Regulation of Supporting Cell Proliferation in the Mouse Cochlea

House Ear Institute
Advancing Hearing Science

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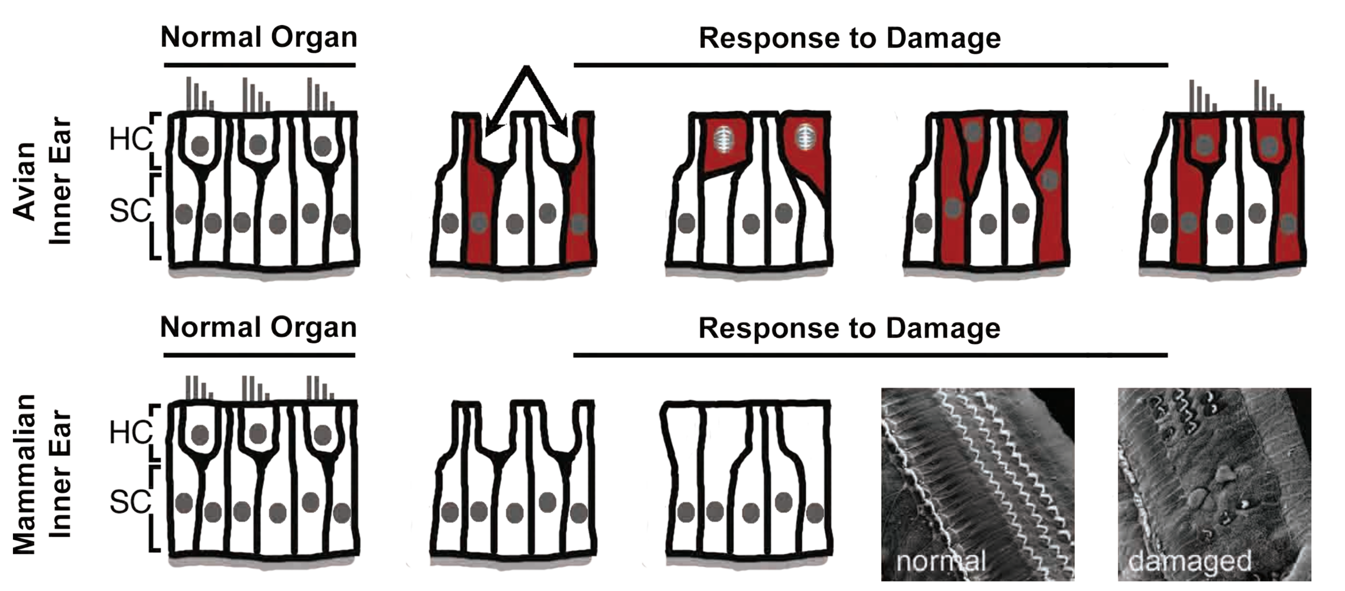
Introduction

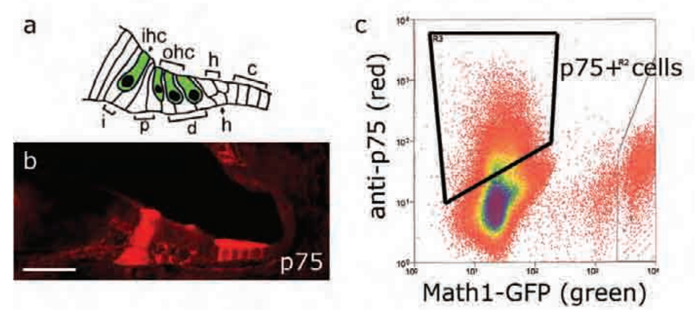
No Regeneration is Observed in the Damaged Mammalian Organ of Corti



We have previously shown that purified mammalian cochlear supporting cells, isolated from either neonatal or juvenile mice, can both transdifferentiate 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 first 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 Purification

Supporting Cells Can Be Purified From Neonatal & Juvenile Mouse Cochlea For Analysis

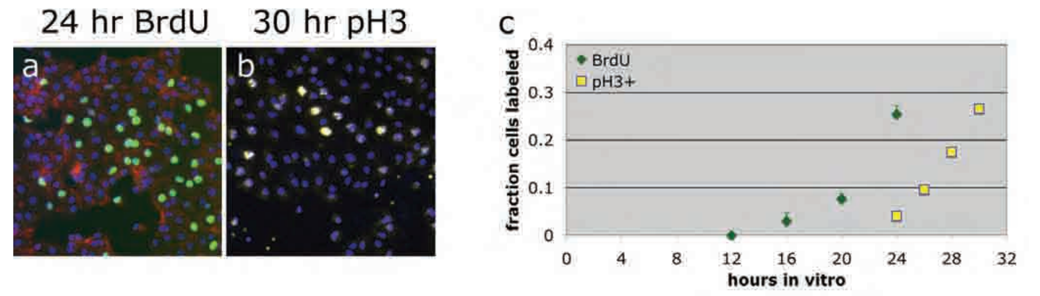


We have previously shown that supporting cells may be isolated from neonatal (P2) cochleae with an antibody to p75 (a, b) using fluorescence-activated cell sorting (c). This subset includes pillar cells (p) Hensen's cells (h), and Claudius cells (c). The box in (c) encloses the p75+ population.

In our previous results, we determined that P2 supporting cells proliferated in defined 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

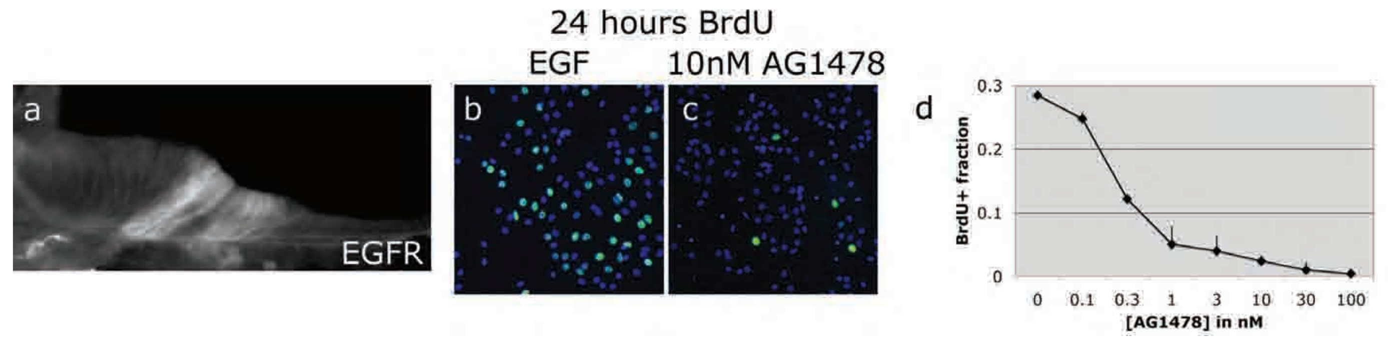
Time Course of Cell Cycle Re-Entry for P2 Supporting Cells



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, fixed at the end of the pulse, and stained for BrdU, E-cadherin, and with Hoechst. Unpulsed cultures were fixed 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.

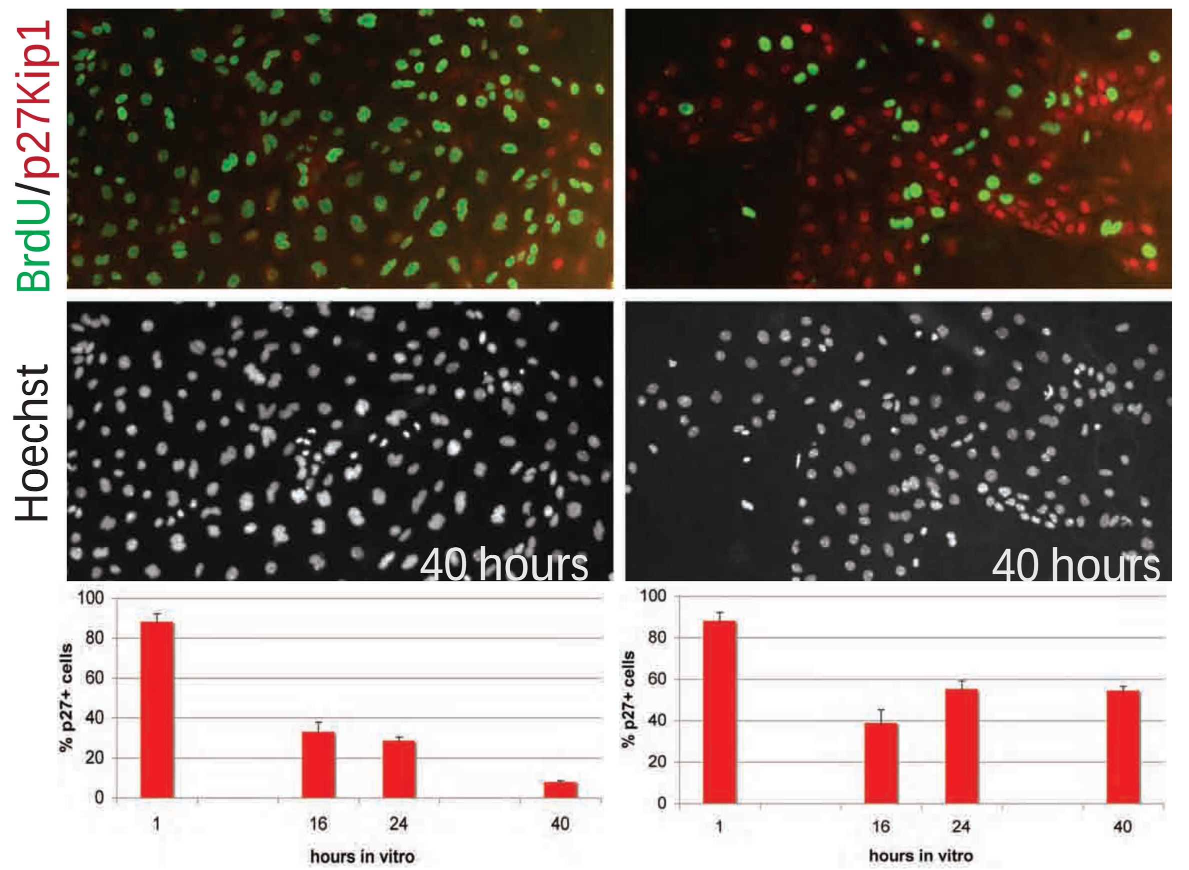
Supporting Cell Re-Entry Requires EGFR/ErbB Signaling



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 purified 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 significantly 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).

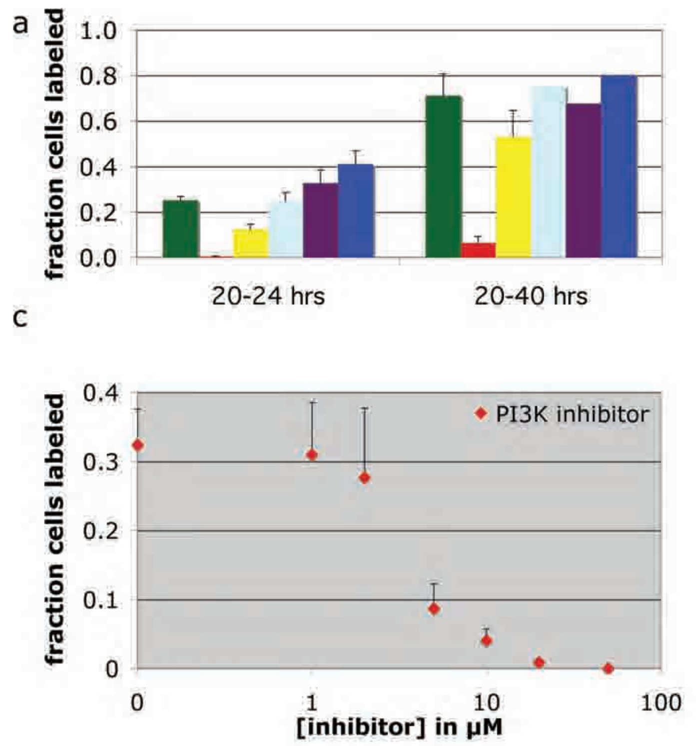
EGFR/ErbB Signaling down-regulates p27Kip1 in culture



In order to determine whether erbB signaling regulates proliferation through p27Kip1, supporting cells were plated in EGF in the absence (left) or presence (right) of the 20 nM AG1478. The cultures shown were also incubated with BrdU between 20 and 40 hours, to label all dividing cells (top panels, green). At 40 hours, the cells were fixed and stained for BrdU (green) and p27Kip1 (red). The red nuclei, therefore, are the cells that maintain p27Kip1 and do not enter S phase.

Such cells are quantified in the graphs. By 40 hours, only 8% of cells cultured in EGF maintain p27Kip1 and do not enter S-phase; in these cultures 87% of the remaining cells label with BrdU. Where erbB signaling is blocked, 54% of cells in sister cultures maintain p27Kip1 expression; 29% of the remaining cells label with BrdU (n=5; p<0.0001)

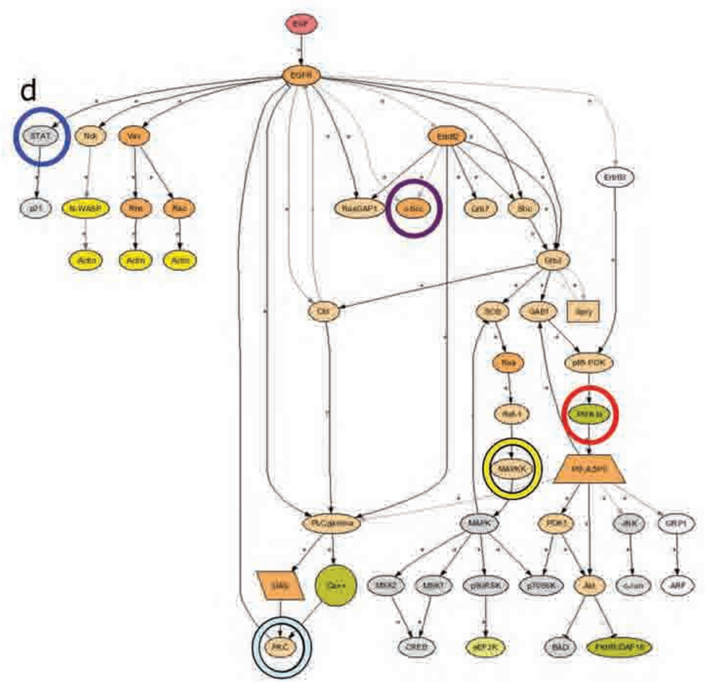
Supporting Cell Re-Entry Requires Phosphoinositide 3-Kinase Activity



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

d



To determine which downstream effectors of erbB signaling are necessary for proliferation, we applied inhibitors to specific pathways to cultures of purified 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 identified 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 significantly 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 effector 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.

Phosphoinositide 3-Kinase Activity regulates Early Gene Expression by Supporting Cells in Culture

To identify what genes are regulated by PI-3K in vitro, P2 supporting cells were purified as described and placed in adherent culture containing either EGF (EGF)or EGF + 20µM LY294003, the PI-3K inhibitor (INH). Cultures were lysed after 6 hours, or in early G1. An equal number of uncultured cells were lysed at the start of the experiment and analyzed simultaneously (C, control). For each experiment, greater than 95% of the supporting cells analyzed expressed E-cadherin, and at least 20% of the cells incorporated BrdU between 20 and 24 hours in sister cultures. Three experiments were performed.

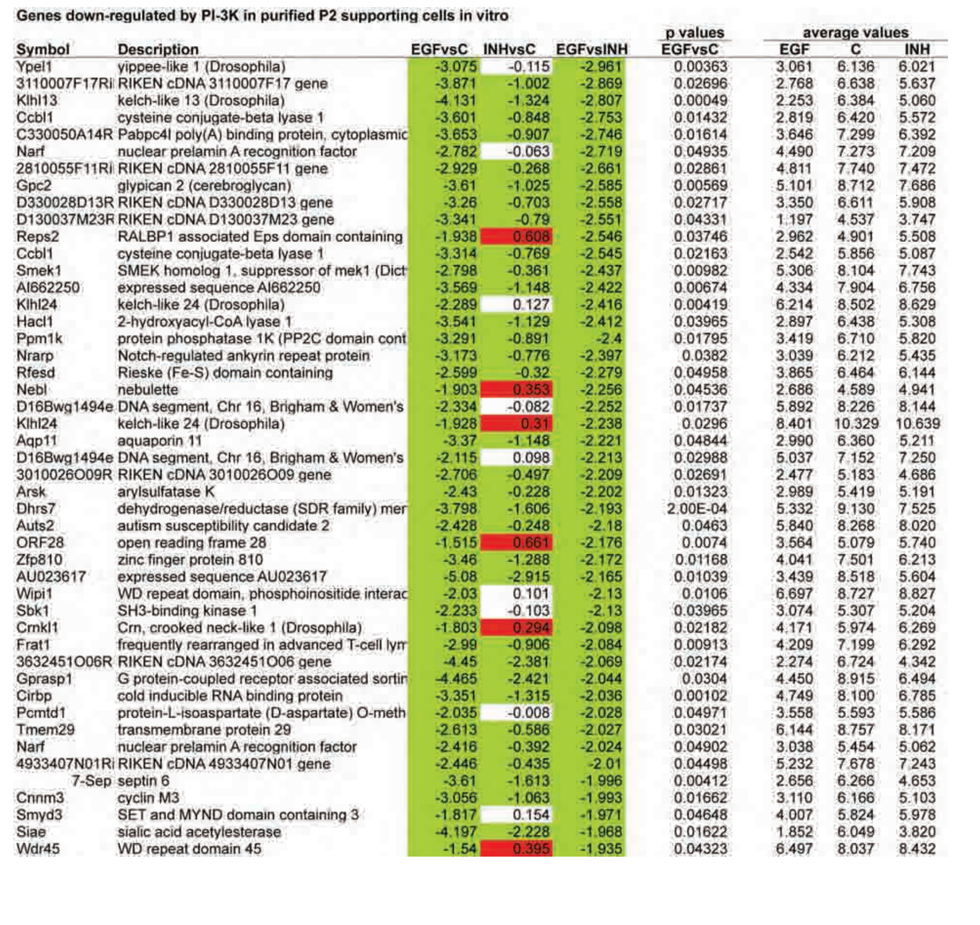
The lysates were analyzed on the Affymetric Mouse 4302 gene array, which has 45101 probes covering most of the mouse genome. The average backgrounds for each sample were similar, and the scale factors were within 3 fold for most samples. The signal was RMA normalized. Clustering based on the Pearson's correlation between arrays showed that the samples with the same condition tended to cluster together. EGF and INH samples formed a group, in that they had more similarity than that of the other comparisons. Overall, with a statistical threshhold of p<0.05, 4268 genes are differentially expressed between the three samples.

We present three tables:

1. Genes up-regulated in EGF compared to control (uncultured) and PI3K inhibitor, which are the genes positively regulated by PI3K
2. Genes down-regulated in EGF compared to control (uncultured) and PI3K inhibitor, which are genes negatively regulated by PI3K
3. Genes up-regulated in EGF compared to control only.

These data are still preliminary. QPCR confirmation of up-regulation and down-regulation are ongoing.

Genes Regulated by PI3K



Genes induced by culture

Conclusions

1. Purified neonatal supporting cells, cultured alone in defined medium, re-enter the cell cycle with S-phase beginning at 16 hours and G2/M at 24 hours. Almost 90% are labeled with BrdU during the first 40 hours.

2. Cell cycle re-entry is dependent on erbB signaling, as the erbB inhibitor AG1478 blocks BrdU uptake in the first 24 hours in a dose-dependent manner.

3. Absent erbB signaling, slightly over half of purified neonatal supporting cells maintain p27Kip1 expression and and this likely plays a role in their failure to divide.

4. Of 5 signaling pathways downstream of EGF, only phosphoinositide 3-kinase was found to be necessary for BrdU incorporation.

5. Purified neonatal supporting cells induce significant transcriptional changes in response to cell culture; investigations into the significance and function of these genes is ongoing.

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