Proliferation of Functional Hair Cells in Vivo in the Absence of the Retinoblastoma Protein

Cyrille Sage,1 Mingqian Huang,1 Kambiz Karimi,2 Gabriel Gutierrez,3 Melissa A. Vollrath,4 Duan-Sun Zhang,4 Jaime García-Añoveros,5 Philip W. Hinds,3 Jeffrey T. Corwin,2 David P. Corey,4 Zheng-Yi Chen1*

1Neurology Service, MGH-HMS Center for Nervous System Repair, Massachusetts General Hospital and Harvard Medical School, Boston MA 02114, USA. 2Department of Neuroscience, University of Virginia School of Medicine, Charlottesville, VA 22908, USA. 3Radiation Oncology, Molecular Oncology Research Institute, Tufts-New England Medical Center, Boston, MA 02111, USA. 4Howard Hughes Medical Institute and Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA. 5Departments of Anesthesiology, Physiology, and Neurology, Northwestern University Institute for Neuroscience, Chicago, IL 60611, USA.

*To whom correspondence should be addressed. E-mail: zhengyi@helix.mgh.harvard.edu

In mammals, hair cell loss causes irreversible hearing and balance impairment because hair cells are terminally differentiated and do not regenerate spontaneously. Profiling gene expression in developing mouse vestibular organs, we identified the retinoblastoma protein (pRb) as a candidate regulator of cell-cycle exit in hair cells. Differentiated and functional mouse hair cells with a targeted deletion of Rb1 undergo mitosis, divide, and cycle, and yet continue to become highly differentiated and functional. Moreover, acute loss of Rb1 in postnatal hair cells caused cell-cycle re-entry. Manipulation of the pRb pathway may ultimately lead to mammalian hair cell regeneration.

In fish, amphibians and birds, regeneration of sensory hair cells through asymmetric cell divisions of supporting cells can contribute to recovery of hearing and balance after hair cell loss caused by trauma or toxicity (1, 2). Mammalian hair cells do not spontaneously regenerate, even though supporting cells in vestibular sensory epithelia retain a limited ability to divide (3, 4). Consequently, hair cell death in mammals often leads to permanent hearing and balance impairment.

As the inner ear develops, hair cell progenitor cells exit from the cell cycle and, like neurons, terminally differentiate. Negative cell-cycle regulators apparently maintain the postmitotic status of hair cells and contribute to their terminal differentiation. Cyclin-dependent-kinase inhibitors, p27Kip1 and p19Ink4d, participate in cell-cycle exit of hair cell progenitors and in hair cell apoptosis, respectively (5, 6). However the key regulators of cell-cycle exit and concomitant hair cell terminal differentiation remain elusive.

The retinoblastoma protein, pRb, encoded by the retinoblastoma gene, Rb1, functions in cell-cycle exit, differentiation and survival (7, 8). pRb is a member of the pocket protein family, which includes p107 (encoded by Rbl1, BC069179) and p130 (encoded by Rbl2, BC020528). Like pRb, p107 and p130 cause cell-cycle arrest when overexpressed (9).

Germline pRb−/− animals die in utero around E13.5, with severe defects in lens development, hematopoiesis, myogenesis, osteogenesis and neurogenesis (7, 10–12). In both the central and peripheral nervous systems, neurons undergo ectopic mitoses and subsequent apoptosis (11, 13). Mice with Rb1 conditionally deleted in the central nervous system show an increase in neuronal number due to aberrant S-phase entry, without apoptosis (14–16). However it is not clear whether these supernumerary neurons are highly differentiated or functional.

To identify molecules involved in cell-cycle regulation during hair cell development we used oligonucleotide microarrays to study gene expression in the developing mouse utricle, a balance organ of the inner ear. We noticed that retinoblastoma family members show a suggestive pattern: From E14.5 to P12, Rb1 expression was constant, Rbl1 showed downregulation, and Rbl2 exhibited upregulation (17). An anti-pRb antibody weakly labeled all cells in the E12.5 otocyst (fig. S1A), and labeling was prominent in all hair cells from embryo to adult (fig. S1, B to F). Therefore pRb could be required to suppress cell division in hair cells.

Because germline pRb−/− mice die around E13.5 (10), when hair cells are extremely immature, we studied a conditional pRb knockout. Mice with lox-P sites flanking exon 19 of the Rb1 gene (Rb1loxp) (18) were crossed with mice carrying cre under the control of the 3.6 kb collagen1A1 (Col1A1) promoter, which express cre-recombinase in a pattern similar to endogenous Col1A1 (19). Because these pRb conditional
knockout mice (Col1A1-pRb−/−) die perinatally, we studied embryos. By in situ hybridization, Col1A1 was detected ubiquitously in the E11.5 otocyst, but later reduced in hair cells and supporting cells (fig. S2). In Col1A1-pRb−/− inner ears, pRb was undetectable in the sensory epithelium (Fig. 1, C and D).

If pRb regulates cell-cycle exit in hair cells, its loss might permit cell-cycle re-entry and increase cell number. We tested this hypothesis by counting cells with hair bundles in E18.5 Col1A1-pRb−/− utricles. Col1A1-pRb−/− utricles had 40% more cells with bundles than littermate controls (Col1A1-pRb+/−: 1406±73 (mean±SD), N=3; Col1A1-pRb−/−: 987±62, N=5; P<0.05) (Fig. 1, E and F). A more dramatic increase in hair number was observed in cochleas. While littermate controls had one row of inner hair cells and three rows of outer hair cells, Col1A1-pRb−/− cochleas had 3-4 rows of inner hair cells and 7-8 rows of outer hair cells. Most Col1A1-pRb−/− cochlear hair cells had bundles, but many were not properly oriented (Fig. 1, G and H).

The increase in hair cell number in Col1A1-pRb−/− ears suggested that new hair cells arose through an increase in differentiation-competent progenitor cells and/or through continuing hair cell division. To study progenitor cell proliferation, E13.5 pregnant mice were injected with BrdU 4 hours prior to embryo harvest. In the primordial organ of Corti, the p27Kip1-positive “zone of non-proliferating cells (ZPNC)” harbors postmitotic sensory precursor cells (20). We found BrdU-positive cells in the p27Kip1-positive region of Col1A1-pRb−/− mice (Fig. 2N), but not in controls (Fig. 2M). Therefore pRb is involved in cell-cycle exit of sensory progenitor cells.

To test hair-cell proliferation specifically, E16.5 pregnant mothers were injected with BrdU and embryos harvested at E18.5. During normal development, mouse hair cells become postmitotic as early as E12.5 (21). As expected, no hair cells or cochlear supporting cells were BrdU-positive in control mice (Fig. 2, I and K). In contrast, many hair cells and cochlear supporting cells were BrdU-positive in Col1A1-pRb−/− mice, indicating that they had entered S-phase (Fig. 2, J and L). BrdU labeling in hair cells tended to be weaker than in supporting cells, suggesting hair cells had further divided, diluting the BrdU (84% of hair cells were weakly labeled vs. 58% of supporting cells, with “weak” considered less than half the level of the brightest supporting cells). We also observed an increased ratio of outer hair cells to Deiters’ cells, suggesting continuous hair-cell division (Col1A1-pRb−/−: OHC/DC: 1.45±0.057, mean±SD, N=51; Col1A1-pRb+/−: 0.79±0.037, N=22; P<0.0001). The proliferation of Col1A1-pRb−/− cochlear supporting cells appeared to be cell-specific (fig. S3), as we saw more Deiters’ cells than in controls (S100A1 labeling) but not more Pillar cells (p75ntr labeling).

We also identified dividing cells with an anti-PCNA antibody (22). In E13.5 and E18.5 Col1A1-pRb−/− utricles but not in controls, most hair cells stained strongly for PCNA (fig. S4). In cochleas as well, Col1A1-pRb−/− hair cells and supporting cells were strongly PCNA-positive, unlike controls (fig. S4, P to R, and fig. S4, M to O). Finally, hair cells in metaphase were observed in E18.5 Col1A1-pRb−/− utricles (Fig. 2O). Staining with DAPI and an antibody to the hair-cell-specific transcription factor Brn-3.1 showed that, for hair cells in M-phase, Brn-3.1 labeling appeared to be cytoplasmic, and separated from DAPI-labeled condensed chromosomes that were segregating into two daughter nuclei during mitosis (Fig. 2O arrows and inset).

Most apical hair cells in E18.5 Col1A1-pRb−/− utricles showed highly differentiated morphology, including pear-shaped cell bodies and intact hair bundles. Hair bundles were labeled with antibodies to espin (an actin crosslinker) and Ptpq (present in highly-differentiated cochlear hair bundles) (Fig. 3, A and B, and Fig. 3, C and D) (23, 24). An anti-tubulin antibody revealed, as in controls, nerve fibers surrounding most Col1A1-pRb−/− hair cells (Fig. 3, E and F), and an antibody to the synaptic vesicle protein synaptophysin showed labeling around many Col1A1-pRb−/− hair cells (fig. S5), suggesting that Col1A1-pRb−/− hair cells can attract axons and form synapses. Other markers of differentiated hair cells were also detected in Col1A1-pRb−/− mice, including Brn-3.1 (Fig. 2O), Lhx3 (Fig. 3, B and D), Gfi1, Math1, calretinin and parvalbumin 3. In contrast to a conditional pRb−/− mouse model where retinal rods failed to differentiate (25), cell fate determination and subsequent differentiation were largely intact in the proliferating Col1A1-pRb−/− hair cells. Therefore Col1A1-pRb−/− hair cells become differentiated without switching off proliferation, indicating that hair cell fate determination and differentiation do not require pRb function.

The sine qua non of hair cell function is mechanosensitivity. FM1-43, a fluorescent dye, enters hair cells through open mechanotransduction channels and so serves as a vital optical assay for mechanosensitivity (26, 27). We observed FM1-43 labeling in bundles and cell bodies of most hair cells in both control (Fig. 4, A to C) and Col1A1-pRb−/− utricles (Fig. 4, D to F). Since most hair cells in Col1A1-pRb−/− utricles are PCNA-positive, FM1-43 entry can occur in cycling hair cells.

We then recorded transduction currents in control and Col1A1-pRb−/− hair cells. Transduction currents were evoked in 4 randomly selected Col1A1-pRb−/− hair cells (Fig. 4, G and H), although currents were smaller than in controls (10-20 pA compared to ~200 pA in controls). Currents might be smaller if bundles had little time to develop between cell divisions, especially with the known delay between bundle formation and transduction (28). Transduction currents showed a normal
activation range and adaptation time course. Thus, specialized hair cell function does not require pRb.

To determine whether apoptosis occurs in Col1A1-pRb−/− hair cells, we assayed for activated caspase-3. We did not detect any caspase-3-positive cells in Col1A1-pRb−/− sensory epithelium, nor in controls (fig. S6). Therefore, loss of pRb itself does not appear to lead to cell death in the inner ear. The prominent expression of Rb1 in postnatal hair cells and the fact that acute loss of pRb causes cell-cycle re-entry in quiescent or senescent cells (29) suggests a role for pRb in maintaining hair cells’ nonproliferative status. To test this hypothesis, floxP-pRb utricles were cultured and infected with adenovirus carrying cre-recombinase, acutely deleting the Rb1 gene in infected hair cells (30). Utricular hair cells are mature at P10 and postmitotic at both stages studied (E17.5 and P10). After continuous culture in the presence of BrdU, no labeling was detected in adenov-GFP-infected (Fig. 5, A to D) or uninfected floxP-pRb hair cells (Fig. 5, F and H, arrows), whereas adenov-recombinase infected hair cells incorporated BrdU (Fig. 5, E to H). There were fewer BrdU-labeled hair cells in P10 cultures than in E17.5 cultures, likely due to lower efficiency of infection of P10 hair cells. Additionally, more pRb was present in infected P10 hair cells following culture, suggesting that cre-mediated recombination or pRb degradation was less efficient in P10 cultures. All the infected hair cells lost hair bundles (30), so we could not test function. Nevertheless, the damaged hair cells re-entered the cell cycle.

Cochleas in Col1A1-pRb−/− mice were studied for supporting-cell to hair-cell conversion. If this was the main pathway for increased hair cell number, we expect p27Kip1-labeled supporting cells would label with Math1, the earliest hair cell marker; or Math1-positive cells would appear in supporting cell regions outside the hair-cell region. No p27Kip1 and Math1 double-positive cells, or Math1-positive cells in the supporting cell region were found. While we cannot completely exclude cell fate conversion, it is most likely that increased hair cell precursors and subsequent hair cell division are primarily responsible for the overproduction of hair cells.

This study demonstrated, for the first time, that differentiated mammalian hair cells can continue to cycle and divide in the absence of pRb, so that functional hair cells can be generated through divisions of preexisting hair cells. Furthermore, acute ablation of pRb in differentiated hair cells led to cell-cycle re-entry.

Demonstration that pRb critically regulates hair cell division opens new opportunities, both for hair cell regeneration and for creating cell lines for hearing research. For hair cell regeneration, it will be important to determine whether a reversible block of pRb function in hair cells might be achieved in place of permanent deletion of the Rb1 gene. Thus, regulated inactivation of pRb through use of siRNA, a small molecule inhibitor of pRb or reversible manipulation of pRb-modifying kinases may result in production of functional hair cells followed by restoration of normal cell-cycle exit. These results also show that an irreversible switch from proliferation is not required for “terminal” differentiation, since cycling cells in the absence of pRb are highly differentiated and functional. Our findings may have implications for regenerating other functional cells through manipulation of negative cell growth control genes.

References and Notes
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Fig. 1. Expression of Rb1 in the inner ear and increased hair cell numbers in the Col1A1-pRb-/- mice. (A, B) An anti-pRb antibody primarily stained hair cells in E18.5 control utricle; an antibody to myosin-7a (Myo7a) marked hair cells. (C, D) pRb was absent in the E18.5 Col1A1-pRb-/- utricle. Note multiple-layer hair cells in Col1A1-pRb-/- utricle. Basal lamina marked with dashed lines. Confocal images of rhodamine phalloidin-labeled hair bundles in the E18.5 utricular macula (E, F), and mid-turn of the cochlea (G, H). The distribution of hair cells in Col1A1-pRb-/- utricle was abnormal, as indicated by clustered hair bundles [round circles in (F)], in contrast to the normal mosaic pattern in control (E). (G, H) Inner hair cells (arrows) and outer hair cells (arrowheads) in cochlea remained separated by pillar cells, which do not have hair bundles. Uniform orientation of the hair bundles was altered in Col1A1-pRb-/- cochlear hair cells (H). Ut-utricle; Coch-cochlea; IHC-inner hair cell; OHC-outer hair cell. Scale bars=25 µm.

Fig. 2. The sensory progenitor cells and hair cells undergoing mitosis in Col1A1-pRb-/- mice. An anti-myosin-7a antibody labels hair cells; confocal images. (A, E, I) In E18.5 control utricular macula, BrdU labeling was not found in hair cells, but appeared in some supporting cells. (B, F, J) In Col1A1-pRb-/- utricular macula, BrdU labeling appeared in both hair cells and supporting cells. (C, G, K) No BrdU labeling in control cochlear hair cells or supporting cells. (D, H, L) BrdU labeling of Col1A1-pRb-/- cochlear hair cells and supporting cells. Overall hair cell labeling was weaker (arrows) than supporting cells (arrowheads) (F,H,J,L). (M) No BrdU labeling was in control progenitor cells in the ZNPC (demarcated by dashed lines) of the primordial organ of Corti at E13.5, whereas BrdU staining was in Col1A1-pRb-/- progenitor cells (N). (O) Hair cells in M-phase of cell cycle, as shown by cytoplasmic like labeling for Brn-3.1 and condensed nuclear labeling by DAPI (arrows). Inset shows a M-phase hair cell with Brn-3.1 alone and Brn-3.1 plus DAPI labeling. Ut-utricle; Coch-cochlea. Scale bars=25 µm (A-N) and 10 µm (O).

Fig. 3. Hair cells labeled with differentiating hair cell markers. (A-D) Lhox3 labels hair cell nuclei. Antibodies to espin labeled hair bundles (arrows) in control (A) and Col1A1-pRb-/- utricles (B). (C, D) Antibodies to Ptprq labeled hair bundles (arrows) in control (C) and Col1A1-pRb-/- cochleas (D). (E, F) Antibodies to tubulin labeled nerve fibers surrounding hair cells marked with myo7a (arrows) in control (E) and the Col1A1-pRb-/- cochleas (F). Note labeling surrounding multiple inner hair cells in the Col1A1-pRb-/- cochlea (F). Scale bars=25 µm.

Fig. 4. Functional mechanotransduction by Col1A1-pRb-/- and control hair cells at E18.5. (A-F) FM1-43 accumulation by utricular hair cells. After a 1 min exposure to FM1-43, most hair bundles (DIC images, A and D) were labeled with FM1-43 (green, B and E) in both control (A-C) and Col1A1-pRb-/- mice, indicating that these cells had functional mechanotransduction channels. Arrows indicate clear-labeled bundles. (G) Transduction currents elicited in control (top) and Col1A1-pRb-/- (middle) littermates by step deflections of the hair bundle (bottom). Adaptations of the transduction currents in response to positive (red) and negative (black) hair bundle deflections were revealed. The wild type response is typical of transduction currents in neonatal mice (31). However, transduction currents in Col1A1-pRb-/- mice were small: peak transducer current (mean ± SEM) was 14.2 ± 2.9 pA (n = 4). (H) Normalized I(X) relations for the control and Col1A1-pRb-/-transduction currents shown in G. These two hair cells had similar operating ranges. Scale bars=10 µm.

Fig. 5. Cell cycle re-entry by postmitotic hair cells, after acute deletion of Rb1 gene. (A, B) E17.5 and (C, D) P10 floxP-pRb utricles were infected with adenovirus carrying GFP as controls, and then cultured with addition of BrdU. All hair cells are pRb positive and BrdU negative. The two BrdU positive cells (A, B) are not hair cells. (E, F) E17.5 and (G, H) P10 floxP-pRb-/- utricles were infected with adenovirus carrying GFP as controls, and then cultured with addition of BrdU. All hair cells are pRb positive and BrdU negative. The two BrdU positive cells are not hair cells. (E, F) E17.5 and (G, H) An antibody to myosin-7a (Myo7a) stained hair cells, as well as supporting cells (arrowheads) (F,H,J,L). (M) No BrdU labeling was in control progenitor cells in the ZNPC (demarcated by dashed lines) of the primordial organ of Corti at E13.5, whereas BrdU staining was in Col1A1-pRb-/- progenitor cells (N). (O) Hair cells in M-phase of cell cycle, as shown by cytoplasmic like labeling for Brn-3.1 and condensed nuclear labeling by DAPI (arrows). Inset shows a M-phase hair cell with Brn-3.1 alone and Brn-3.1 plus DAPI labeling. Ut-utricle; Coch-cochlea. Scale bars=25 µm (A-N) and 10 µm (O).