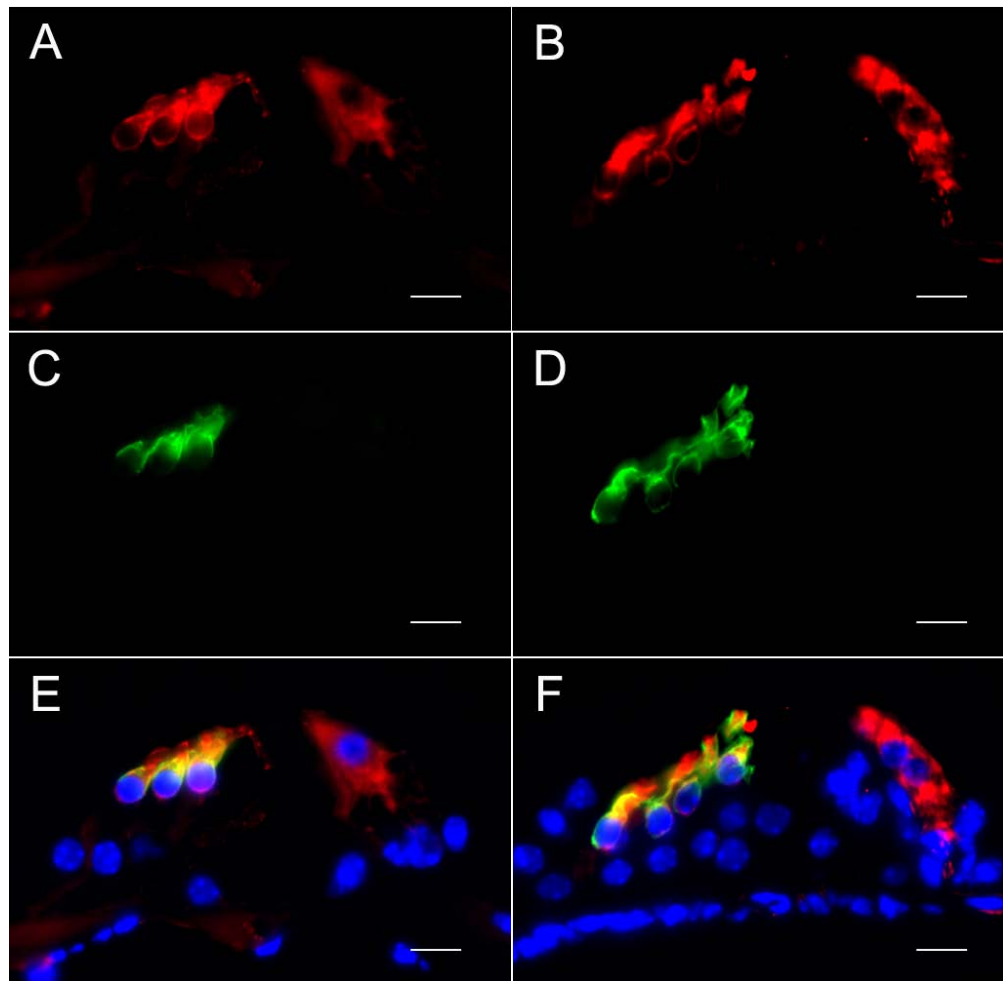


Fig. (2). Cross sectional analysis of mechanotransduction and cell motility markers in p27 +/+ and -/- mouse cochlea. Normal cellular uptake of the mechanotransduction indicating dye AM1-43 (Red) for (A) p27 +/+ and (B) p27 -/- genotypes in both inner and outer hair cells. Immunostaining for the outer hair cell motor protein Prestin (Green) shows normal staining pattern in Outer Hair Cells for (C) p27 +/+ and (D) p27 -/- genotypes. Merged images for AM1-43 uptake (Red) and Prestin immunostaining (Green) shows a normal staining pattern for (E) p27 +/+ and (F) p27 -/- genotypes. Nuclear DNA was stained with DAPI. Super-numary inner hair cells present in the p27-/- cochlea have normal uptake of the mechanotransduction dye AM1-43 (B) but do not express the Outer Hair Cell motor protein Prestin (D, F). Scale bar = 25 μ m.

Table 2. BrdU Positive Nuclei in the p27-/- Organ of Corti Increase After BrdU/Amikacin Versus BrdU Alone

Cell Type	BrdU with Amikacin		BrdU Alone		Normalized Ratio
	BrdU (+) Cells	Normalized BrdU (+) Cells	BrdU (+) Cells	Normalized BrdU (+) Cells	
IP	176	212	66	67	3.2
IHC	27	33	10	10	3.2
PC	30	36	43	43	0.8
OHC	5	6	1	1	6
DC	9	11	5	5	2.1
HC	128	154	30	30	5.1
Sum	375	451	155	157	2.9
# Sections	831	1000	990	1000	1

Quantification of BrdU positive nuclei of different cell types in the organ of Corti of p27Kip1-/- mice treated with BrdU/amikacin (Experimental group) or BrdU only (Control group). Normalized values reflect the number of cells expected in the organ of Corti based on 1000 sections at 4 micron thickness. Inner phalangeal cell (IP), inner hair cell (IHC), pillar cell (PC), outer hair cell (OHC), Deiter's cell(DC), and Hensen cell (HC).

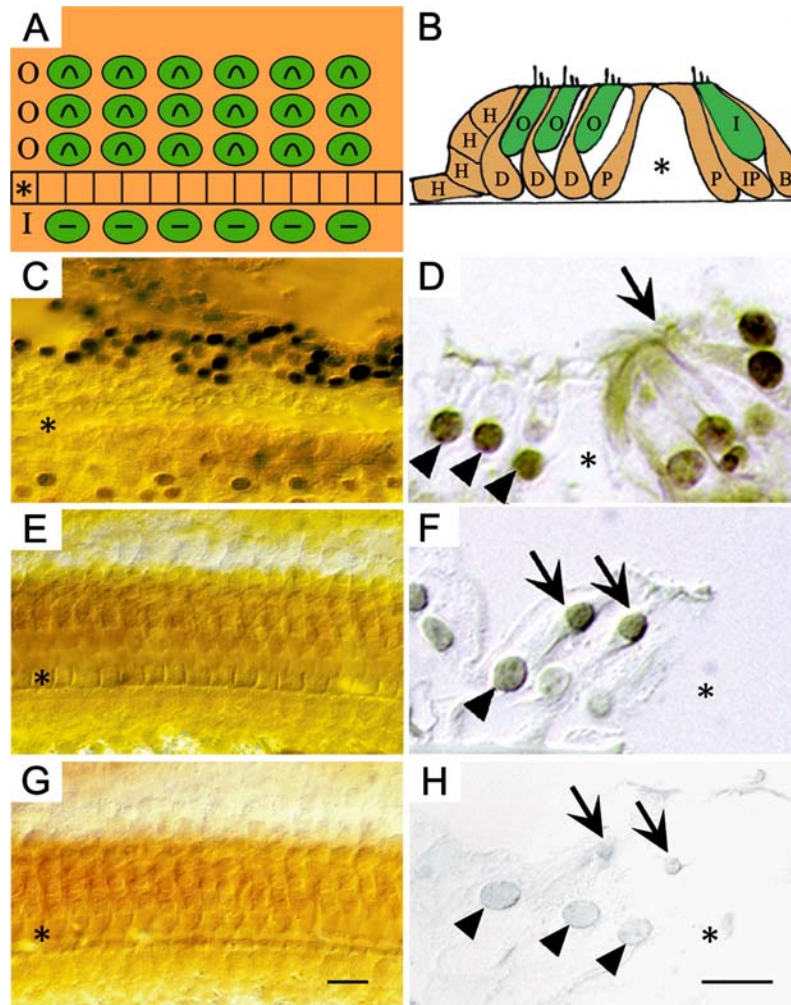


Fig. (3). Spontaneous and induced cell proliferation in the organ of Corti of p27 deficient mice. Schematic of the organ of Corti (A) when viewed as a whole mount shows three rows of the outer hair cells (O), one row of inner hair cells (I) and the intervening tunnel of Corti (*). (C, E, G) Whole mount images of organ of Corti stained with immunoperoxidase to detect BrdU incorporation in newly synthesized DNA. Cochlea from p27^{-/-} mice have BrdU labeled supporting cells (C), while p27^{+/-} (E), and p27^{+/+} (G) show no spontaneous BrdU incorporation in their DNA. (B) Schematic of the organ of Corti in transverse section shows the Inner (I) and Outer (O) hair cells (O), and the five interdigitating supporting cell types: Hensen’s cells (H), Deiter’s cells (D), pillar cells (P), inner phalangeal cells (IP), and border cells (B). Also shown is the location of the tunnel of Corti (*). (D, F, G) Transverse section images of organ of Corti stained with immunoperoxidase to detect BrdU incorporation after amikacin treatment. The cochlea from a p27^{-/-} mouse (D) has multiple labeled supporting cells flanking the tunnel of Corti including three BrdU-positive Dieter’s cells (arrow heads), one BrdU-positive inner hair cell (arrow), and three BrdU-positive supporting cells near the labeled IHC (one above and two below). The cochlea from a p27^{+/-} (F) after amikacin treatment has a BrdU-positive Deiter cell (arrow head) and two BrdU-positive OHCs (arrows). The cochlea from a p27^{+/+} (H) is devoid of BrdU-positive hair cells and supporting cells following amikacin treatment. Noted are two BrdU-negative pyknotic OHC nuclei (arrows) associated with the amikacin treatment and three BrdU-negative supporting cell nuclei (arrowheads). Scale bar: 25 µm (G); 10 µm (H).

To characterize the timing of auditory hair cell production, cochlea from p27^{-/-} mice injected with BrdU from P10-P12, were double immunostained for both BrdU and myosin-VIIa, a hair cell specific marker. By P26, BrdU-positive cells that were also myosin-VIIa positive were identified in the organ of Corti (Fig. 5). These cells often appeared in a doublet, where one cell was BrdU/Myosin VIIa double labeled and the other was BrdU-positive only. The majority of these double-labeled cells were in the IHC region. In p27^{+/-} and ^{+/+} cochlea of mice injected with BrdU only on P10-P12, no BrdU-positive cells were observed at either P14 or P26. This observation along with their normal cochlear morphology and auditory function

indicates that hair cells and supporting cells in the p27^{+/-} cochlea have completed the normal process of terminal differentiation. However, in p27^{+/-} mice injected with amikacin and BrdU, a small number of BrdU positive cells were observed in the organ of Corti at P14 (4.7 ± 7.6 cells/ 1 mm, $P > .05$) and at P26 (10.5 ± 12.0 cells/ 1mm, $P < .001$, Table. 2). The level of proliferation observed in p27^{+/-} mice was 7.6% of that observed in identically treated p27^{-/-} littermates. These data support the hypothesis that cellular proliferation in p27^{-/-} cochlea leads to the generation of new hair cells and that these cells are derived from the proliferation of adjacent supporting cells as well.

Expression of cyclins and cyclin dependent kinases in the mouse cochlea was studied to determine which cyclin is responsible for allowing supporting cell and hair cell regeneration in p27 knockout mice. In our survey of cyclin expression in the mouse cochlea, CyclinE was remarkable in that it was highly expressed in supporting cells at hearing onset and into adulthood (Figs. 7, 8). The expression pattern and intensity of CyclinE did not appear to change in p27^{-/-} mice suggesting that it is the dominant cyclin involved in cell-cycle re-entry in p27^{-/-} supporting cells.

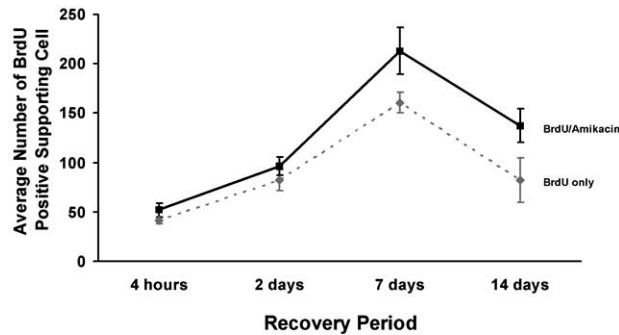


Fig. (4). Cell proliferation in the organ of Corti of p27^{-/-} mice. The time course for supporting cell proliferation in p27Kip1^{-/-} cochlea after the last BrdU injection on P12. Littermates received either BrdU or amikacin plus BrdU and were sacrificed at 4 hours, 2 days, 7 days, or 14 days of recovery. The number of BrdU-positive supporting cells were counted from a 1 mm length of the organ of Corti per cochlea (mean + s.d.).

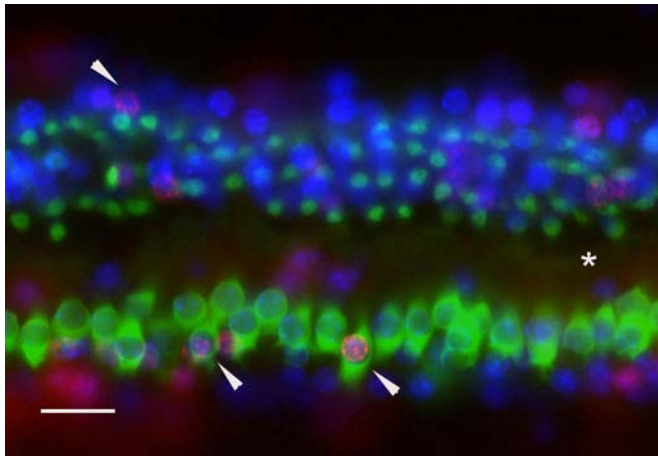


Fig. (5). Evidence of *in vivo* hair cell differentiation in the organ of Corti of p27^{-/-} mice. *De novo* hair cell production was determined in cochleae from p27^{-/-} mice given BrdU (P10-P12) by immunolabeling with anti-BrdU (red) and anti-myosinVIIa (green) antibodies. By P26, BrdU and myosin-VIIa double-labeled inner and outer hair cells were observed (arrowheads). In most cases, BrdU-positive, myosinVIIa negative supporting cells (red nuclei) were observed adjacent to the double-labeled hair cells. * denotes the location of the tunnel of Corti with inner hair cells below and outer hair cells above in the image shown. Scale bar = 25 μm.

DISCUSSION

Terminal differentiation is the process of irreversible cell cycle withdrawal coupled to the development of specialized morphological and functional attributes. In the mouse organ of Corti permanent cell cycle exit normally occurs at E14.

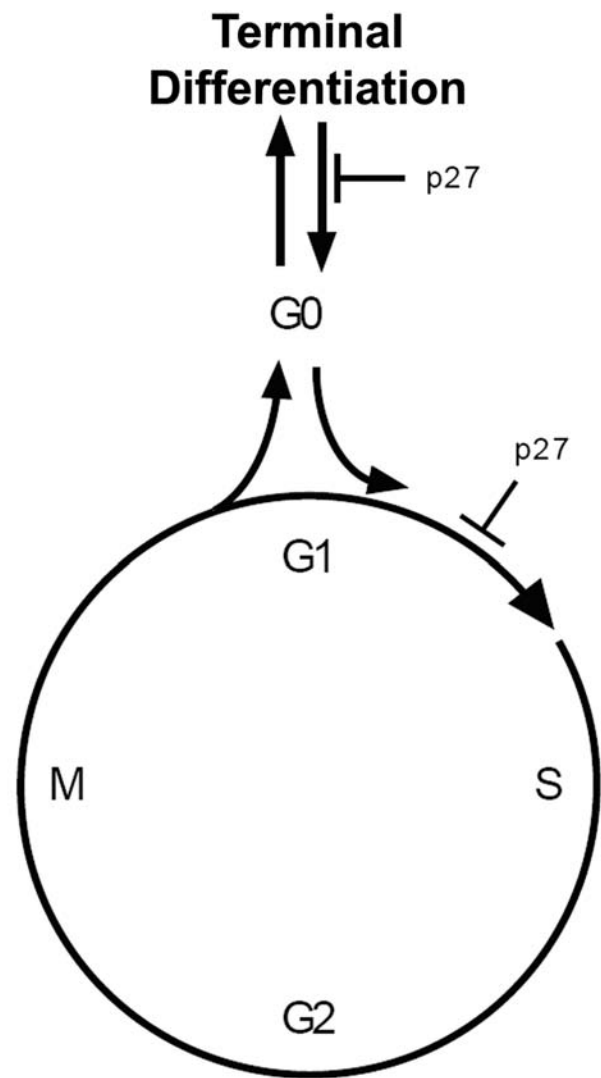


Fig. (6). A schematic illustrating the role of p27 in terminal differentiation and cell cycle re-entry. The site of p27 activity in the G1 phase as it has been described from prior *in vitro* and *in vivo* studies. Also indicated is the site of activity of p27 in the prevention of cell cycle re-entry from the terminally differentiated state which our current data suggests. The G0 state is not well defined in the sensory cells of the mammalian cochlea. A temporary G0 state may normally exist during embryonic or early postnatal development prior to cellular differentiation or the onset of auditory function. Transit from G0 back into G1 may depend on p27-independent mechanisms which are also responsible for the decreased proliferation observed in older p27^{-/-} animals.

It has been shown that mice lacking the CKI, p27Kip1, demonstrate ongoing proliferation in the supporting cell population of the organ of Corti in the postnatal and adult period [17, 19]. Thus, p27 is part of the machinery that controls cell cycle exit during the development of this organ. However, the mechanism responsible for cell cycle exit during embryogenesis is not necessarily identical to that which prevents cell cycle reentry in adulthood. This may be particularly true if cells enter a G0 or quiescent state during the postnatal period. Our current results indicate that p27 is required to prevent cell cycle re-entry in terminally

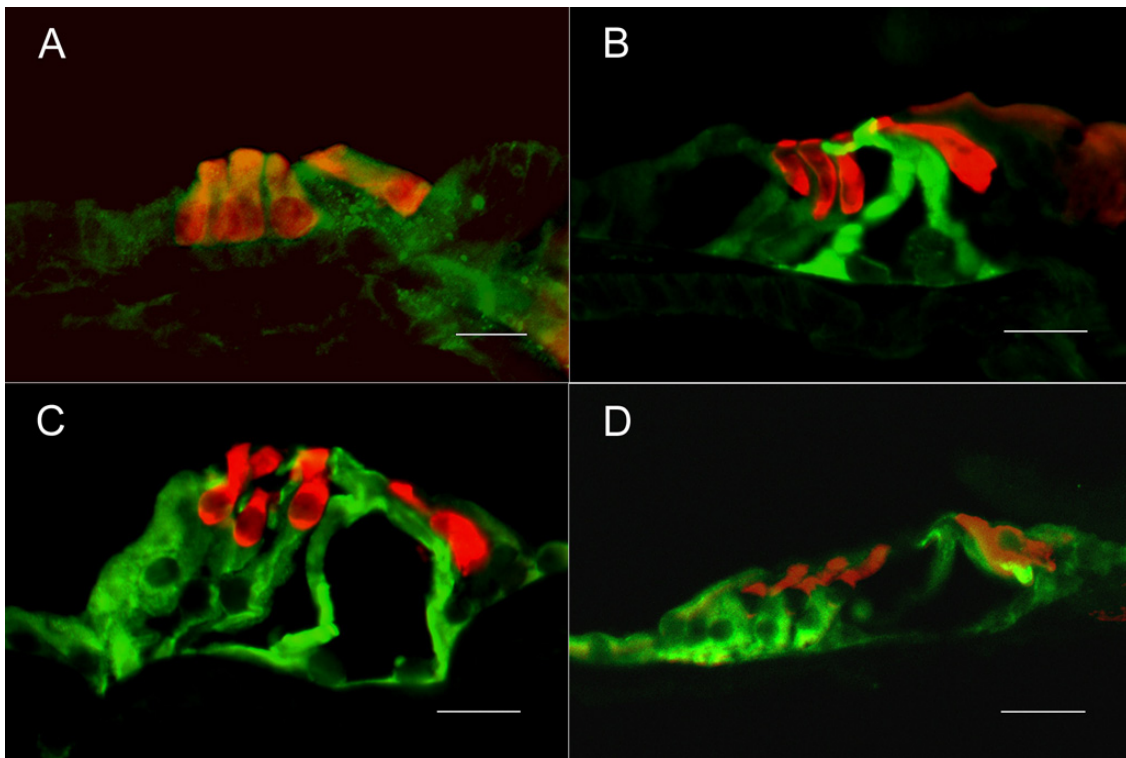


Fig. (7). Expression of Cyclin E in supporting cells is associated with the development of Organ of Corti. Weak but detectable cyclin E staining (green) is observed in supporting cells at birth (P0) in mouse cochlea (A). By postnatal day 7 (P7), robust cyclin E expression is observed in pillar cells with weak staining in the Deiter's cells (B). At P14, Cyclin E expression is easily detected in all supporting cells (C). In P30 and older mice, Cyclin E expression is strongest in Deiter's and Hensen's cells (D). Cyclin E immunostaining was not observed in the nuclei of supporting cells during these time points. Hair cells are stained with anti calbindin antibody (red). Scale Bar = 20 μ m.

differentiated supporting cells of the mature mammalian organ of Corti (summarized in Fig. 6) Cellular injury, by means of ototoxic antibiotics, which are known to provide a proliferative stimulus in non-mammalian cochlea [Reviewed in 26], also increased this novel regenerative phenotype in p27^{-/-} mice. We also show that a subset of these newly proliferating supporting cells further differentiate and display molecular and morphologic criteria that are characteristic of auditory hair cells. This is the first *in vivo* example of terminally differentiated cells re-entering the cell cycle, proliferating and then re-adopting a terminally differentiated phenotype. The majority of these proliferating cells survive beyond a four week period after entering S-phase.

In contrast to Rb^{-/-} hair cells [27, 28] or p27^{-/-}, p19^{-/-} neurons in the central nervous [29], cell cycle reentry in the terminally differentiated supporting cells of the p27^{-/-} organ of Corti is not coupled to apoptosis. In the retina of p27^{-/-} mice, cytotoxic injury induces proliferation of Mueller glia without immediate apoptosis. However, Mueller glial cells are not post-mitotic, and do not differentiate into photoreceptor, bipolar interneuron or retinal ganglion cells after cell cycle re-entry [30]. Thus auditory supporting cells possess the ability to become pluripotent after dedifferentiation without the addition of an exogenous factor.

One might ask whether the supporting cells of the organ of Corti are auditory stem cells. The fact that supporting cells of the mammalian organ of Corti do not have the capacity to proliferate and regenerate after embryogenesis argues against this characterization. Instead they are

considered terminally differentiated because of their loss of proliferation and their significant anatomical and functional specialization. Unlike the neocortex of mice where neurogenesis occurs or can be induced from the proliferation of non-terminally differentiated cells [6], the regenerated hair cells and supporting cells reported in this study arose from terminally differentiated supporting cells that are normally incapable of either spontaneous or growth factor induced proliferation. This suggests that despite their post-mitotic state, supporting cells contain adequate levels of cyclins and CDKs to catalyze a new round of cell replication following p27 deletion.

Our current data indicates that the process of cell cycle withdrawal in the terminally differentiated cells of the organ of Corti is dependent on more than one mechanism. The diminished proliferation that occurs in p27 knockout mice as they age between two weeks and two months may reflect a drop in the expression of cyclins or cyclin dependent kinases. Cyclin D1 has been shown to be dynamically expressed in supporting cells and hair cells in the early postnatal (P0) organ of Corti and tapering off by P15, with no observed expression in the adult [31]. This observation has led to speculation that over expression of Cyclin D1 in the adult organ of Corti may be advantageous in driving supporting cell proliferation as a means to stimulate hair cell regeneration. Our data suggests that Cyclin E is the dominant cyclin that mediates cells cycle progression in postnatal and adult supporting cells.

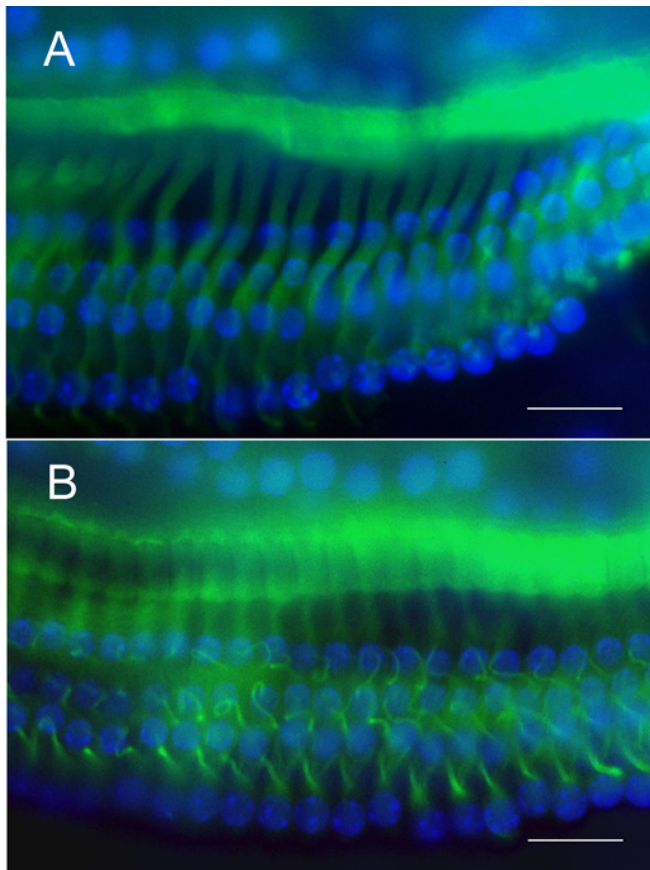


Fig. (8). Whole mount view of Cyclin E expression in an adult mouse organ of Corti. Focal plane near top of sensory epithelium in the organ of Corti with Cyclin E expression (green) present in the apical processes of pillar cells and Deiter's cells (A). At a deeper focal plane, Cyclin E expression is maintained in the cytoplasm of supporting cells including pillar and Deiter's cells but is not detected in the nuclei of these cells (B). Nuclear DNA is stained blue by DAPI. Scale bar = 20 μm .

Additionally, contact mediated inhibition to cell proliferation is partially relieved by cytotoxic injury to the cells resulting in a level of proliferation not normally observed in the p27 knockout mouse. This suggests that there are at least three molecular pathways (cyclin/CDKs, CKIs, and contact inhibition) involved in this novel proliferative regeneration phenomenon in the organ of Corti. Our data demonstrate that p27 and Cyclin E are expressed in supporting cells into adulthood. This finding should prove useful in the future study of supporting cell proliferation and differentiation in normal and injured mouse organ of Corti.

It is noteworthy that both Rb and p27 have been implicated in the suppression of cell cycle re-entry of terminally differentiated cells. This suggests a general role for the Rb pathway in the maintenance of the G_0 state in terminally differentiated cells. However, Rb knockout mice possess hair cells that can re-enter the cell cycle [27], and for the most part rapidly enter apoptosis [28]. In contrast, deletion of p27 does not induce hair cells to re-enter the cell cycle and proliferate. This is consistent with the lack of p27 and Cyclin E expression in hair cells and the continuous expression of p27 and Cyclin E in adult supporting cells. Interestingly, proliferating supporting cells in p27 $-/-$ mice can differentiate into either hair cells or supporting cells, a

unique phenomenon. However, in differentiated myocytes where p27 expression is normally high, the deletion of p27 is not sufficient to allow their re-entry into S-phase as was reported with Rb $-/-$ myotubes [32].

Alternative mechanisms of CDK regulation, which may compensate for p27 deletion in other tissues, include altered phosphorylation, expression of other CDK inhibitors, or diminished cyclin protein expression. Mice lacking both p27^{Kip1} and p19^{Ink4d} demonstrate postnatal neuronal cell proliferation resulting in cell death [29]. Mice lacking both p27^{Kip1} and p57^{Kip2} have lens fiber cells that fail to withdraw from the cell cycle appropriately [33]. However, in the organ of Corti, deletion of p27 is sufficient to stimulate post-mitotic supporting cell and hair cell regeneration through a process of supporting cell proliferation and differentiation. These are three normal characteristics of cellular regeneration in the organ of Corti that may be critical in the restoration of hearing for the deaf or hearing impaired.

Our efforts targeting p27 inhibition in adult guinea pigs with experimentally induced hearing loss involve the local injection of siRNA. This method appears to be well tolerated and allows for Adult Stem Cell Induction *in situ* (ASCI) as a potential clinical therapy for hearing regeneration. The ASCI technology could be applied to the diseased eye, spinal cord, and brain of patients where cellular regeneration is necessary to return function.

MATERIALS AND METHODS

p27Kip1 $-/-$ Mice

All protocols and procedures involving mice utilized in this work were reviewed and approved by the Fred Hutchinson Cancer Research Center IACUC or by the Sound Pharmaceuticals IACUC with veterinary oversight and were in compliance with the Animal Welfare Act and Animal Welfare Regulations. Adult p27Kip1 $-/-$, $-/+$, $+/+$ used in this study were mice bred from a founder colony kindly provided by Drs. James Roberts at the Fred Hutchinson Cancer Research Center, Seattle WA. Mice were housed in an SPF facility at Sound Pharmaceuticals and provided access to food and water ad lib.

AM1-43 Uptake in Hair Cells *via* Mechanotransduction Channels

Bony cochleae were exposed immediately after the animal was euthanized in a CO₂ chamber. A hole was made on the tip of cochlea, 100 μL of AM1-43 (InvitroGen Corp.) at 20 μM was infused gently *via* round window over a two minute period. Then the cochlea was infused with PBS briefly, followed with 4% paraformaldehyde (PFA) to fix the tissue *in situ*. After being dissected out, the cochlea was kept in 4% PFA for 30 minutes, then stored in PBS at 4 degree C.

Immunocytochemical Staining of Cochlear Tissues

For Prestin immunostaining on whole mount tissues, the dissected membranous cochleae were blocked, incubated with goat anti-Prestin (N-20) antibody (1:100) overnight at 4 degree C (Santa Cruz Biotechnology). After rinsing in PBS, tissues were incubated with Cy5-donkey anti-goat IgG antibody (1:100) for 30 minutes RT (Jackson Immuno

Research). Tissues were mounted intact a mounting medium with DAPI on glass slides for whole mount analysis.

For cross section observation of Prestin immunostaining, the bony cochlea was decalcified in EDTA at RT. After cryo-protection in 30% sucrose, the cochlea was embedded in OTC and frozen sectioned. The sections were blocked, incubated with goat anti Prestin (N-20) antibody (1:100) for 3 hours RT. After rinsing, the tissues were reacted with Cy5-donkey anti-goat IgG antibody (1:100) for 30 minutes RT. Then tissues were mounted in a mounting medium with DAPI for further analysis. Epifluorescent images were captured with a CCD camera using a QED imaging system.

For whole mount and cross sectional analysis of CyclinE immunostaining, polyclonal rabbit anti-cyclin E antibody (1:200), (Santa Cruz Biotechnology, Inc. Catalog number M-20 SC-481) was used as the primary antibody. Secondary detection was performed with biotinylated goat anti-rabbit IgG antibody and fluorescein-Avidin D (1:200) (Vector Laboratories Inc). For whole mount and cross sectional analysis of calbindin, polyclonal rabbit anti-calbindin antibody was used as the primary antibody (1:160) (Chemicon International, Inc). Secondary detection was performed using rhodamine conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc). Staining times and conditions were similar to those described for Prestin immunodetection.

TUNEL Assay

Cochlea from 6 month old p27 $+/+$ and $-/-$ mice were harvest and dissected for analysis. Bony cochlea capsule was removed, and the sensory epithelium dissected out on ice. Tissues were TUNEL stained per manufacturers' instructions (Apoptag Kit Rhodamine, Millipore). Tissues were then post fixed with paraformaldehyde and immunostained with rabbit anti-Myosin VIIa (1:500 AbCam antibody AB3481), followed by FITC-Goat anti-Rabbit immunostaining. Samples were counter stained with DAPI, mounted, and visualized on a Nikon E800 microscope under UV fluorescence. TUNEL positive cells in the sensory epithelium were analyzed for the apical 180 degrees of each cochlea. Analysis did not include the stria vascularis, spiral ligament, or the modiolus as these tissues were removed during dissection.

Amikacin Sulfate and BrdU Proliferation Assay

p27Kip1 littermates on a 129/Sv inbred background were given amikacin sulfate, an ototoxic antibiotic, starting on postnatal day 7 through P12 (500-1000 mg/kg/d subcutaneous (s.c.) for 6 consecutive days). The nucleoside analogue, bromodeoxyuridine (BrdU), was also administered daily from P10 through P12 (30 mg/kg/d s.c. for 3 consecutive days). Mice were sacrificed either 4 hrs, 2 days, 7 days or 14 days post last injection (i.e. on P12, P14, P19 or P26) and their cochleae were fixed with 4% paraformaldehyde for 30 min. The cochleae were further dissected and immunostained for the presence of BrdU using biotinylated sheep anti-BrdU antibody (1:300, BioDesign International) plus a Streptavidin-Texas Red fluorescent secondary (1:500, Vector Labs) or an avidin-biotin HRP complex plus diaminobenzidine (Vector Labs). Cell counts were performed on whole mounts under epifluorescence and Nomarski (DIC) light microscopy. BrdU labeled cells in the Hensen's cell region through

the border cell region adjacent to the inner hair cell row were counted along a 1000 μ m continuous length of the organ of Corti starting at 90 degrees from the apical tip of the cochlea. To confirm the position of these labeled cells within the organ of Corti and to eliminate the possibility of counting mesenchymal cells below the basilar membrane, selected specimens were embedded in plastic and cross-sectioned at four μ m thickness and viewed under DIC optics.

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