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Sonic hedgehog initiates cochlear hair cell regeneration through downregulation of retinoblastoma protein

Na Lu [a,b,1], Yan Chen [c,1], Zhengmin Wang [a,d], Guoling Chen [a], Qin Lin [a,e], Zheng-Yi Chen [b,*], Huawei Li [a,d,*]

[a] Otology Skull Base Surgery Department, Hearing Research Institute, Eye and ENT Hospital of Shanghai Medical School, Fudan University, Shanghai 200031, China
[b] Department of Otolaryngology and Program in Neuroscience, Harvard Medical School and Eaton Peabody Laboratory, Massachusetts Eye and Ear Infirmary, Boston, MA 02114, USA
[c] Central Laboratory, Hearing Research Institute, Eye and ENT Hospital of Shanghai Medical School, Fudan University, Shanghai 200031, China
[d] Institute of Biomedical Sciences, Fudan University, Shanghai 200032, China
[e] Department of Otolaryngology, First Affiliated Hospital of Fujian Medical University, Otolaryngology Institute of Fujian Province, Fuzhou, China

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ABSTRACT

Cell cycle re-entry by cochlear supporting cells and/or hair cells is considered one of the best approaches for restoring hearing loss as a result of hair cell damage. To identify mechanisms that can be modulated to initiate cell cycle re-entry and hair cell regeneration, we studied the effect of activating the sonic hedgehog (Shh) pathway. We show that Shh signaling in postnatal rat cochleae damaged by neomycin leads to renewed proliferation of supporting cells and hair cells. Further, proliferating supporting cells are likely to transdifferentiate into hair cells. Shh treatment leads to inhibition of retinoblastoma protein (pRb) by increasing phosphorylated pRb and reducing retinoblastoma gene transcription. This results in upregulation of cyclins B1, D2, and D3, and CDK1. These results suggest that Shh signaling induces cell cycle re-entry in cochlear sensory epithelium and the production of new hair cells, in part by attenuating pRb function. This study provides an additional route to modulate pRb function with important implications in mammalian hair cell regeneration.

1. Introduction

Hair cells in the inner ear play an essential role in converting mechanical sound movement to neural signals for hearing and balance. Unlike lower vertebrates, mammalian cochlear hair cells cannot regenerate spontaneously after damage, although the vestibular system maintains limited hair cell regeneration capacity [1–3]. As a result, hair cell loss is the major cause of permanent sensorineural hearing loss.

Hair cells and supporting cells of the inner ear are derived from sensory progenitor cells [4]. Their development involves permanent exit from the cell cycle, cell fate determination, and differentiation. Differentiated mammalian hair cells remain in a permanent quiescent state throughout life. Although cochlear supporting cells in newborn mice can be induced to divide and regenerate new hair cells *in vitro* [5], spontaneous auditory hair cell regeneration *in vivo* has not been observed after hair cell loss. Cell cycle exit by progenitor cells and maintenance of the quiescent status of differentiated hair cells and supporting cells are controlled by negative cell growth proteins, including p27kip1, p19ink4d, pRb, and p21cip1.

The retinoblastoma gene (Rb1) is a potent tumor suppressor gene, and its protein product (pRb) plays essential roles in cell cycle exit of sensory progenitor cells, maintenance of hair cell and supporting cell postmitotic states, and survival of differentiated hair cells. When Rb1 is conditionally deleted at embryonic day 10, sensory progenitor cells are overproduced in the sensory primordium [6]. The overproduced progenitor cells differentiate into supporting cells and functional hair cells, demonstrating that cell cycle exit and early functional maturation of hair cells occur in the absence of Rb1 [6]. Conditional deletion of Rb1 from an embryonic stage or acute deletion at an early postnatal stage causes cochlear hair cells to rapidly re-enter the cell cycle and subsequently undergo apoptosis, demonstrating that survival of postnatal cochlear hair cells is pRb-dependent [6–8]. Further, acute Rb1 deletion leads to proliferation of pillar and Deiters' cells indicating a role for pRb in the maintenance of supporting cell quiescence [9]. Because pRb is essential for the maintenance of the postmitotic

Abbreviations: BrdU, bromodeoxyuridine; Cdk, cyclin-dependent kinase; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; Myo7a, myosin VIIA; P2, postnatal day 2; Pax2, paired box 2; pRb, retinoblastoma protein; Ptc, patched; Rb1, retinoblastoma 1; Shh, sonic hedgehog; Smo, smoothened; Sox2, SRY (sex-determining region Y)-box 2.

* Corresponding authors. Address: Otology Skull Base Surgery Department, Hearing Research Institute, Eye and ENT Hospital of Shanghai Medical School, Fudan University, Shanghai 200031, China. Fax: +86 21 64377151 (H. Li), fax: +1 617 720 4408 (Z.-Y. Chen).

E-mail addresses: Zheng-yi_chen@meei.harvard.edu (Z.-Y. Chen), hwli@shmu.edu.cn (H. Li).

[1] These authors contributed equally to this work.

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state of hair cells and supporting cells, and for the survival of mature hair cells, transient inhibition of pRb may allow for hair-cell regeneration in damaged cochlear sensory epithelia [10].

Sonic hedgehog (Shh) is an essential signaling molecule in inner ear sensory epithelia development, and in Shh knockout mice the cochlear sensory organ and spiral ganglion cells are not formed [11]. We have previously shown that Shh can promote mouse inner ear progenitor cell proliferation and hair cell differentiation [12]. In the current study we have activated Shh signaling in cultured neonatal rat cochleae and shown that Shh treatment leads to cell cycle re-entry in cochlear hair cells and supporting cells, some of which further differentiate into hair cells. By reverse transcription PCR (RT-PCR) and Western blot, we show that Shh activation results in suppression of pRb and hypothesize that this is a mechanism underlying cell cycle re-entry.

2. Materials and methods

2.1. Tissue culture

Cochlear sensory epithelium was dissected from anaesthetized postnatal day 2 (P2) Sprague–Dawley rats in PBS at pH 7.4. The stria vascularis and surrounding epithelial tissue and remains of the nerve fiber were removed and transferred onto poly-L-lysine-coated cover slides (Sigma–Aldrich) in a 35 mm dish filled with 2 mL serum-free DMEM/F12 medium (mixed 1:1) supplemented with N2 and B27 solutions (media and supplements were from Invitrogen). Neomycin (1 mM) was added for 24 h to kill hair cells. Shh (5 nM, R&D Systems) or cyclopamine (2.5 μM, Sigma–Aldrich) was added to the media for the next 5 days. For proliferation analysis, BrdU was added to the media to a final concentration of 10 μg/mL. Half of the media was replaced every second day.

2.2. Immunostaining

For immunolabeling, we used monoclonal anti-BrdU (1:50 dilution, ABD Serotech), polyclonal anti-myosin VIIA (Myo7a) (1:200, Proteus Biosciences), polyclonal anti-paired box 2 (Pax2) (1:200, Covance), or polyclonal anti-SRY (sex-determining region Y)-box 2 (Sox2) (1:200, Santa Cruz Biotechnology) antibodies. The labeling was visualized with secondary antibodies including donkey anti-mouse antibody conjugated to 594 (1:500, Jackson ImmunoResearch Lab Inc.), donkey anti-rat IgG antibody conjugated to Alexa Fluor 488 (1:500), or donkey anti-rabbit antibody conjugated to Alexa Fluor 647 (1:200). Counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) (1:800, Sigma–Aldrich) allowed visualization of cell nuclei. Negative controls were performed as above by omitting the primary antibodies. Details of the immunolabeling protocol were described previously [6].

2.3. RT-PCR and Western blot

Reverse transcription with Superscript II reverse transcriptase (Invitrogen) used 2 μg total RNA treated with RNase-free DNase (Roche) and the PCR primers and conditions listed in Table 1. The identities of the PCR products were confirmed by sequencing. Control reactions lacking reverse transcriptase did not produce products. The optimized conditions were held constant for each sample to assure valid comparison of the results, and the data are from at least four independent experiments.

Protein extracts were obtained using RIPA buffer. Proteins were separated on 8% SDS–PAGE gels and transferred to nitrocellulose membranes in 20% methanol buffer at 4 °C. Protein concentrations were determined with the BCA assay kit (Pierce). The proteins were immunodetected by anti-pRb antibody (1:1000, BD Pharmingen) that detects both unphosphorylated and phosphorylated pRb. Bound primary antibodies were detected with horseradish peroxidase-conjugated antibody to rabbit IgG (1:2000, Amersham Pharmacia Biotech). Detection was performed with chemiluminescence substrate (Pierce) and X-Omat X-ray film (Kodak), according to the manufacturer's instruction.

2.4. Quantification and statistical analysis

Specimens were examined by confocal microscopy (Leica SP5, Leica) using a 63× lens with 1 μm between optical sections. Images were processed with Leica software. Cells labeled with BrdU or cell markers were counted in one optical section that best represented the visual field. Cells were only counted when the nucleus comprised more than 50% of the cell area. Quantification was from at least five random areas over the length of an entire cochlea and data were presented as mean ± SEM. Statistical comparisons were made using one-way ANOVA with post hoc Tukey's tests or Student's two-tailed unpaired *t*-tests. Differences between groups were considered significant when $P < 0.05$.

3. Results

3.1. Shh promoted proliferation of postnatal cochlear sensory epithelial cells

We hypothesized that activation of Shh signaling could enhance proliferation and result in production of new hair cells in postnatal cochleae. We activated Shh signaling by adding Shh to neomycin-treated P2 rat cochlear organ cultures in the presence of BrdU for 5 days. Controls were cochlear cultures without any treatment or treated with neomycin or Shh alone, and we performed whole mount immunohistochemistry with anti-hair cell (Myo7a) and anti-supporting cell (Sox2) antibodies together with BrdU labeling.

Virtually no Myo7a-BrdU or Sox2-BrdU double-positive cells were detected in controls (Fig. 1A–O and E′). In the neomycin and Shh-treated group, however, there was a significant increase in the number of Myo7a-BrdU and Sox2-BrdU cells (Fig. 1P–E′). Double labeling of Myo7a-BrdU and Sox2-BrdU suggests that hair cells and supporting cells re-entered the cell cycle and incorporated BrdU. We also identified Myo7a-Sox2-BrdU triple-labeled cells, particularly in the region of outer hair cells (Fig. 1 P–T, Z–D′). We further tested if the Shh effect was dose-dependent. Treatment with 5, 10, or 50 nM Shh produced 16.53 ± 2.60, 17.59 ± 4.89, and 8.90 ± 0.47 ($n = 3$ for each group) Myo7a-BrdU-positive cells per millimeter of the cochlea, respectively. Significantly fewer of such cells were seen with 50 nM Shh ($P < 0.05$), but this number was still significantly greater than control or neomycin alone-treated cells indicating a general role of Shh in promoting proliferation in early postnatal cochlear epithelial cells. In explants treated with Shh and the Shh inhibitor cyclopamine, the number of Myo7a-BrdU cells was significantly reduced and this demonstrated that proliferation was Shh-dependent and could be specifically inhibited (Fig. 1E′).

Myo7a-BrdU-positive hair cells could result from hair cells re-entering the cell cycle, or from proliferating supporting cells that transdifferentiate into hair cells. A hair cell that re-enters the cell cycle should produce two Myo7a-BrdU-labeled cells, but new hair cells derived from transdifferentiation of dividing supporting cells could be labeled with the supporting-cell marker in addition to Myo7a-BrdU. During embryonic development young hair cells express Sox2, but this ceases in postnatal cochlear hair cells [13]. In addition to Myo7a-BrdU-positive cells that likely derived from hair-cell division (Fig. 1U–Y), we found Myo7a-Sox2-BrdU-positive cells (Fig. 1P–T, Z–D′) in the Shh-treated group that likely arose

Table 1
The oligonucleotid primer sequences and corresponding information.

Gene	Oligonucleotide primer sequence		Annealing temperature (°C)	Cycle number	Product length
Ptc1	F	5′TTCAGACTCCAAAAGAAGAAGGCG3′	56	35	494
	R	5′CAAAACAAGGGCCACATCAAGAG3′			
Ptc2	F	5′GCTTGCTGGTTCTAACTTTGAC 3′	56	35	297
	R	5′ACGTATGCTCCAGGTAGTGGT 3′			
Smo	F	5′TGCACAGTTACATCGCAGCCTTCG 3′	56	35	451
	R	5′TGAGCCACAGCAAGGATTGCCA 3′			
Gli1	F	5′CAACTCCACGAGCACACAGGATCA 3′	56	35	389
	R	5′TATAAGGCTTCTCACCGGTGTGCG 3′			
Gli2	F	5′AGGAGCAGCTGGTGCATCACATCA 3′	56	35	467
	R	5′ACGGAGGTGCACATCATTACGCTG 3′			
Gli3	F	5′TATGGACTACATCCGCTCCTTGC3′	52	35	450
	R	5′TAGGTGAAGCTCAAAGCAGGGC3′			
Pax2	F	5′ TCTGCCTCCCCATGGATATGCA 3′	50	35	319
	R	5′ GCTTGTATTCAGCAATCTTGTCCAC 3′			
Rb1	F	5′-TCTACCTCCCTTTCCCTGTTT-3′	50	35	549
	R	5′-AGTCATTTTTGTGGGTGTTGG-3′			
Cyclin B1	F	5′ CCGATGTGATTCTTGCAGTGAGTG 3′	57	30	372
	R	5′ AAGCGAAGTCACCGATCTCTGGAG 3′			
Cyclin D1	F	5′-TGGAGCCCCTGAAGAAGAG-3′	60	30	423
	R	5′-AAGTGCGTTGTGCGGTAGC-3′			
Cyclin D2	F	5′CGCTACCTCCCGCAATGTTCCTAT3′	56	35	449
	R	5′ATGAAGGTCTGCGCATGCTTGC3′			
Cyclin D3	F	5′AGGATGTCTTCCCTCTGGCTATG3′	50	35	374
	R	5′AGGGTACATCGCAAAGGTGTAATC3′			
Cyclin E	F	5′-CTGGCTGAATGTTTATGTCC-3′	58	32	386
	R	5′-TCTTTGCTTGGGCTTTGTCC-3′			
CDK1	F	5′ TCAGCCTGCAGGATGTACTCATGC 3′	58	30	384
	R	5′ TCCCTATGCTCCAGATGTCAACCG 3′			
GAPDH	F	5′ ATTGTTGCCATCAACGACC 3′	58	30	450
	R	5′ CATGGACTGTGGTCATGAGC 3′			

F, forward primer; R, reverse primer.

from transdifferentiation of proliferating Sox2-positive supporting cells into hair cells. No Myo7a-Sox2-BrdU-positive cells were seen in any of the control groups, suggesting that their appearance is Shh-dependent (Fig. 1E′). In triple-labeled cells, the levels of Myo7a and Sox2 were variable, including cells expressing robust Myo7a and weak Sox2 (arrows in Fig. 1Z–D′) and cells expressing weak Myo7a and relatively normal levels of Sox2 (arrowheads in Fig. 1Z–D′). Such variations likely represent different stages of transdifferentiation and are consistent with recent studies indicating that Sox2 downregulation correlates with differentiation of hair cells [14].

We also studied the progenitor cell marker Pax2 in Shh-induced proliferation. Pax2 is primarily expressed in the developing sensory primordium and early postnatal mouse cochlear hair cells, and disappears within the first week after birth [15]. By semi-quantitative RT-PCR, we found that Pax2 mRNA was upregulated upon Shh treatment (Fig. 2M). We performed double labeling of Myo7a and Pax2 (Myo7a-Pax2) to determine if Shh-induced hair cells express Pax2. We found a significant increase in the number of Myo7a-Pax2 double-positive cells in neomycin and Shh-treated samples (Fig. 2I–L, N) compared to controls (Fig. 2A–H). The number of Myo7a-Pax2-positive cells was significantly decreased after Shh inhibition (Fig. 2N). These results are consistent with the Myo7a-Sox2-positive cells observed in the Shh-treated group, and provide additional evidence that the induction of supporting cell proliferation can lead to transdifferentiation into hair cells.

3.2. Shh activated downstream targets in postnatal cochleae

Shh signaling acts through a series of downstream targets including the receptors patched (Ptc) and smoothened (Smo) and the Gli family of transcription factors (Gli1–3). Semi-quantitative RT-PCR identified the downstream targets involved in Shh-induced proliferation in postnatal cochleae. Five days after treatment with Shh, we found upregulation of a subset of Shh downstream genes, including Ptc1, Gli1, and Gli3. Ptc2 was downregulated and other Shh downstream genes, including Smo and Gli2, showed no significant changes in expression (Fig. 3A). Thus Ptc1, Gli1, and Gli3 were specifically activated by Shh in rat cochlear explants and likely contribute to the promotion of cochlear hair cells and proliferation of supporting cells.

3.3. Shh promoted proliferation through inhibition of pRb function

Rb1 is required for cell cycle exit by both hair cells and supporting cells, and inhibition of pRb leads to sustained proliferation of both cell types [6,7,9]. To determine if Shh plays a role in the context of pRb function, and thus in renewed proliferation, we analyzed Rb1 mRNA and protein expression levels. Both Rb1 mRNA and pRb protein were downregulated in neomycin and Shh-treated cochleae (Fig. 3B). The function of pRb depends on its phosphorylation status, which is regulated by cyclin-dependent kinases (Cdks). Unphosphorylated pRb suppresses the cell cycle regulator E2f1 forcing cell cycle exit and preventing cell cycle re-entry. Upon phosphorylation by Cdks, pRb releases E2f1 allowing E2f1 to activate its target genes to initiate proliferation [16,17]. Consistent with the pRb state, immunoblotting detected a larger band corresponding to phosphorylated pRb (ppRb) in the sample treated with Shh, but only unphosphorylated product (pRb) was detected in controls (Fig. 3B). This suggests that Shh signaling not only inhibits pRb by reducing mRNA and protein levels, but also inhibits pRb through phosphorylation.

During proliferation cell cycle genes, including cyclins and cyclin-dependent kinases, are activated. Fig. 3C shows that the mRNA levels of cyclin B1, cyclin D2, cyclin D3, and CDK1 were upregulated in the Shh-treated cochleae, but cyclin D1 and cyclin E mRNA levels remained constant.

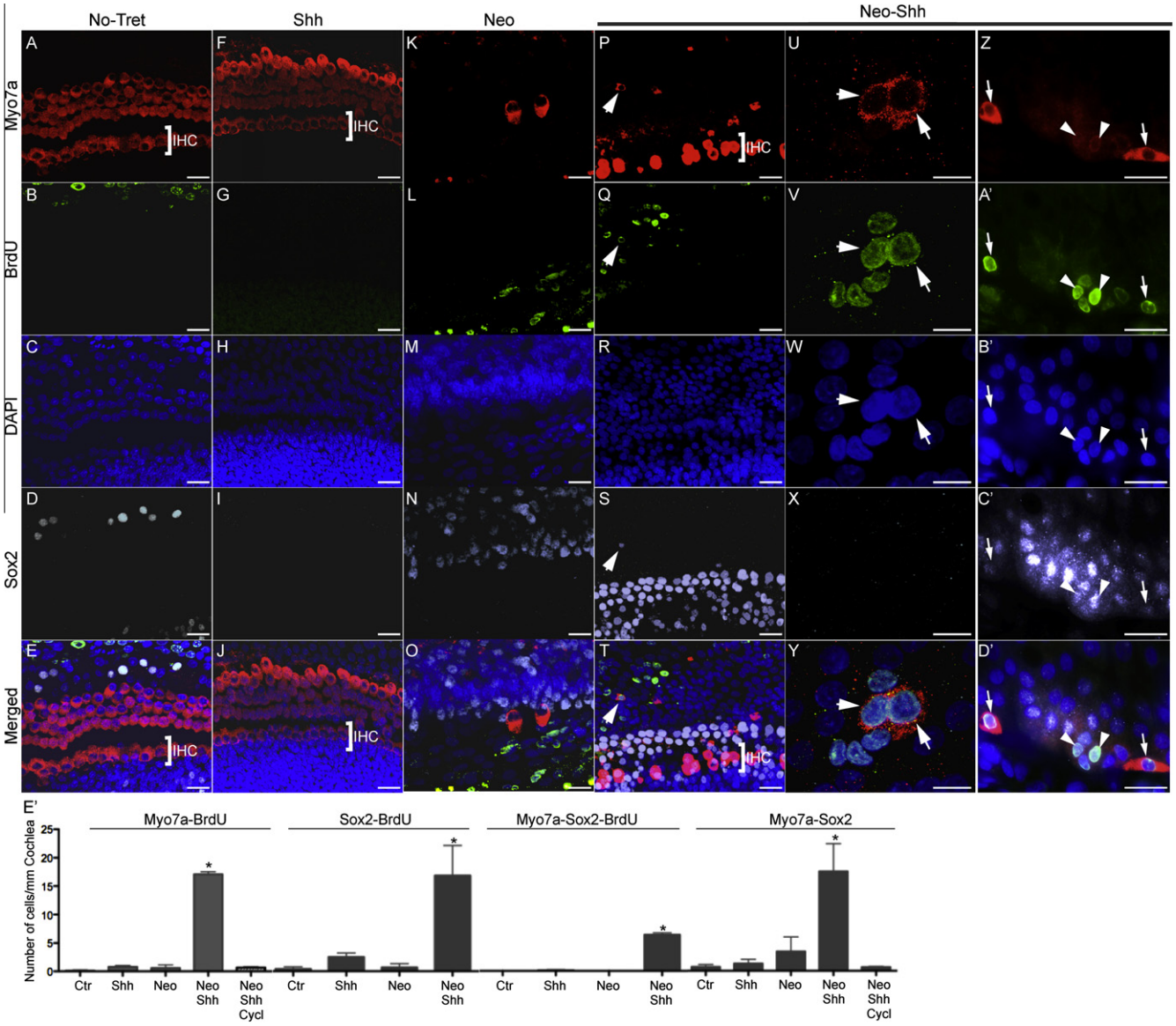

Fig. 1. Shh promotes proliferation of hair cells and supporting cells. In cultured untreated (A–E) and Shh-treated (F–J) P2 rat cochleae, no BrdU-positive hair cells or supporting cells were seen. In the Shh-treated control sample, the image was focused on the hair cell layer (F). (K–O) In cultured P2 cochleae treated with neomycin, no Sox2-BrdU or Myo7a-BrdU cells were seen in the sensory region. The number of hair cells was greatly reduced due to neomycin's effect (K). (P–D′) A significant increase in the number of Myo7a-BrdU and Sox2-BrdU cells was seen upon neomycin and Shh treatment. (P–T) An example of Myo7a-Sox2-BrdU is indicated (arrow) in the vicinity of the outer hair cell region that was opposite the inner hair cell region. (U–Y) A Myo7a-BrdU doublet was seen (arrows) that was not labeled with Sox2 (X), an indication of a hair cell division. (Z–D′) A cochlear region showed two BrdU cells with strong Myo7a and weak Sox2 (arrows), and two BrdU cells with weak Myo7a and relatively normal Sox2 (arrowheads). Scale bars: 20 μm. (E′) Shh treatment enhanced proliferation after hair cell damage as evidenced by a significant increase in Myo7a-BrdU and Sox2-BrdU cells. There was also a significant increase in the number of Myo7a-SOx2-BrdU cells, an indication of supporting cells transdifferentiating into hair cells. Upon treatment with cyclopamine (Cycl), an Shh inhibitor, the number of Myo7a-BrdU ad Myo7a-Sox2 cells was significantly reduced ($P < 0.05$ vs. control groups in pairwise comparisons, Student's t-test, $N \geqslant 4$ in each group). The number of cells was the average from at least five 700-μm regions over the length of an entire cochlea.

4. Discussion

In situ cochlear hair cell regeneration should be explored as one of the main approaches to restore hearing that is lost due to hair cell loss. A growing body of evidence suggests that postnatal cochlear supporting cells can divide and differentiate or transdifferentiate into hair cells [5,18,19]. Not all supporting cells have the capacity to proliferate, but a subset of cells in postnatal cochleae are capable of proliferating and differentiating into hair cells when isolated and cultured *in vitro* [5,20]. We found that Shh could trigger postnatal rat cochlear epithelial cell proliferation and production of hair cells after ototoxic damage. Some of the Shh-induced hair cells expressed early hair cell markers Sox2 and Pax2, indicating that they likely resulted from transdifferentiation that followed developmental sequences. However, we cannot rule out the possibility that upon Shh treatment existing hair cells divide along with induction of progenitor genes. Future lineage-tracing experiments using a transgenic mouse model to permanently mark supporting cells could address this question unequivocally.

The hedgehog pathway is essential for inner ear development, but the exact roles of this signaling are not fully understood. In Shh knockout mice the auditory compartment, including Kölliker's organ and spiral ganglion cells, does not form. This indicates that auditory cell fates in otic vesicles are established by the direct action of Shh [11]. Nevertheless, in a mouse model of Pallister–Hall syndrome that produces only the truncated, repressor form of

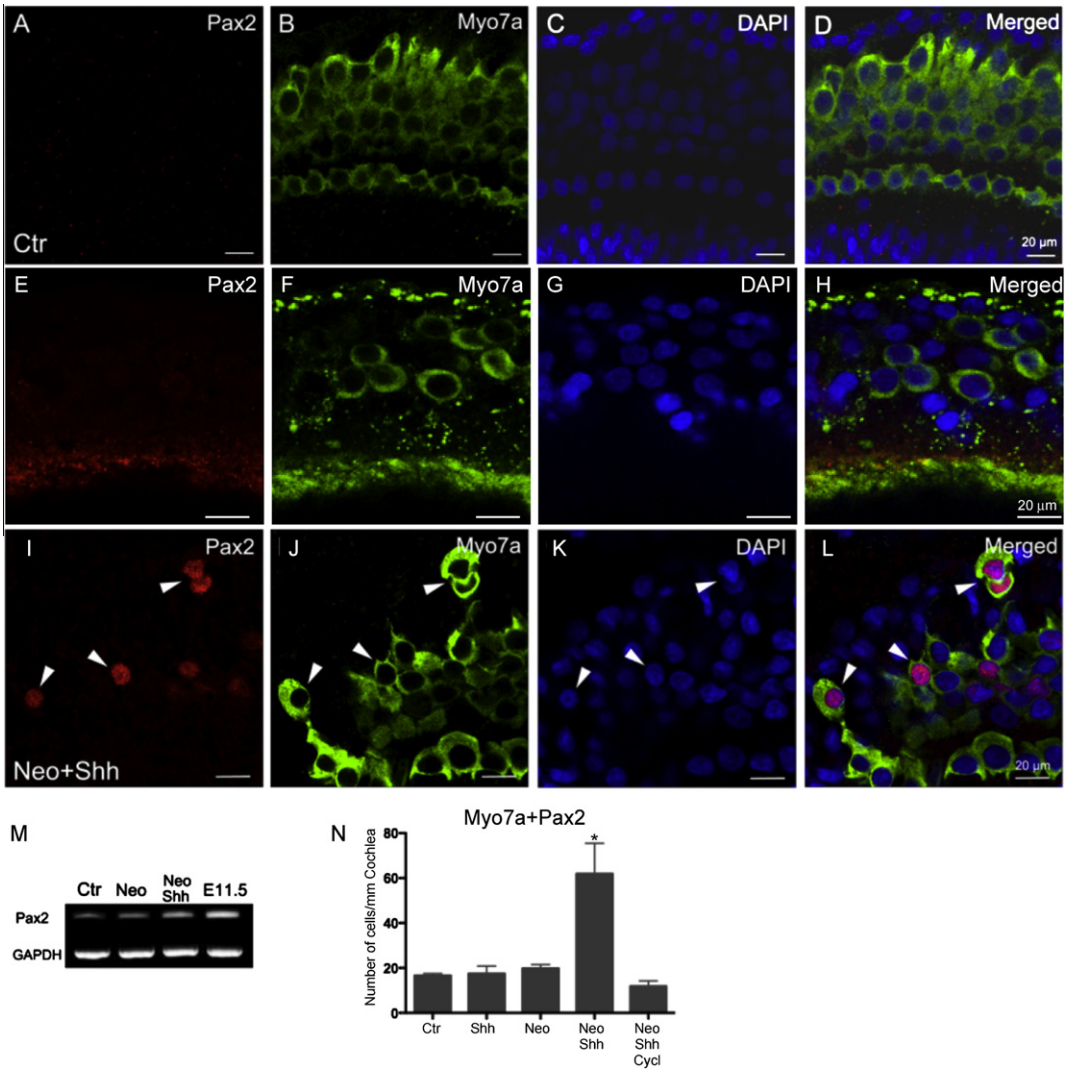

Fig. 2. Shh treatment increases Pax2 expression. (A–D) In the untreated control cochleae, Pax2 was not expressed in hair cells after culturing for 5 days. (E–H) In the cochleae treated with neomycin alone, few Myo7a-Pax2 cells were seen after culturing for 5 days. (I–L) In Shh-treated neomycin-damaged cochleae, an increase in the number of Myo7a-Pax2 double-positive cells was observed (arrowheads indicate representative Myo7a-Pax2 cells). (M) RT-PCR showed upregulation of Pax2 mRNA after Shh treatment to a level that was comparable to the otocyst at embryonic day 11.5, but the Pax2 level was lower in neomycin-treated and untreated P2 cochleae. (N) The number of Myo7a-Pax2 cells was significantly increased in the Shh-treated cochleae, but the number was greatly reduced after Shh inhibition by cyclopamine. Scale bars: 20 μm. ($P < 0.01$ vs. untreated or neomycin-treated control groups. $N = 4$ for each group).

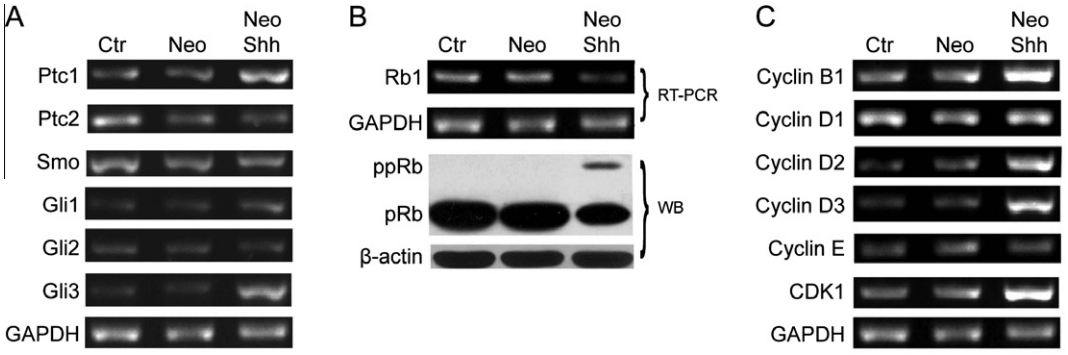

Fig. 3. Regulation of gene expression and phosphorylation by Shh. (A) RT-PCR showed that Shh treatment resulted in upregulation of downstream genes including Ptc1, Gli1, and Gli3. Ptc2 was downregulated and expression of Smo and Gli2 was unchanged, (B) RT-PCR and Western blots (WB) showed that Shh treatment decreased the expression levels of Rb1 mRNA and pRb protein, and led to an increase in phosphorylated pRb (ppRb) and (C) RT-PCR showed that Shh increased the mRNA levels of cyclin B1, cyclin D2, cyclin D3, and CDK1, but had no obvious effect on cyclin D1 or cyclin E levels.

Gli3 (a downstream effector of the Shh pathway), larger ectopic sensory patches in Kölliker's organ are observed [21]. Furthermore, exogenous Shh played different roles in cochlear progenitor cell proliferation and prosensory formation according to their state. When the inner ear progenitor cells were dissolved and isolated from otic vesicles, exogenous Shh promoted proliferation and subsequent differentiation into hair cells [12].

It has been shown that enhanced differentiation from progenitor cells into hair cells upon Shh pathway activation involves upregulation of Atoh1 that leads to Pou4f3 activation [22]. Our data support a role for Shh in promoting proliferation and differentiation. However, Shh has also been shown to inhibit prosensory formation and hair cell differentiation when newly formed cochlear epithelia are cultured in whole mounts [21]. It remains to be determined where the differences come from in those studies, but one possibility is that Shh plays different roles that are stage-dependent. In the studies in which Shh promoted hair cell differentiation, the effects were observed in neonatal tissue, but the inhibitory effect was observed in early embryonic tissue at day 13. Systematic evaluation of Shh function at different developmental stages may shed light on this matter.

Activation of Shh represents a novel way to modulate pRb function in the context of proliferation and hair cell differentiation. Rb1 is required for the quiescent postmitotic state as well as survival of hair cells and supporting cells, and our results showed that pRb could be effectively and temporally inhibited by Shh at the transcription, protein, and phosphorylation levels leading to up-regulation of cell cycle positive regulators cyclin B1, cyclin D2, cyclin D3, and CDK1. This conclusion is consistent with our recent microarray expression profiles of hair cell-specific Rb1 deletion mice in which both cyclins B1 and D2 were upregulated [23]. Thus upregulation of some cyclins in response to Shh treatment resembles upregulation in the Rb1 knockout inner ear, strongly suggesting that Shh may directly inhibit pRb function.

Our results are also consistent with recent studies on the role of Shh in proliferation in different tissues. Shh interacts with Atoh1 to promote proliferation of granule neuron precursors via Gli2 during development of medulloblastoma [24]. Further, Shh has been shown to activate E2f1, promote lipogenesis, and downregulate pRb, which leads to proliferation in medulloblastoma [25]. Thus reduction in Rb1 expression and pRb activity by Shh probably contributes similarly to proliferation of cochlear supporting cells and hair cells, which leads to the production of new hair cells. Although complete deletion of Rb1 leads to proliferation and cell death in cochleae, it is possible that transient inhibition of pRb could lead to renewed proliferation yet maintain the survival of proliferating cells. Our study thus offers an opportunity to explore how Shh signaling can transiently modulate pRb function to induce cell cycle re-entry and hair cell regeneration in the mature mammalian inner ear.

Acknowledgments

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