Regulation of Supporting Cell Proliferation in the Mouse Cochlea

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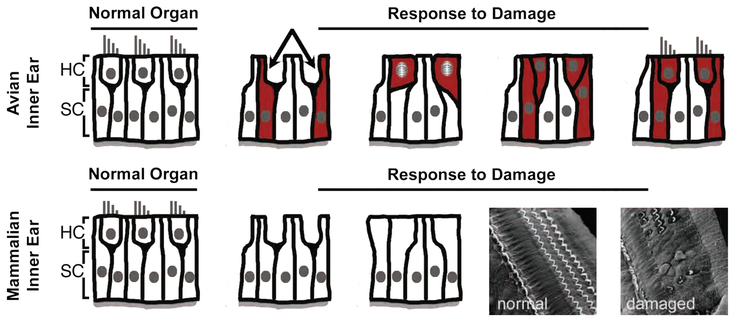
Introduction

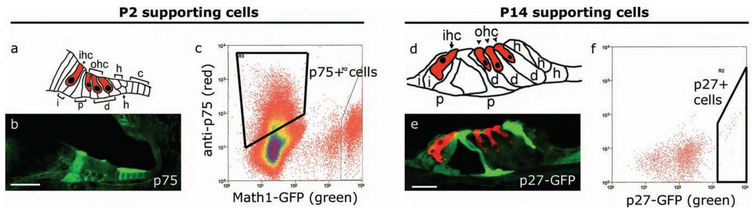
No Regeneration is Observed in the Damaged Mammalian Organ of Corti



We have previously shown that puri□ed mammalian cochlear supporting cells, isolated from either neonatal or juvenile mice, can both transdi□erentiate into sensory hair cells, but that only neonatal, not juvenile supporting cells, could respond to culture with embryonic mesenchymal cells with proliferation. Here we address how proliferation is regulated. As proliferation is the □rst response made by regenerating tissue, understanding its regulation could yield important insights into the entire process. Moreover, any therapy designed to stimulate regeneration of sensory hair cells from supporting cells will need to include some way of replacing the lost cells in the damaged area.

Methods of Cell Puri□cation

Supporting Cells Can Be Puri□ed From Neonatal & Juvenile Mouse Cochlea For Analysis

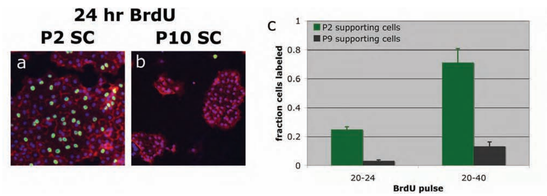


We have previously shown that supporting cells may be isolated from neonatal (P2) cochleae with an antibody to p75 (a, b) using □uorescence-activated cell sorting (c). This subset includes pillar cells (p) Hensen's cells (h), and Claudius cells (c). A similar subset of supporting cells can be isolated from juvenile (P14) mice using the p27-GFP BAC transgenic mouse (d-f): in this case we can obtain pillar cells, Hensen's cells, and interphalangeal cells (i). Boxes in (c) & (f) enclose the populations expressing either p75, for the neonatal, or p27-GFP, for the juvenile.

In our previous results, we determined that P2 supporting cells proliferated in de□ned medium when cultured with embryonic periotic mesenchyme, but that P14 supporting cells did not. In order to analyze the signal(s) regulating cell cycle re-entry in this system, we wanted to reduce its complexity while preserving whatever conditions allowed P2 supporting cells to proliferate.

Results

Puri□ed Neonatal Supporting Cells Retain the Age-Dependent Ability to Proliferate in the Absence of Periotic Mesenchyme



d

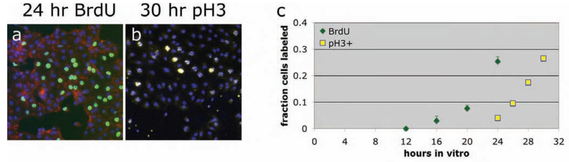
Cell Recovery	24 hours	40 hours
P2	86% +/- 6%	117% +/- 13%
P9	49% +/- 12%	49% +/- 7%

P2 and P10 supporting cells were collected as described, and placed in adherent culture alone in de□ned medium containing EGF (a, b). Cultures were pulsed with BrdU as indicated in (c), □xed, and stained for BrdU (green), E-cadherin (red) and with Hoechst (blue). E-cadherin was used to con□rm that the cells analyzed were epithelial cells. 25.2% +/- 1.7% of P2 supporting cells incorporated BrdU between 20-24 hours, and 71.1% +/- 9.6% incorporated BrdU between 20 and 40 hours (c, green bars). Moreover, more cells were observed at 40 hours compared to 24 hours, suggesting that some cells had completed one round of division.

In contrast to P2 supporting cells, supporting cells taken from P10 animals did not re-enter the cell cycle in signi□cant numbers. 3.3% +/- 0.7% of P10 supporting cells took up BrdU by 24 hours, and 13% +/- 2.9% incorporated it by 40 hours in vitro (c, black bars). There was no signi□cant change in P10 cell numbers over this period (d).

These data demonstrate that a cell-intrinsic change occurs in pillar and Hensen's cells between the neonatal and juvenile stage that prevents cell cycle re-entry, and is not related to the mesenchymal cells used in previous experiments.

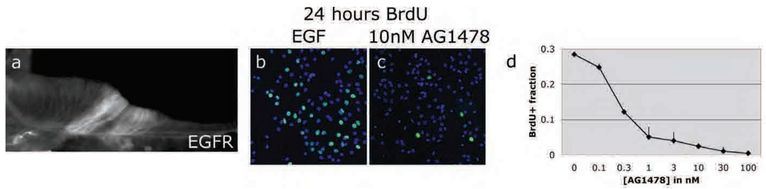
Time Course of Cell Cycle Re-Entry for P2 Supporting Cells



The time course of cell cycle re-entry was determined for P2 supporting cells. Cells were pulsed with BrdU from 0-12, 12-16, 16-20, or 20-24 hours, □xed at the end of the pulse, and stained for BrdU, E-cadherin, and with Hoechst. Unpulsed cultures were □xed at 24, 26, 28 and 30 hours and stained for phosphohistone-3 to reveal cells entering G2/M.

These data indicate that signaling events between 0 and 16 hours are implicated in cell cycle re-entry.

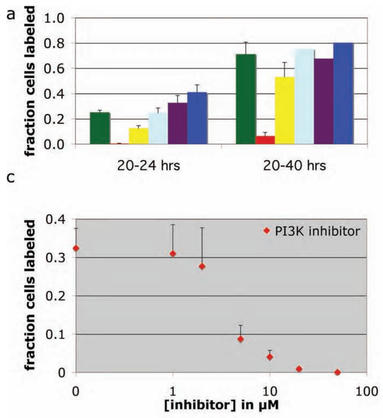
Supporting Cell Re-Entry Requires EGFR/ErbB Signaling



All four members of the ErbB family of receptors are expressed in the neonatal and adult mouse cochlea (Hume et al, JARO 4(3): 422-441 2003). We show antibody staining for EGFR (a) for demonstration purposes. We have found through quantitative PCR that puri□ed P2 and P10 supporting cells express similar amounts of EGFR and ErbB2 mRNA (data not shown). Nonetheless, blockade of erbB receptor signaling through the use of 10nM AG1478 completely inhibits BrdU uptake without changing cell survival signi□cantly at 24 hours (88.5% +/- 6.1% for control vs 73.5% +/-5.1% for 10nM, n=3, p=0.13, Student's t-test; survival at at 30 and 100 nM were similar or better).

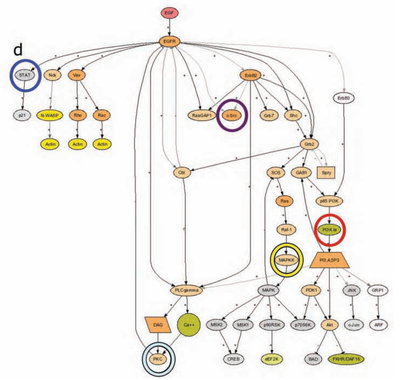
These data implicate signaling through erbB family members in cochlear supporting cell proliferation in the mouse. These data are consistent with other reports implicating erbB family members in utricular supporting cell proliferation. (Yamashita & Oesterle, PNAS 92:3152-55 1995; Zheng et al J. Neurosci 17:216-26 1997; Montcouquiol & Corwin J. Neurosci 21:570-80 2001).

Supporting Cell Re-Entry Requires Phosphoinositide 3-Kinase Activity



b

Target Protein	Inhibitor	Concentration
PI3K	LY294003	20 μM
MEK	PD98059	20 μM
PKC	BIM1	10 μM
c-SRC	SU6656	25 μM
STAT3	STAT3 inhibitory peptide	100 μM



To determine which downstream e□ectors of erbB signaling are necessary for proliferation, we applied inhibitors to speci□c pathways to cultures of puri□ed P2 supporting cells and assayed BrdU incorporation at 24 hours and 40 hours as indicated (a, green bars are EGF-only controls). The pathways are identi□ed by colors as shown in (b), with reagents and concentrations used. At 24 hours, we observed a reduction in BrdU incorporation when cultures were incubated with inhibitors to either phosphoinositide 3-kinase (PI3K) or mitogen-activated protein kinase kinase 1 (MEK). Curiously, blocking STAT3 potentiated proliferation at 24 hours. (overall ANOVA p<10E-12; individual ttests for PI3K, MEK, and STAT3 p<0.001, n=4 or more). Only PI3K inhibition signi□cantly reduced proliferation by 40 hours (ANOVA p=0.0001; PI3K ttest p<0.001, MEK p=0.17, n=4).

These results suggest that only one downstream e□ector of erbB signaling, PI3K, is necessary for mitosis. Indirectly, they suggest erbB signaling might also interact with other pathways, namely MAPK and STAT3, which may change the rate at which cells re-enter the cell cycle.

Conclusions

1. Mammalian cochlear supporting cells, puri□ed by p75 expression from neonatal animals, proliferate when cultured alone in de□ned medium. The same subset of cells puri□ed by p27-GFP expression from young juvenile animals does not.

2. Mammalian cochlear supporting cells initiate S-phase, determined by BrdU incorporation, starting at 16 hours in vitro, and begin G2/M, determined by phospho-histone3 expression, about 8 hours later. By 40 hours, at least some of the cells have completed mitosis.

3. Cell cycle re-entry requires erbB signaling; however, both neonatal and juvenile supporting cells express similar levels of EGFR and erbB2.

4. Among the pathways tested that are downstream of erbB signaling, only PI3K is necessary for cell-cycle re-entry.

Given these results, we suggest that in puri□ed neonatal supporting cells, erbB signaling connects to PI3K and is required for cell cycle re-entry. This signaling pathway is somehow inaccessible or inhibited in the same cells puri□ed from P10 cochleae, as well as in intact neonatal tissue.

Experiments to determine the activation state of these signaling molecules in P2, P10, and undissociated cochlear epithelial tissue are ongoing, as are experiments to test the su□ciency of activating PI3K or its downstream target AKT in overcoming the mitotic block in juv enile cells.