Published in final edited form as:

Eur J Neurosci. 2018 November ; 48(10): 3299–3316. doi:10.1111/ejn.14183.

ERBB2 signaling drives supporting cell proliferation *in vitro* and apparent supernumerary hair cell formation *in vivo* in the neonatal mouse cochlea

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Abstract

In mammals, cochlear hair cells are not regenerated once they are lost, leading to permanent hearing deficits. In other vertebrates, the adjacent supporting cells act as a stem cell compartment, in that they both proliferate and differentiate into *de novo* auditory hair cells. Although there is evidence that mammalian cochlear supporting cells can differentiate into new hair cells, the signals that regulate this process are poorly characterized. We hypothesize that signaling from the epidermal growth factor receptor (EGFR) family may play a role in cochlear regeneration. We focus on one such member, ERBB2, and report the effects of expressing a constitutively active ERBB2 receptor in neonatal mouse cochlear supporting cells, using viruses and transgenic

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Author Contributions. JZ, AE, and PMW designed experiments; JZ, QW, and DAA performed experiments; JM designed and constructed the viruses; JZ, QW, AE and PMW analyzed data; and AE and PMW wrote the paper.

Competing Interests.

AE is a co-founder and scientific advisor to Decibel Therapeutics.

Data Accessibility.

All original data files are available on request to the contributing author, Patricia White.

expression. Lineage tracing with fluorescent reporter proteins was used to determine the relationships between cells with active ERBB2 signaling and cells that divided or differentiated into hair cells. *In vitro*, individual supporting cells harboring a constitutively active ERBB2 receptor appeared to signal to their neighboring supporting cells, inducing them to down-regulate a supporting cell marker and to proliferate. *In vivo*, we found supernumerary hair cell-like cells near supporting cells that expressed ERBB2 receptors. Both supporting cell proliferation and hair cell differentiation were largely reproduced *in vitro* using small molecules that we show also activate ERBB2. Our data suggests that signaling from the receptor tyrosine kinase ERBB2 can drive the activation of secondary signaling pathways to regulate regeneration, suggesting a new model where an interplay of cell signaling regulates regeneration by endogenous stem-like cells.

Graphical Abstract

Keywords

cochlear regeneration; SOX2; WS3; WS6

Introduction

Hearing loss affects 12% of individuals over the age of twelve, or around 30 million Americans (NIDCD, 2010). The likelihood of having bilateral hearing loss doubles each decade after the age of fifty (Bainbridge & Wallhagen, 2014), to the point that over 60% of people aged 70 or older have hearing loss (Lin *et al.*, 2011). Outer hair cell loss is a significant factor in many kinds of hearing loss (Crowe *et al.*, 1934; McGill & Schuknecht, 1976), largely because these specialized acoustic amplifying cells do not regenerate their numbers if they die (Chardin & Romand, 1995). The goal of this work is to test a candidate signaling pathway for its ability to drive the early cellular activities of cochlear regeneration: proliferation of supporting cells and their trans-differentiation into hair cells (Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Brignull *et al.*, 2009), with an emphasis on outer hair cell trans-differentiation.

Ligands and receptors from the epidermal growth factor (EGF) family were implicated in mouse utricular supporting cell proliferation in early experiments (Kuntz & Oesterle, 1998; Hume *et al.*, 2003). In other epithelial organs, members of the epidermal growth factor receptor (EGFR) family can be activated through tissue stretch damage to induce proliferation (Vermeer *et al.*, 2003), making this signaling pathway an attractive candidate for sensing acoustic injury in the cochlea. *In vitro*, EGF promotes hair cell differentiation from dissociated embryonic cochlear precursors (Doetzlhofer *et al.*, 2004) and promotes the proliferation of dissociated mouse cochlear supporting cells (White *et al.*, 2012). Moreover, avian auditory supporting cells require signaling from the EGFR family for proliferation in regeneration experiments *in vitro* (White *et al.*, 2012). Other experiments have implicated canonical downstream signaling molecules of the EGFR family such as PI3K (Montcouquiol & Corwin, 2001). However, there are no reports that adding exogenous EGF family ligands affects proliferation in mouse cochlear organ cultures. Thus, it is unclear what role, if any, that signaling from the EGFR family may play in stimulating the cellular activities of cochlear regeneration.

To test the hypothesis that signaling through the EGFR family can effect either proliferation or hair cell differentiation in supporting cells, we use multiple tools to drive such signaling in mouse cochlear supporting cells. First, we generated a custom adenovirus that drives expression of a mutated member of the EGFR family, ERBB2, with constitutive activity (CA-ERBB2). We used this virus to infect neonatal mouse cochleae *in vitro*. We assayed these cultures for supporting cell marker expression and proliferation. Second, we used a tetracycline inducible transgene (TET-ON), also encoding a constitutively active ERBB2 receptor, to promote signal transduction in neonatal mouse supporting cells *in vivo*. We assayed these mice for supporting cell marker expression, proliferation, and supernumerary hair cell marker expression. Finally, we tested two small molecules that activate signaling from a second member from the EGFR family, ERBB3 (Shen *et al.*, 2013; Swoboda *et al.*, 2013), on neonatal mouse cochlear cultures. These cultures were then assayed for supporting cell proliferation and hair cell marker expression in neonatal mouse cochlear cultures.

Materials and Methods

Mice.

The following mouse strains were used. *Fgfr3-iCre* (Cox *et al.*, 2012) was a kind gift from Dr. Jian Zuo. CD1 mice, *Atoh1-GFP* (Lumpkin *et al.*, 2003), *CA-ErbB2* (Xie *et al.*, 1999), *ROSA-floxed-rtTA/GFP* (Belteki *et al.*, 2005), *ROSA-floxed-tdTOMATO* (Ai14, (Madisen *et al.*, 2010), and *Sox2-CreER* (Suh *et al.*, 2007) were all purchased from Jackson Laboratories. Crosses between *Fgfr3-iCre*, *ROSA-floxed-rtTA/GFP*, and *CA-ErbB2*, and crosses between *Sox2-CreER*, *ROSA-floxed-rtTA/GFP*, and *CA-ErbB2* mice were used for *in vivo* experiments. Crosses between *Sox2-CreER* and *ROSA-floxed-tdTOMATO* were used for Sox2-lineage tracing *in vitro* experiments. Finally, unmodified CD1 mice and *Atoh1-GFP* mice were used for the remaining *in vitro* experiments. In all, 171 mice were euthanized in the course of these experiments, including transgenic littermates that were not used for controls.

The University Committee for Animal Resources (UCAR) approved all mouse experiments at the University of Rochester, and the Massachusetts Eye and Ear Infirmary Institutional Animal Care and Use Committees (IACUC) approved all mouse experiments at the MEEI. Both male and female mice were used equally throughout these experiments. Mice were maintained on a 12 hour light-dark cycle, housed no more than five adults per cage with food and water available ad libitum. Mice also received ample nesting supplies and small houses. The day that pups were found was designated P0. Genotyping primers and protocols are available upon request.

Administration of substances to mice.

Substances were administered to genetically modified mice, in order to activate gene expression and label dividing cells for *in vivo* experiments only. This includes doxycycline food (200 mg/kg of chow, BioServ #S3888), which replaced the dam's normal chow. Additionally, three substances were administered via injections to post-natal day 0 to post-natal day 2 (P0-P2) pups intramuscularly, in the upper thigh, using Ultrafine insulin syringes (Becton-Dickinson 31G #08290–328468). These included doxycycline hyclate (DOX: 100 mg/kg body weight, Sigma Aldrich #D9891), which was freshly prepared as 10 mg/ml stock in 0.9% sterile saline. 5-ethynyl-2'-deoxyuridine (EdU: 0.01 mg/kg, Invitrogen #A10044) was made as a 10 mM stock solution in DMSO and diluted for injection to 40% strength in 0.9% sterile saline. Tamoxifen (75 mg/kg, Sigma, #T5648) was dissolved in corn oil (Sigma, #C8267) at 5 mg/kg.

Antibodies.

The following antibodies were used: ERBB2 (Neu C-18, rabbit polyclonal, Santa Cruz Biotechnology #SC284); phosphor-ERBB2 (P-Neu Try1248, rabbit polyclonal, Santa Cruz #SC12352); phosphor-PI3K (P-PI3-Kinase P85 α , rabbit polyclonal, Santa Cruz #SC12929); β -ACTIN (BA3R, mouse monoclonal, ThermoFisher Scientific #MA5–15739); SOX2 (Y-17, goat polyclonal, Santa Cruz #SC17320); MYO7A (H-60, rabbit polyclonal, Santa Cruz #SC25834); JAG1 (C-20, goat polyclonal, Santa Cruz #SC6011); GFP (chicken polyclonal, Abcam, ab13970); RFP (rabbit polyclonal, Rockland #600-401-379); OCM (goat polyclonal, N-19, Santa Cruz #SC7446); PVALB (mouse monoclonal, EMD Millipore #MAB1572). Secondary antibodies were purchased from Jackson Immunoresearch. We used fluorescently-conjugated donkey secondaries and horseradish peroxidase (HRP) conjugated goat secondaries.

Western blotting.

To obtain fibrocytes, P3 mouse brains were minced in Dulbecco's Modified Essential Media with Glutamax (DMEM, Gibco, #10569–044), trypsinized (0.25% trypsin/EDTA, Gibco, #25200056) for 3 minutes at 37°C, neutralized with 10% fetal bovine serum (FBS, Hyclone #SH30088) in DMEM, triturated, filtered through a 40 μ m nylon mesh (Falcon cell strainers, #352340), and plated on uncoated plates in DMEM Glutamax, with 10% FBS, 1% penicillin and streptomycin supplement (Gibco #15140122) and 25 mM HEPES (Gibco #15630080). Cultures were fed every 2 days. After reaching confluency (around 6–7 days), the cells were re-plated in 6-well plates at 10⁶ cells/well. To assay adenovirus activity, wild-type fibrocytes were infected for 24 hours and then extracted in RIPA buffer (10 mM Tris, 100 mM NaCl, 2

mM EDTA, 0.1% SDS, 1% Triton X100, 100 µg/ml phenylmethylsulfonyl fluoride) supplemented with HALT protease and phosphatase inhibitors (Thermo Scientific, #78430 & #78420, respectively). To assay transgene activity, fibrocytes were isolated from transgenic mice, and cultured similarly. They were stimulated with freshly prepared 2 µg/ml DOX (Fisher, #BP2653) prior to extraction in RIPA buffer with HALT protease and phosphatase inhibitors.

Extracts were sonicated and quantified (Micro BCA Protein Assay Kit, Thermo, #23235). 20 µg of protein per lane were boiled with Laemmli buffer, subjected to polyacrylamide gel electrophoresis/PAGE (12% Mini PROTEAN Gels, BioRad, #4561043), and transferred to a nitrocellulose membrane (Sigma, GE #10600016) using ice cold transfer buffer (25 mM Tris base, 200 mM glycine, and 25% methanol). Blots were probed with primary antibodies (1:1000) in Tris-buffered saline (TBST, 10 mM Tris, 150 mM NaCl) at pH 7.5, with 0.5% Tween-20 and 5% nonfat milk, overnight at 4°C. Secondary antibodies (anti-mouse and anti-rabbit, Jackson Immunoresearch, #115-035-003 and #111-035-003) conjugated with HRP were further incubated with the blot in the same buffer for 1 hour at room temperature. Signal was revealed with SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific, #34087) and X-ray film (Kodak BioMax, #829–4985).

Tissue processing.

P8 and P14 mice were euthanized with carbon dioxide and decapitated, and P2-P3 pups were decapitated. Inner ears were fixed at least overnight in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) at 4°C. P8 and P14 inner ears were further decalcified for 3 days in 100 mM EDTA. For sectioning, inner ears were cryoprotected in 30% sucrose, embedded in OCT compound (Andwin Scientific #25608930), frozen in liquid nitrogen, and sectioned at 20 microns onto Superfrost Plus slides (Fisher, #22–037-246). For whole mount, P8 and P14 cochleae, fixed and decalcified as described above, were dissected into three large pieces (Montgomery & Cox, 2016).

Construction of the ErbB2 adenoviruses.

Plasmid #16259 (human HER2, V654E) and plasmid #16258 (human HER2, K753M) were purchased from Addgene and sequenced. Both plasmids were originally in the vector pcDNA3 with 5' and 3' HindIII cloning sites. They were cloned into the HindIII site of pAdTrack-CMV (He *et al.*, 1998). These constructs were recombined with pAdEasy in BJ5183 competent cells (Agilent Technologies). Subsequently, Adenovirus5 (Ad5) was produced and titrated using standard methods. Western blot experiments were used to verify the activity of the constructs, as described in the Results section. The GFP virus was purchased from Vector Biolabs. All viral protocols were reviewed by the URM Biosafety committee to ensure the safety of staff and the environment.

Cochlear culture and infection.

P1-P2 pups were decapitated and their cochleae dissected into DMEM/F12 media (Gibco 11330–032) buffered with 15 mM HEPES (Gibco, 15630–080). Cochleae were cultured on Lab-Tek CC2 chamber slides (Nunc 154917) coated with 0.5 mg/ml poly-D-lysine (Sigma P6407) and 2 µg bovine fibronectin (Sigma F1141). After removal of the tectorial

membrane, the apical and basal turns were cut away to obtain the middle turn. These were placed in the slide wells with a minimal amount of media and incubated for 10 minutes at 37°C in 5% CO₂ to facilitate attachment. Middle turns were cultured overnight in DMEM/F12 with 15 mM HEPES, 1 mg/ml penicillin G (Sigma P3032), 2% B27 supplement (Gibco 17504–044), 25 ng/ml EGF (Sigma E1257) and 1% FBS (HyClone SH30088). The next day, the media was replaced with similar culture media, except that it now lacked FBS and contained 2 µM EdU (Invitrogen A10044). Note that in other studies, higher concentrations of EdU have been associated with DNA damage in various stem cell types (Kohlmeier *et al.*, 2013). In preliminary experiments, we found that the inclusion of 1% FBS facilitated organ attachment, but its presence during adenovirus infection increased the basal level of supporting cell proliferation. Middle turns were infected with 1–3 × 10⁷ particles per 500 µl culture in a dedicated virus lab using BSL2+ precautions. For Sox2-Cre lineage tracing, 10 µM (z)-4-hydroxytamoxifen (Sigma H7904) was added on the day of isolation to stimulate CRE activity. All viral procedures were reviewed and approved by the University of Rochester's Institutional Biological Safety Committee. After incubations, cultures were fixed overnight in fresh 4% paraformaldehyde in PBS at 4°C.

Immunostaining.

Sections were dried at 50°C prior to staining. For cochlear cultures on LabTek chamber slides, the wells were removed prior to staining without drying. Both sections and cultures were stained with identical procedures. They were first washed three times in PBS (Gibco, 14190–144) at room temperature for ten minutes each. If the analysis involved assessment of proliferating cells by EdU incorporation, the EdU Click-It kit (Invitrogen C10339 or C10340) was used next. This reaction was performed prior to antibody staining according to the manufacturer's instructions. Then sections and cultures were both blocked with 5% donkey serum in PBS with 0.5% Triton X100 for 1 hour at room temperature, and incubated overnight in block containing primary antibodies diluted to 1:500 at 4°C. After the primary incubation, they were washed three times in PBS for ten minutes each, and then were incubated for 2 hours in secondary antibodies diluted to 1:500 at room temperature. After the secondary incubation, sections and cultures were washed three times in PBS for ten minutes each and mounted in ProLong Gold (Invitrogen #P36930).

Whole mount cochlear tissue was first washed three times in PBS at room temperature for twenty minutes each with gentle rocking. Next, the EdU Click-It kit was used according to the manufacturer's instructions. The tissue was then boiled in 10 mM citric acid (pH 6.0) for 15 minutes in a microwave on low power, allowed to cool slowly to room temperature for one hour, washed three times in PBS at room temperature for twenty minutes each with gentle rocking, and blocked with 5% donkey serum in PBS with 0.5% Triton X100 for 1 hour at room temperature with gentle rocking. Tissue was then incubated overnight in block containing primary antibodies diluted to 1:500 at 4°C. After the primary incubation, tissue was washed three times in PBS for twenty minutes each, and then incubated for overnight in secondary antibodies diluted to 1:500 at 4°C. After the secondary incubation, sections and cultures were washed three times in PBS for twenty minutes each. Tissue was mounted in ProLong Gold (Invitrogen #P36930) between two 50 mm long coverslips, using two 25mm square coverslips at either side to make a small well for the mounting medium.

Cochlear culture with Pa2g4/EBP1 inhibitors.

Cochleae were isolated from postnatal day 1- day 2 wild type or *Atoh1*-nGFP mice (Lumpkin *et al.*, 2003). The organ of Corti was isolated from the otic capsule, and the nerve tissue and stria vascularis were removed. The organ of Corti was plated on a glass coverslip coated with a 1:10 mixture of Matrigel and DMEM/F12 to promote attachment. Cochlear explants were cultured in a serum-free 1:1 mixture of DMEM and F12, supplemented with Glutamax, N2, and B27. For the treated cochlea, small molecules were added to this culture medium, while the control cochlea was cultured with medium containing DMSO at the same concentration used in the treatments. To measure proliferation, explants were treated with EdU (10 µg/ml) along with the drug or DMSO. Drug-treated explants were cultured for 3 days then fixed in 4% paraformaldehyde for 30 minutes.

Confocal microscopy and image analysis.

All imaging was done on an Olympus FV1000 laser scanning confocal microscope using the Fluoview software package. ImageJ 64 (NIH) was used to Z-project maximal brightness in confocal stacks. Photoshop (Adobe) was used to set maximal and background levels of projections for the construction of figures.

Experimental Design and Statistical Analysis.

Data fields were blinded and randomized using a deck of cards prior to quantification. Virally infected cochleae were imaged at 20x on a confocal microscope using the stitching function of the FV1000 to obtain a composite of the entire field (3000 px by 3000 px). With only the supporting cell marker channel visible, one individual positioned 200 µm rectangles along the supporting cell region (usually 5–7 per middle turn) and then used the channel as a mask to reveal EdU+ cells. The rectangles were cropped out of the original images, exported as TIFs, and renamed using a deck of cards. Another individual blinded to condition counted the EdU+ cells. After unblinding, the rectangles were averaged to obtain a biological replicate and the average of these replicates is presented in the text.

For EdU+ and MYO7+ cells in P8 and P14 confocal stacks, an individual blinded to genotype counted EdU+ nuclei and supernumerary MYO7+ cells in P8 and P14 *Fgfr3-iCre/CA-ErbB2* image files by examining stack side views. ANOVA was used to establish statistical significance for data groups and a Student's two-tailed t-test with Bonferroni correction was used to establish pair-wise significance.

To quantify WS3 and WS6-treated cochleae, the length of the sensory epithelium was measured using ImageJ software with the overall length determined from the hook to the apex in each sample. The number of myosin VIIa-positive cells or Edu positive cells in the supporting cell region were manually counted. The total number of cells was counted in each of four cochlear segments of 1200–1400 µm (apical, mid-apical, mid-basal, and basal), density (cells per 100 µm) was then calculated for each segment. Statistical analyses were performed using Prism version 7.0 software, comparisons among groups were made by one-way ANOVA followed by Dunnett's multiple-comparisons test for comparing the mean of each group with the mean of a control group.

Results.

***In vitro*, CA-ErbB2 drives cochlear supporting cell proliferation in a non-cell autonomous manner that correlates with the down-regulation of Sox2**

To determine if supporting cells with active ERBB2 signaling proliferate, we constructed adenoviruses to drive expression of mutated ERBB2 in conjunction with GFP. In many human cancers, mutated HER2/ERBB2 harbors a charged glutamic acid residue in place of a hydrophobic valine located in the transmembrane region (Fig. 1A, asterisk). This glutamic acid facilitates the dimerization of mutated ERBB2 polypeptides, enabling phosphorylation of intracellular tyrosine residues (Stern *et al.*, 1988; Weiner *et al.*, 1989) and activation of the downstream effector PI3K via sub-unit phosphorylation (p185 / PI3K α). In previous studies regarding supporting cell proliferation after dissociation, inhibitors of PI3K blocked BrdU incorporation in a dose-dependent fashion (White *et al.*, 2012). We used two ErbB2 constructs derived from human cancer studies (Li *et al.*, 2004): *CA-ErbB2*, containing this activating mutation, and *I-ErbB2*, in which the valine is instead mutated to isoleucine; this mutation fails to drive auto-phosphorylation. We tested both constructs, along with a GFP-only control, in fibrocyte culture by Western blot (Fig. 1B-E). While ERBB2 immunoreactivity was detected in culture extracts infected with either virus (Fig. 1B, α -ERBB2), downstream events such as phosphorylation of ERBB2 or the PI3K regulatory unit were only observed consequent to *CA-ErbB2* infection (Fig. 1C, D, α -pERBB2 and α -pPI3K α). Semi-quantitative analysis of the blots illustrates the profound increase in downstream signaling for CA-ERBB2 (Fig. 1F, green) compared to I-ERBB2 (Fig. 1F, red, around 6-fold for pERBB2 and 40-fold for pPI3K) and GFP (Fig. 1F, blue, around 30-fold for pERBB2 and 1000-fold for pPI3K).

We infected cultures of neonatal cochlear middle turns with the three adenoviruses. Twenty-four hours later, we assayed proliferation by EdU incorporation (Fig. 2, white). In previous experiments, 24 hours was sufficient for dissociated supporting cells to re-enter the cell cycle but not long enough for them to complete more than one cycle (White *et al.*, 2012). As a consequence, counting cells at 24 hours does not over-estimate the occurrence of cell cycle re-entry. Infected cells expressed GFP (Fig. 2, green), and anti-JAG1 (Fig. 2A, C, E, red) was used to label supporting cells. EdU incorporation was focal, and it was not evenly distributed through the organ (Fig. 2E). To avoid field selection bias, we quantified proliferation in these middle turn cultures from stitched confocal images, positioning 200 μ m long rectangles on these images with only the JAG1 channel visible. A schematic of this strategy is shown in Fig. 2G, where rectangles indicate example fields cropped from a large, stitched image for quantification. We then blinded and randomized the resulting sections of EdU+ cells revealed through the JAG1 mask. Increased proliferation was observed in CA-ErbB2-infected cultures. The overall results, expressed as EdU+ cells/mm, were modest, but significant ($F = 3.8$, $T_{GFP} = 39.5$, $T_I = 37.6$, $T_{CA} = 72.9$, $P = 0.040$, $n = 24$, ANOVA). Proliferation within the JAG1+ supporting cell region did not differ between GFP-infected and *I-ErbB2* infected cultures ($T_{GFP} = 39.5$, $T_I = 37.6$, $P = 0.89$, $n_{GFP} = 8$, $n_I = 6$, two-tailed t-test). However, a significant increase in EdU incorporation was observed in the supporting cell region marked by anti-JAG1 after *CA-ErbB2* infection compared to GFP only ($T_{GFP} = 39.5$, $T_{CA} = 72.9$, $P = 0.04$, $n_{GFP} = 8$, $n_{CA} = 10$, two-tailed t-test).

Since virally infected cells express the lineage tracer GFP, we examined images of these cultures to determine if infected cells proliferate (Fig. 2E). Surprisingly, EdU incorporation (Fig. 2E, red) was observed in the supporting cell region in the cells adjacent to the *CA-ErbB2* infected cells (Fig. 2E, green), indicating a non-cell autonomous effect. We replicated these experiments using anti-SOX2 to mark the supporting cells (Fig. 2B, D, F, cyan). Remarkably, co-localization of EdU with SOX2 was rarely observed in cultures infected with *CA-ErbB2* (Fig. 2F, cf. white and cyan).

With the lack of co-localization between EdU+ and SOX2+ cells, we wondered if SOX2 protein is down-regulated in supporting cells when they begin mitosis. Previous studies have implicated SOX2 in the regulation of *Cdkn1b* in cochlear supporting cells (Liu *et al.*, 2012). In order to determine if these *in vitro* proliferating cells were originally SOX2+, we crossed a *Sox2-CreER* knock-in line to a *ROSA-floxed tdTomato* line and used the progeny for infection experiments. We induced Td-TOMATO (TOM) expression with 4-hydroxytamoxifen in culture prior to viral infection (Fig. 3). Cultures were assayed for infection, marked by GFP (green staining, pink arrows), Sox2-lineage TOM expression (red staining), SOX2 protein expression (cyan staining), and EdU incorporation (white staining, yellow arrows). Little proliferation was observed in the *GFP*-infected cochleae at 24 hours (Fig. 3A, white). Consistent with previous experiments, we observed non-cell autonomous proliferation from CA-ERBB2 infection; e.g. EdU+ cells are GFP-negative (Fig. 3C, D, cf. white staining/yellow arrows with green staining/pink arrows). We quantified EdU+/TOM+ cells and EdU+/TOM+/SOX2+ cells in 10 fields from 4 infected cochleae. $79.4\% \pm 4.6\%$ of all TOM+/EdU+ cells did not express SOX2 protein (Fig. 3C cf. 3C', yellow arrows, cf. white and cyan, n = 566 total cells). These cells are in the TOM+ region (Fig. 3C, cf. white nuclei with red cell bodies). EdU+/SOX2- nuclei were interspersed in the SOX2+ nuclear layer at 32 hours after infection (Fig. 4D). These data are consistent with a down-regulation of SOX2 as proliferation was initiated in cochlear supporting cells.

Constitutively activated ErbB2 does not promote cochlear supporting cell proliferation *in vivo*.

To determine if ERBB2 activation could drive proliferation in cochlear supporting cells *in vivo*, we employed a Tet-On system to drive a *CA-ErbB2* transgene encoding a constitutively active rat ERBB2 protein, which harbors the same valine to glutamic acid mutation used previously (Xie *et al.*, 1999). We validated the *CA-ErbB2* transgene in protein extracts from cultures of fibrocytes derived from mice harboring both a *CA-ErbB2* transgene and a functional *ROSA-rtTA* knock-in gene (Fig. 4A, lanes a), comparing them to extracts isolated from fibrocyte cultures with *ROSA-rtTA* alone (Fig. 4A, lanes b). Twenty-four hours after doxycycline (DOX) addition, ERBB2 protein was evident on Western blots (Fig. 4A, α -ERBB2, cf. lanes a and b). Both ERBB2 and the regulatory unit of PI3K were phosphorylated (Fig. 4A, α -pERBB2, α -pPI3K, cf. lanes a and b). Probing with anti- β -ACTIN revealed similar protein amounts in both extracts (Fig. 4A, α - β -ACTIN, cf. lanes a and b). To determine the onset of PI3K phosphorylation, we harvested sister cultures from *CA-ErbB2/ROSA-rtTA* derived fibrocytes at 2, 4, 6 and 8 hours after DOX addition. Phosphorylation of the PI3K regulatory unit was evident at 8 hours (Fig. 4B). These data

indicate that expression of the *CA-ErbB2* transgene indeed resulted in phosphorylation of ERBB2 and its downstream target, PI3K.

To express CA-ERBB2 in cochlear supporting cells, we used the *Fgfr3-iCre* knock-in to activate a floxed *ROSA-rtTA/GFP* knock-in gene in supporting cells in neonatal *CA-ErbB2* mice (Fig. 4C, (Cox *et al.*, 2012)). All mice shown for these experiments harbor the floxed *ROSA-rtTA-GFP* knock-in gene. The injection schedule for inducing CA-ERBB2 and labeling proliferating cells is shown in Fig. 4F. Cochleae from pups sacrificed at P3 were analyzed for p-ERBB2 immunoreactivity (Fig. 4D, E, red) and GFP expression (Fig. 4D, E, green). Co-localization of GFP and p-ERBB2 was readily apparent in animals harboring both *Sox2-CreERT* and *CA-ErbB2* (note Fig. 4D', inset). At this time point, we found that in *Sox2-CreERT* triple transgenic mice, 96% of GFP+ cells also expressed p-ERBB2, and 98% of p-ERBB2+ cells also expressed GFP (n = 281 cells). Similarly, in *Fgfr3-iCre* triple transgenic mice 96% of GFP+ cells also express p-ERBB2, and 89% of p-ERBB2+ cells also express GFP (n = 172 cells).

Since SOX2 protein was not detected in most supporting cells as they re-entered the cell cycle, we examined SOX2 protein by immunofluorescence in mice that harbored either *Sox2-CreERT* or *Fgfr3-iCre* in addition to *CA-ErbB2* and *ROSA-flox-rtTA-GFP* modifications. Exposure-matched images of p-ERBB2 (Fig. 4G-I, red) and SOX2 protein (Fig. 4G-I, cyan) show an apparent reduction in the numbers of SOX2+ cells when compared to control *CA-ErbB2/ROSA-flox-rtTA-GFP* mice alone. Per 200 μ m segment, control organs contained significantly more SOX2+ cells compared to both CA-ERBB2-expressing genotypes ($F = 5.9$, $T_{con} = 212$, $T_{Sox2} = 74$, $T_{Fgfr3} = 49$, $P = 0.013$, n=6 blinded fields per genotype, ANOVA).

Using the treatment schedule illustrated in Fig. 4F, we tested if supporting cells proliferated *in vivo* after CA-ERBB2 induction (Fig. 5). We co-labeled cochleae from each of the three genotypes for SOX2 (Fig. 5A-C, cyan), EdU (Fig. 5A-C, white) and p-ERBB2 (Fig. 5A-C, red). EdU+ nuclei were clustered in and near cells containing phosphorylated ERBB2 (Fig. 5B, C, cf. red and white). We assessed the numbers of EdU+ cells at P8 and P14, using blinded confocal stacks (Fig. 5D) labeled for DAPI (blue), GFP (green), EdU (red) and MYO7 (white). No significant differences were seen in the numbers of EdU+ cells/mm at either P8 ($T_{con} = 21.0$, $T_{CA} = 21.9$, $P = 0.88$, n = 4 per genotype, student's two tailed t-test) or P14 ($T_{con} = 8.6$, $T_{CA} = 12.5$, $P = 0.065$, n = 3 per genotype, student's two tailed t-test), indicating that the activation of CA-ERBB2 is not sufficient to drive significant increases in proliferation *in vivo*.

Although approximately 1 in 4 pups analyzed at P3 or P8 harbored both *Fgfr3-iCre* and *CA-ErbB2*, we obtained only 3 in 34 mice at P14. Similar experiments performed with *Sox2-CreERT* mice yielded no mice with both a *Cre* gene and *CA-ErbB2* (out of 39 generated, data not shown). Moreover, surviving *Fgfr3-iCre+/CA-ErbB2+* mice were small, sickly, and hairless, with wrinkled skin (not shown).

Supernumerary MYO7+ cells *in vivo* consequent to ErbB2 activation

In our analyses of P8 and P14 mice, we were surprised to discover many supernumerary Myosin7+ (MYO7+) cells located in the supporting cell regions of CA-ERBB2 cochleae (Fig. 6). Mice harboring *Fgfr3-iCre/ROSA-flox-rtTA* (control) transgenes had normal complements of hair cells, identified with antibodies against MYO7a and Oncomodulin (OCM), juxtaposed to GFP-containing CRE+ cells (Fig. 6A, B). Normal organization was observed in both the mid-base (Fig. 6A) and apical regions (Fig. 6B). Animals additionally harboring a *CA-ErbB2* transgene, in contrast, had supernumerary MYO7+ cells that co-expressed OCM, suggesting outer hair cell differentiation (Fig. 6C, D yellow arrows). Such cells were present in the mid-base turn (Fig. 6C, arrows) and in apical regions (Fig. 6D, arrow). Occasionally, supernumerary MYO7+ cells near inner hair cells were also observed in the apical regions (Fig. 6E, arrow). Supernumerary MYO7+ cells also expressed another hair cell marker, Parvalbumin (PVALB) (Fig. 6E, cyan). They did not express the supporting cell marker SOX2 (Fig. 6F, cyan, arrow). They were typically located near GFP+ cells (Fig. 6C, D, cf. red with green) but did not express GFP.

We quantified supernumerary MYO7+ cells (Fig. 6G-I). Supernumerary MYO7+ cells were readily observed in the supporting cell layer in blinded confocal stacks, as indicated with yellow arrows (Fig. 6G, H). The frequency of supernumerary MYO7+ cells near outer hair cells ranged from 10 to 30 cells/mm of cochlea, to a maximum of 117 total new MYO7+ cells in one organ (Fig. 6I). Few supernumerary cells were observed in control animals (Fig. 6I), and the increase was statistically significant ($T_{\text{con}} = 0.73$, $T_{\text{CA}} = 13.7$, $P = 0.018$, $n = 4$ per genotype, student's two tailed t-test). These data suggest that ERBB2 signaling is upstream of a short-range paracrine signal that drives the initial events of hair cell differentiation in young animals *in vivo*.

Small molecules that activate ERBB2 pathway promote supporting cell expansion and supernumerary MYO7+ cell generation

Our findings with the conditional ERBB2 activation animal models have shown that the constitutive activation of ERBB2 can result in supporting cell expansion and ectopic MYO7+ cell generation. To further validate the crucial role of ERBB2 activation, we turned to small molecules to pharmacologically activate the EGFR family pathway and determine their effect on supporting cell expansion and hair cell differentiation. Two compounds, WS3 and WS6, were chosen, as these two analogues promoted cell proliferation in growth arrested cells such as islet β cells and retinal pigment epithelial cells (Shen *et al.*, 2013; Swoboda *et al.*, 2013). Through their action on ERBB3 binding protein 1 (EBP1/PA2G4), a component of the EGFR family signaling pathway, these compounds reduced the anti-proliferative role of EBP1 and upregulated several cell cycle-activated genes (Squatrito *et al.*, 2004; Shen *et al.*, 2013; Swoboda *et al.*, 2013). To test the effect of these drugs on supporting cells, we applied them to cochlear explants derived from *Atoh1-nGFP* reporter mice, in which hair cells express nuclear GFP (Lumpkin *et al.*, 2003). With WS3 or WS6 treatment, additional *Atoh1-nGFP*-positive cells near outer hair cells were seen (Fig. 7A and B; $F = 3.9$, $P = 0.0076$, ANOVA). The effect was concentrated in the apex, where the number of MYO7+ cells in the apex was increased by 20 cells/100 μm (apex: $T_{\text{con}} = 53.9$, $T_{\text{WS6}} = 75.1$, $T_{\text{WS3}} = 73.8$, $P_{\text{WS6}} = 0.0024$, $P_{\text{WS3}} = 0.010$; mid-apex: $T_{\text{con}} = 56.2$, $T_{\text{WS6}} =$

65.4, $T_{WS3} = 72.7$, $P_{WS6} = 0.13$, $P_{WS3} = 0.046$; Mid-base: $T_{con} = 59.2$, $T_{WS6} = 59.1$, $T_{WS3} = 70.8$, $P_{WS6} = 0.99$, $P_{WS3} = 0.042$; $n = 3$ per group, Dunnett multiple comparisons).

To determine if the drug treatments promoted supporting cell proliferation, we applied them to cochlear explants from wild type mice at P1-P2 with EdU present to label the proliferating cells. A large number of EdU⁺ cells were observed in the SOX2⁺ supporting cell region in both WS3 and WS6-treated cochleae (Fig. 8A and B; $F = 13.0$, $P = 0.0000016$, ANOVA), with a gradient from apex to base (apex: $T_{con} = 4.1$, $T_{WS6} = 22.9$, $T_{WS3} = 29.0$, $P_{WS6} = 0.011$, $P_{WS3} = 0.00059$; mid-apex: $T_{con} = 3.0$, $T_{WS6} = 14.8$, $T_{WS3} = 17.4$, $P_{WS6} = 0.00057$, $P_{WS3} = 0.00077$; Mid-base: $T_{con} = 0.85$, $T_{WS6} = 6.2$, $T_{WS3} = 7.7$, $P_{WS6} = 0.0098$, $P_{WS3} = 0.0049$; $n = 3$ per group, Dunnett multiple comparisons). These results were consistent with the enhanced supporting cell proliferation observed with the *CA-ErbB2* viral transduction *in vitro* (Fig. 2). To confirm that the effect of the drugs was mediated through ERBB2 activation, the ERBB2 phosphorylation was analyzed using the protein lysates from cochlear explant. Due to the limited protein quantity from the primary tissue, we were not able to detect the ERBB2 protein signal by western blot (data not shown); in the breast cancer cell line MCF-7, however, p-ERBB2 level was elevated in response to drug treatment (Fig. 8C), indicating that the ERBB2 signaling pathway was activated by WS3 or WS6. The data suggest that pharmacological activation of EGFR family signaling by small molecules promoted supporting cell proliferation and increased MYO7⁺ cell generation *in vitro*, similar to what we had observed in the transgenic animal model *in vivo* and further validating the crucial role of EGFR family activation for the inner ear regeneration.

Discussion.

The mammalian cochlea lacks the regenerative capacity of non-mammalian counterparts. Here we test intrinsic ERBB2 signaling as a candidate regulator of mammalian cochlear regeneration. We find that neonatal mouse supporting cells expressing a constitutively activated ERBB2 receptor (CA-ERBB2) promote supporting cell proliferation *in vitro*. Moreover, cochleae with CA-ERBB2 expression develop supernumerary MYO7⁺ cells *in vivo*. Both proliferation and MYO7⁺ induction are observed when small molecule effectors stimulate ERBB3 signaling *in vitro*. These data suggest ERBB2 signaling as a candidate pathway in regulating the regeneration response.

Our findings are summarized in Fig. 9. Each method used to modulate EGFR family signaling had similar, but not identical results. The use of the virus or transgenic technology to drive CA-ERBB2 activity enabled lineage tracing of transduced cells (dark green). Strikingly, we find that it is the cells nearby these transduced cells that respond, either by modulating SOX2 expression (cyan), proliferating (red), or inducing MYO7 (white). That ERBB2 signaling is non-cell autonomous implies the existence of downstream signals that regulate these activities, a completely unexpected result. SOX2 modulation was observed with CA-ERBB2 expression both *in vitro* (Fig. 2, 3) and *in vivo* (Fig. 4–6), but not after ERBB2/ERBB3 activation with small molecules. Similarly, proliferation was observed with both *in vitro* systems, but not *in vivo*. This finding suggests that additional constraints provided by cochlear structure *in vivo* could play a role in preventing proliferation, for example by limiting cell growth. Finally, we observed significant levels of MYO7 induction

in the two systems where it could be assessed. Although not shown here, in preliminary experiments we found that infected cochlear explants became too disorganized and spread out to accurately quantify MYO7+ cells within two days of viral transduction (Fig. 9, “Not assayed”). Importantly, we observe supernumerary MYO7+ cells throughout the cochlea in vivo (Fig. 6). This rules out the possibility that ERBB2 activation affects secondary processes such as convergent extension. Convergent extension is complete in the basal and middle cochlea by birth (Chen *et al.*, 2002), when ERBB2 activation is initiated. In concert, these findings strongly implicate EGFR family signaling in the regulation of cochlear regeneration events.

We note that each *CA-ErbB2* transgene used in this study was derived from carcinoma cells (Xie *et al.*, 1999; Li *et al.*, 2004). In many tumors, CA-ERBB2 acts cell-autonomously to promote proliferation. Surprisingly, constitutively active ERBB2 signaling in certain non-proliferating tumor cells drives a change in their secretome that promotes neighboring cells to change fate and become metastatic (Angelini *et al.*, 2013). Our data strongly imply that CA-ERBB2 triggers expression of regenerative signals for responding neighbor cells, which parallels the second mechanism. Recently, others have investigated heart regeneration using this CA-ERBB2 transgene (D’Uva *et al.*, 2015). Transient induction of CA-ERBB2 following myocardial ischemic injury can improve heart function, by the proliferation and de-differentiation of cardiomyocytes via β -CATENIN accumulation (D’Uva *et al.*, 2015). Findings in both mammalian cochlear and heart regeneration suggest CA-ERBB2 is driving specific regeneration activities rather than oncogenic transformation.

We report that SOX2 protein expression is reduced in both proliferating and trans-differentiating supporting cells after ErbB2 transduction (Figs. 2–6). Our proliferation data is consistent with reports that SOX2 directly activates *Cdkn1b*, a cell cycle inhibitor in supporting cells. Targeted deletion of *Sox2* in postmitotic supporting cells leads to inner pillar cell (a subtype of supporting cell) proliferation (Liu *et al.*, 2012). During development, ectopic expression of SOX2 can drive both supporting cell and hair cell markers (Pan *et al.*, 2013; Puligilla & Kelley, 2017). SOX2 binds to the *Atoh1* promoter and increases its expression levels (Neves *et al.*, 2012; Kempfle *et al.*, 2016; Puligilla & Kelley, 2017). Interestingly, SOX2 also drives expression of ATOH1 repressors, including *Hes* genes and *Id1* (Neves *et al.*, 2012; Neves *et al.*, 2013). In this so-called incoherent feed-forward loop (Alon, 2007), such contradictory effects of the inducer SOX2 drive a pulse-like accumulation of the target, ATOH1, which is sufficient for hair cell differentiation (Zheng & Gao, 2000). Our data support the model that prolonged SOX2 expression may maintain the post-mitotic supporting cell phenotype.

Recent efforts from other laboratories describe potential candidates for CA-ERBB2’s as yet unknown downstream signal. Supernumerary hair cells are observed when NOTCH1 signaling is reduced in supporting cells (Lanford *et al.*, 1999; Yamamoto *et al.*, 2006; Mizutari *et al.*, 2013). As NOTCH signaling maintains SOX2 expression in supporting cells during the neonatal period (Lanford *et al.*, 1999; Kiernan *et al.*, 2006; Pan *et al.*, 2013), this pathway fits well with our data. Stabilization of the WNT effector β -CATENIN in neonatal supporting cells promotes their proliferation (Chai *et al.*, 2012; Shi *et al.*, 2013; Kuo *et al.*, 2015). This treatment also increases *Atoh1* expression, likely through direct interactions of

β -CATENIN with the *Atoh1* enhancer (Shi *et al.*, 2010). SHH treatment also promotes rodent supporting cell proliferation *in vitro* (Lu *et al.*, 2013). ERBB2 binds to other EGFR family proteins and various receptor tyrosine kinases (Jones *et al.*, 2006). ERBB2 heterodimers with other EGFR family proteins are the active receptors for growth factor ligands and amplify EGFR family signaling by slowing endocytosis and decreasing the receptor-recycling period [reviewed in (Bertelsen & Stang, 2014)]. This would enhance the existing growth factor signaling in our CA-ERBB2 model. Further experiments will be required to determine which of these pathways may act downstream of CA-ERBB2. Further experiments will also be necessary to determine if there is heterogeneity in the responses of supporting cells to intrinsic CA-ERBB2 signaling, which supporting cells produce factors to induce regeneration-like responses, if regeneration-like responses have a concentration dependence, and if inhibitor molecules that limit the scope of regeneration are also produced.

In addition to investigating ERBB2 in transgenic models, we identify two small molecules, WS6 and WS3, that exhibited similar regenerative potential by regulating ERBB2 signaling (Fig. 7 & 8). WS6 treatment increases β -cell mass in a rodent diabetes model by promoting cell proliferation (Shen *et al.*, 2013), while WS3 expands retinal pigment epithelium cells and preserves vision when the cells are transplanted into a retinal degeneration model (Swoboda *et al.*, 2013). Here we assess effects of WS6 and WS3 in cochlear explant culture. While WS6 and WS3 activate ERBB2 in MCF-7 cells (Fig. 8), we could not confirm this activation in the cochlear culture. Besides direct activation of ERBB2 phosphorylation, these two EBP1 inhibitors may alter the EGFR family signaling cascade through other means. EBP1/PA2G4 is expressed throughout the sensory region of the P0 mouse cochlea (Hertzano & Orvis, 2016). EBP1 negatively regulates ERBB2 mRNA and protein level via transcriptional mechanisms (Ghosh *et al.*, 2013). A recent study showed that EBP1 interacts with the EGFR family downstream molecule PI3K and inhibits its kinase activity (Ko *et al.*, 2014). Our EBP1 inhibitor might increase PI3K activity and enhance the EGFR family signaling. Our results provide a potential approach using drugs to enhance hair cell regeneration in future studies.

We note that during normal cochlear homeostasis, EGFR family signaling is implicated in the regulation of spiral ganglion neuron innervation. Expression of a dominant negative ERBB2 variant in supporting cells shows that this signaling is crucial for the survival of spiral ganglion neurons (Stankovic *et al.*, 2004). How then, can EGFR family signaling be tasked with two completely separate functions: regeneration and innervation? The intracellular signaling sites on ERBB2 are highly promiscuous, strongly interacting with at least 17 distinct protein domains (Jones *et al.*, 2006). We speculate that mammalian supporting cells may express different levels of specific EGFR family interactants compared to bird or fish supporting cells. Our work implicates EBP1, a regulator of ERBB2/ERBB3 signaling, in blocking supporting cell regeneration activities. It is possible that evolution may have redirected EGFR family signaling in mammals towards facilitating innervation and away from regeneration.

In summary, we demonstrate a potential role of EGFR family signaling in stimulating supporting cell proliferation and supernumerary MYO7+ cell differentiation in neonatal

mouse cochlea. Using multiple methods to activate EGFR family signaling, we find that its constitutive activation promotes regeneration processes in neighboring supporting cells. Taken together with ERBB2's role in detecting stretch damage in other epithelial tissues, our findings suggest that within some supporting cells, EGFR family signaling may initiate a cascade of downstream signaling pathways that enhance regeneration activity.

Acknowledgements.

We thank Ms. Felicia Gilels, Mr. Gavin Jenkins, Mr. Aaron Rushdeen, and Ms. Luoying Yang for technical assistance; Ms. Brooke Burgess for colony management assistance; Dr. Steve Paquette for assistance in imaging and quantification; Dr. Jian Zuo for the *Fgfr3-iCre* mouse strain, Dr. Peter Schultz for WS3 and WS6; Dr. Steve Dewhurst for biosafety assistance; and the URM C Confocal and Conventional Microscopy Core. We also thank Dr. Amy Kiernan, Dr. Joseph C. Holt, and Dr. Kenneth Henry for critically reading the manuscript. PMW was supported by NIH R01 DC014261, and AE was supported by NIH R01 DC014089.

Abbreviations.

ANOVA	Analysis of variance
Atoh1	Atonal homologue 1
β-actin	actin beta
B27	B-27 Serum-free supplement for neural culture
BrdU	Bromodeoxyuridine
CA-ERBB2	Constitutively active ERBB2 receptor
Cdkn1b	Cyclin dependent kinase inhibitor 1B
CRE	Cre recombinase protein
DMEM	Dulbecco's Modified Essential Media
DOX	Doxycycline hyclate
EBP1	ERBB3 binding protein 1, also called PA2G4
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
ERBB2	Erb-b2 receptor tyrosine kinase 2 (EGFR family member)
ERBB3	Erb-b3 receptor tyrosine kinase 3 (EGFR family member)
FBS	Fetal bovine serum
FGFR3	Fibroblast growth factor receptor 3
GFP	Green fluorescent protein

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human erb-b2 receptor tyrosine kinase 2
HES	Hairy Enhancer of Split
HRP	Horseradish peroxidase
Id1	Inhibitor of differentiation 1
I-ERBB2	Inactive ERBB2 receptor
JAG1	Jagged1
kDA	Kilodalton (molecular weight)
MCF-7	Michigan Cancer Foundation cell line 7
MYO7	Myosin7
OCM	Oncomodulin
P0	Post-natal day 0 (age of mice in days)
PBS	Phosphate buffered saline
PI3K	Phosphoinositide-3-kinase
PI3Kreg	Phosphoinositide-3-kinase regulatory subunit (p185)
PVALB	Parvalbumin
RFP	red fluorescent protein, also called TOMATO
rtTA	Tetracycline-regulated transactivator
ROSA	Gene trap ROSA 26
SHH	Sonic Hedgehog
SOX2	SRY-box 2
TBST	Tris buffered saline with Tween
TET-ON	Tetracycline-on
TOM	tdTomato
WNT	Wnt oncogene analogue

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Figure 1. Viral CA-ERBB2 transduction drives ERBB2 phosphorylation and downstream signaling.

(A) A schematic of CA-ERBB2 receptors shows the mechanism of dimerization. The asterisk indicates approximate region of mutation, where a valine is transformed to a glutamine in the transmembrane domain. This alteration drives constitutive interactions between receptors and subsequently kinase activity in the absence of ligand.

(B-E) Mouse brain fibrocytes were isolated from control mice and separately infected with 3 viral constructs, *GFP* (1), *I-ErbB2* (2) and *CA-ErbB2* (3) for 24 hours. Their protein extracts were analyzed in western blots with antibodies against ErbB2 (B), phosphor-ERBB2 (C), phosphor- PI3Kreg (regulatory unit of PI3K) (D), and β -ACTIN (E). β -ACTIN was used as a loading control. All four panels were processed at the same time and exposed together. ERBB2 protein has an approximate size of 190 kDa, PI3Kreg has an approximate size of 185 kDa, and β -actin has an approximate size of 42 kDa. Only *CA-ErbB2* infected fibrocytes demonstrate activation of both down-stream phosphorylation events.

(F) Semi-quantitation of western blots (ImageJ). Values for *GFP* (circles), *I-ErbB2* (triangles) and *CA-ErbB2* (squares) are shown. The y axis shows arbitrary units.

Figure 2. Viral CA-ERBB2 transduction in P2 cochlear cultures drives non-cell autonomous proliferation in JAG1+ supporting cells, but not in cells that maintain SOX2 expression.

P2 cochlear middle turns were infected *in vitro* by each of the 3 viruses: *GFP* (A, B), *I-ErbB2* (C, D), and *CA-ErbB2* (E, F). Antibodies against JAG1 (A, C, E, red) and SOX2 (B, D, F, cyan) are used to identify supporting cells. Staining for GFP (green) and EdU (white) reveal infected and proliferating cells, respectively. Similar numbers of JAG1+/EdU+ cells were seen in GFP-infected and I-ErbB2 infected cultures ($T_{GFP} = 39.5$, $T_I = 37.6$, $P = 0.89$, $n_{GFP} = 8$, $n_I = 6$, two-tailed t-test), but significantly more were seen after CA-ERBB2 infection ($T_{GFP} = 39.5$, $T_{CA} = 72.9$, $P = 0.04$, $n_{GFP} = 8$, $n_{CA} = 10$, two-tailed t-test). However, few SOX2+/EdU+ nuclei are observed in a similar region of the cochlea (cf. E and F). Scale bar: 200 microns. To quantify these explants without bias, the whole organ was imaged at 20X using the confocal “stitching” function (G, blue) and closely situated rectangles representing 200 microns (G, gray) were used to crop fields for blinded, randomized counting.

Figure 3. Sox2 down-regulation in cochlear supporting cells during proliferation *in vitro*. Cochleae derived from *Sox2-Cre^{ert}/ROSA-floxed-Tomato* pups were first cultured with 6-hydroxytamoxifen, to genetically label SOX2+ supporting cells with TOM protein, and then infected with either *GFP* virus (A) or *CA-ErbB2* virus (B-D). Cultures were allowed to incubate with EdU for 24 (A-C) or 32 hours (D) before fixation. Various combinations of staining are displayed, with EdU (white), TOM (designating the Sox2 lineage, red), GFP (designating viral infection, green), and SOX2 protein (cyan) as indicated. Pink arrows indicate infected cells; yellow arrows indicate EdU+ cells. Projections from confocal stacks with side views are presented to place EdU+ nuclei in the context of TOM and SOX2 (C, D). 79.4% of all EdU+/TOM+ nuclei were negative for SOX2 protein ($n=566$ cells surveyed). (C) shows projections from the area indicated with an arrow in (B); (D) shows an additional image from a separate experiment fixed at 32 hours. At this time, EdU+ nuclei line up well with SOX2+ nuclei (D''), indicating that they are in the supporting cell layer. Scale bar: 50 microns.

Figure 4. Activation of CA-ErbB2 in cochlear supporting cells at neonatal stages drives SOX2 down-regulation *in vivo*.

(A) Western analysis of lysates obtained from brain fibrocytes from *ROSA-rtTA*⁺/*CA-ErbB2*⁺ mice (lane a) and *ROSA-rtTA* mice (lane b) cultured for 24 hours after DOX addition, which stimulates the production of the CA-ERBB2 protein. Antibodies specific for ERBB2, phosphor-ERBB2, β -actin, and phosphor-PI3Kreg proteins (clockwise from upper left) were used to probe blots made with the two lysates. β -ACTIN serves as a loading control. L indicates the molecular weight ladder run on the blot. All four panels were run at the same time and exposed together. ERBB2 protein has an approximate size of 190 kDa, PI3Kreg has an approximate size of 185 kDa, and β -actin has an approximate size of 42

kDa. Only ROSA-rtTA+/ *CA-ErbB2* fibrocytes demonstrate activation of both down-stream phosphorylation events.

(B) Western analysis of CA-ErbB2 protein induction. Only *CA-ErbB2/ROSA-rtTA* derived fibrocytes are shown. Samples were harvested 2, 4, 6 and 8 hours after DOX addition and probed with the same four antibodies as (A). Anti-phosphor-PI3Kreg signal is evident at 8 hours.

(C) Example breeding scheme used to generate mice for these experiments. *Fgfr3-iCre* is shown; *Sox2-CreERT* mice were similarly bred. A red X is placed over symbols for genes if the protein cannot be expressed in that genetic combination. Only mice harboring all three modifications can express CA-ERBB2. Note that the *ROSA-flox-rtTA* modification includes an *IRES-GFP* (not shown), which enables lineage tracing of cells where that locus is recombined after CRE activation.

(D, E) GFP produced along with TA protein from the ROSA locus (green) co-localizes with p-ERBB2 in mice harboring both *Sox2-CreERT*, *CA-ErbB2*, and *ROSA-flox-rtTA* genes (D, red) but not *CA-ErbB2* and *ROSA-flox-rtTA* alone (E, red). Inset in D' shows co-localization. Scale bar: 20 microns. Anti-phosphor-ERBB2 staining confirms that CA-ERBB2 is active in GFP+ supporting cells.

(F) A schematic of experimental design depicts the timing of tamoxifen (amber), DOX (pink), and EdU (black) injections into pups. EdU analysis is presented in Fig. 5.

(G-I) Mice harboring *CA-ErbB2* (G), *Sox2-CreERT/CaErbB2* (H), and *Fgfr3-iCre/CA-ErbB2* (I), in conjunction with the *ROSA-flox-rtTA* modification, were treated as shown in (F). P3 cochleae were isolated and analyzed for phosphor-ERBB2 (red) and SOX2 protein (cyan). SOX2+ cells were significantly reduced in number after CA-ERBB2 activation: Per 200 μ m segment, control organs (G) contained significantly more SOX2+ cells compared to both CA-ERBB2-expressing genotypes ($F = 5.9$, $T_{con} \equiv 212$, $T_{Sox2} \equiv 74$, $T_{Fgfr3} \equiv 49$, $P \equiv 0.013$, $n=6$ per genotype, ANOVA). Scale bar 100 microns.

Figure 5. Activation of CA-ErbB2 *in vivo* does not drive significant proliferation
 (A-C) Mice harboring *CA-ErbB2* (A), *Sox2-CreERT/CA-ErbB2* (B), and *Fgfr3-iCre/CA-ErbB2* (C), in conjunction with the *ROSA-flox-rtTA* modification, were treated with the schedule displayed in Fig. 4F. P3 cochleae were isolated and analyzed for SOX2 (cyan), p-ERBB2 (red) and EdU (white). Occasional EdU+ cells are seen in CA-ERBB2+ cochleae at P3. Scale bar: 50 microns.
 (D) *Fgfr3-iCre/CA-ErbB2/ROSA-flox-rtTA* mice were treated with the schedule displayed in Fig. 4F, except that they were fixed at P14. No mice harboring *Sox2-CreERT* and the other transgenes survived past P6. An EdU+ cell (red fluorescence, yellow arrow) was observed in the supporting cell compartment (green) among MYO7a+ hair cells (white). Side panels display cross sections of the confocal stack at the yellow lines. Scale bar: 50 microns.
 (E) *Fgfr3-iCre/CA-ErbB2/ROSA-flox-rtTA* mice (Fg+ CA+) and *Fgfr3-iCre/ROSA-flox-rtTA* (Fg+, control) were treated with the schedule displayed in Fig. 4 F and fixed at P8 or P14. Blinded quantification of confocal stacks, similar to (D), shows little difference in numbers of EdU+ cells between genotypes at either P8 ($T_{con} = 21.0$, $T_{CA} = 21.9$, $P = 0.88$, $n = 4$ per genotype, student's two tailed t-test) or P14 ($T_{con} = 8.6$, $T_{CA} = 12.5$, $P = 0.065$, $n = 3$ per genotype, student's two tailed t-test). Circles represent data from individual cochleae; the line represents the mean.

Figure 6. Activation of CA-ErbB2 in cochlear supporting cells at neonatal stages drives the formation of supernumerary hair cell-like cells *in vivo*.

(A-B) Mice harboring *Fgfr3-iCre/ROSA-flox-rtTA* (control) or were injected with tamoxifen and DOX with the schedule displayed in Fig. 4F and allowed to survive to P14, when they were analyzed for hair cell markers. Hair cells in control cochleae (A, B) are revealed with Myo7 (red) and Ocm (purple) immunoreactivity, near GFP+ supporting cells (green) in both the mid-base (A) and apical (B) regions. Scale bar: 50 microns.

(C-D) Mice harboring *Fgfr3-iCre/CA-ErbB2/ROSA-flox-rtTA* contain supernumerary MYO7+ cells (red) that also co-localize with anti-Ocm (purple), as indicated with yellow arrows. Supernumerary hair cell-like cells are present in the mid-base (C) and apical (D) turns.

(E) Supernumerary MYO7+ cells in *Fgfr3-iCre/CA-ErbB2/ROSA-flox-rtTA* cochleae co-localize with PVALB, another hair cell marker.

(F) Supernumerary MYO7+ cells in *Fgfr3-iCre/CA-ErbB2/ROSA-flox-rtTA* cochleae do not co-localize with the supporting cell marker Sox2 (I, cyan, arrow).

(G, H) Examples of supernumerary MYO7+ cells near inner hair cells (G) and outer hair cells (H) are indicated with yellow arrows in the side views of whole mount confocal stacks. Scale bar: 50 microns.

(I) Supernumerary MYO7+ cells were quantified on blinded stacks, similar to the examples shown in (G, H). P8 mice harboring *Fgfr3-iCre/ROSA-flox-rtTA* (control, blue) are shown in contrast to *Fgfr3-iCre/CA-ErbB2/ROSA-flox-rtTA* mice at P8 (red) or P14 (orange). The increase in supernumerary MYO7+ cells in *Fgfr3-iCre/CA-ErbB2/ROSA-flox-rtTA* at P8 compared to *Fgfr3-iCre/ROSA-flox-rtTA* controls is significant ($T_{\text{con}} = 0.73$, $T_{\text{CA}} = 13.7$, $P = 0.018$, $n = 4$ per genotype, student's two tailed t-test). Quantification of supernumerary Myo7+ cells near outer hair cells (top graph) and near inner hair cells (bottom graph) are shown.

Figure 7. WS3 or WS6 treatment enhances MYO7+ cell generation *in vitro*.

A. Explant cultures of the organ of Corti from postnatal mice (P1-P2) cultured for 48–72 hours in the presence of DMSO, WS3 (0.01 μ M) or WS6 (0.5 μ M) had extra MYO7+ cells (red) in the outer hair cell region. Images are taken from the apex region. The supporting cell marker SOX2 (blue) and hair cell marker Atoh1-GFP (green) are shown. IHC: inner hair cell; OHC: outer hair cells; scale bar: 25 microns.

A' Cross section of the organ of Corti at the yellow line in (A) to show organization of supernumerary MYO7+ cells.

B. MYO7+ cell counts in the apex, mid-apex, mid-base and base region. Outer hair cell counts (squares) and inner hair cell counts (circles) are presented for each cochlea, along with means $\pm$ SD per 100 μ m (bars). Significantly more MYO7+ cells were observed in the WS3 or WS6-treated cochlea than in the controls at the apex region (apex: $T_{con} = 53.9$, $T_{WS6} = 75.1$, $T_{WS3} = 73.8$, $P_{WS6} = 0.0024$, $P_{WS3} = 0.010$; mid-apex: $T_{con} = 56.2$, $T_{WS6} = 65.4$, $T_{WS3} = 72.7$, $P_{WS6} = 0.13$, $P_{WS3} = 0.046$; Mid-base: $T_{con} = 59.2$, $T_{WS6} = 59.1$, $T_{WS3} = 70.8$, $P_{WS6} = 0.99$, $P_{WS3} = 0.042$; $n = 3$ per group, Dunnett multiple comparisons).

Figure 8. WS3 or WS6 treatment enhances supporting cell proliferation *in vitro*.

A. Explant cultures of the organ of Corti from postnatal mice (P1-P2) cultured for 48–72 hours in the presence of EdU (blue), in addition to DMSO, WS3 (0.01 μM) or WS6 (0.5 μM) displayed co-localization of EdU with the supporting cell marker SOX2 (red) among MYO7+ hair cells (green). Images are taken from the apex region. IHC: inner hair cells; OHC: outer hair cells, scale bar: 25 microns.

A' Cross section of organ of Corti from A at the yellow line.

B. Quantification of EdU+ cells in the supporting cell region, showing significantly more EdU+ supporting cells in the WS3 (triangles) or WS6-treated (squares) cochleae compared to controls (circles; $F = 13.0$, $P = 0.0000016$, ANOVA), with a gradient from apex to base (apex: $T_{con} = 4.1$, $T_{WS6} = 22.9$, $T_{WS3} = 29.0$, $P_{WS6} = 0.011$, $P_{WS3} = 0.00059$; mid-apex: $T_{con} = 3.0$, $T_{WS6} = 14.8$, $T_{WS3} = 17.4$, $P_{WS6} = 0.00057$, $P_{WS3} = 0.00077$; Mid-base: $T_{con} = 0.85$, $T_{WS6} = 6.2$, $T_{WS3} = 7.7$, $P_{WS6} = 0.0098$, $P_{WS3} = 0.0049$; $n = 3$ per group, Dunnett multiple comparisons).

C. Western blot analysis of ERBB2 pathway activation was conducted using anti-p-ERBB2 antibody (Y1248) in MCF-7 cells. Cells were treated with WS3 or WS6 for 15 minutes.

Figure 9. Summary of findings from activation of EGFR family signaling in supporting cells. Diagram of the cochlea (top) shows three outer hair cells and one inner hair cell, all in white, surrounded by pink supporting cells with blue SOX2+ nuclei. Three different methods of activation were employed (arrows): *in vitro* viral transduction, *in vivo* transgene induction, and *in vitro* drug manipulation. The top line shows where signaling is induced: the first two methods employed cell lineage tracing (bright green) which marked the CA-ERBB2-expressing supporting cells, and in the third method, ERBB3 activation is presumed throughout supporting cells (light green). The second line shows SOX2 modulation, in that SOX2 was down-regulated in cells neighboring those that express CA-ERBB2 (cyan changes to grey, *in vitro* virus and *in vivo* transgene). No SOX2 modulation is observed after drug treatment. The third line indicates supporting cell proliferation (red nuclei), which was observed with both *in vitro* methods but not *in vivo*. The fourth line indicates MYO7 induction (pink cells convert to white), which was observed *in vivo* and after drug manipulation.