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

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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.

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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