



FGF20-FGFR1 signaling through MAPK and PI3K controls sensory progenitor differentiation in the organ of Corti.

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Key Findings:

1. PI3K and MEK1/2 are necessary for hair cell differentiation while p38 and PLC γ are not.
2. pAKT is necessary but not sufficient for hair cell differentiation.

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3. PTEN inhibition partially rescues *Fgf20* knockout phenotype.
4. dpERK is downstream of FGF20-FGFR1 signaling.

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ABSTRACT

Background: FGF20-FGFR1 signaling is essential for cochlear hair cell (HC) and supporting cell (SC) differentiation. In other organ systems, FGFR1 signals through several intracellular pathways including MAPK/ERK, PI3K, PLC γ , and p38. Previous studies implicated MAPK and PI3K pathways in HC and SC development. We hypothesized that one or both would be important downstream mediators of FGF20-FGFR1 signaling for HC differentiation. **Results:** By inhibiting pathways downstream of FGFR1 in cochlea explant cultures, we established that both MAPK and PI3K pathways are required for HC differentiation while PLC γ and p38 pathways are not. Examining the canonical PI3K pathway, we found that while AKT is necessary for HC differentiation, it is not sufficient to rescue the *Fgf20*^{-/-} phenotype. To determine whether PI3K functions downstream of FGF20, we inhibited Phosphatase and Tensin Homolog (PTEN) in *Fgf20*^{-/-} explants. Overactivation of PI3K resulted in a partial rescue of the *Fgf20*^{-/-} phenotype, demonstrating a requirement for PI3K downstream of FGF20. Consistent with a requirement for the MAPK pathway for FGF20-regulated HC differentiation, we show that treating *Fgf20*^{-/-} explants with FGF9 increased levels of dpERK.

Conclusions: Together these data provide evidence that both MAPK and PI3K are

important downstream mediators of FGF20-FGFR1 signaling during HC and SC differentiation.

INTRODUCTION

The organ of Corti (OC) is a specialized sensory epithelium within the cochlea that is responsible for converting sound waves into electrical signals that the brain can interpret. The OC is comprised of sensory hair cells (HCs) organized as one row of inner hair cells (IHC) and three rows of outer hair cells (OHC) spanning the length of the cochlea. Supporting cells (SCs) are located beneath the rows of HCs. Two rows of pillar cell, a specialized type of SC, forms the tunnel of Corti separating the medial IHCs from the lateral OHCs.¹

During mouse cochlear development, sensory progenitor cells exit the cell cycle in a wave that initiates near the apex of the cochlea around embryonic day 12.5 (E12.5) and progresses to the base by ~E14.5. Cell cycle exit is used as a marker of the completion of prosensory specification. Differentiation of HCs and SCs follows cell cycle exit and specification progressing in the opposite direction, initiating around E14.5 in the mid-base and traveling to the far base and apex.²⁻⁴ Differentiation is a critical step in OC development and is in part regulated by Fibroblast Growth Factor 20 (FGF20) activation

of FGF receptor 1 (FGFR1).^{4,5} However, the intracellular signaling mechanisms that govern HC and SC differentiation are poorly defined.

Fgfr1 is expressed in the floor of the cochlear duct throughout development and in the prosensory region of the cochlear epithelium during the initiation of differentiation (E14.5-E16.5).^{4,6-8} Conditional inactivation of *Fgfr1* in the cochlear epithelium dramatically reduces the number of HCs and SCs and disrupts the ordered rows of HCs leaving gaps in the sensory epithelium.^{4,6,9,10}

Fgf20 expression in the prosensory domain progresses in a basal to apical wave at around E14, similar to the progression of differentiation.⁷ FGF20 is likely the primary ligand that signals to FGFR1 during the differentiation of specified progenitor cells, based on similarities in phenotype between cochleae of mice lacking *Fgf20* and those lacking *Fgfr1*.^{4,11} In vitro inhibition of FGF20 results in fewer total cochlear HCs while genetic inactivation of *Fgf20* leads to a 70% reduction in OHC number.^{4,7,11} In a Sox2 hypomorphic background, loss of *Fgf20* also resulted in loss of IHCs.⁴ However, loss of IHCs was attributed to effects of Sox2 on earlier FGFR1 signaling, consistent with IHC loss with early (*FoxG1-Cre*) but not late (*Emx2-Cre*) inactivation of *Fgfr1*.¹⁰

In support of a specific role for FGF20-FGFR1 regulation of progenitor cell differentiation, genetic induction of FGF9 (a ligand closely related to FGF20) during the period of differentiation (E13.5-15.5) completely rescued the *Fgf20*^{-/-} cochlea phenotype. Additionally, when FGF9 was induced at progressively later days between E13.5-15.5, the phenotypic rescue occurred at progressively more apical regions of the cochlea consistent with the normal base to apex wave of sensory progenitor differentiation.⁴

Although FGF20 signals through FGFR1 to regulate HC and SC differentiation, the signaling pathways that regulate sensory progenitor differentiation downstream of FGF20-FGFR1 are poorly defined. FGFR signaling activates multiple intracellular pathways including phosphoinositide 3-kinase (PI3K), p38, mitogen activated protein kinase (MAPK), phospholipase C γ (PLC γ) and signal transducer and activator of transcription 1 (STAT1).¹²⁻¹⁴ FGFR has been shown to use one or more downstream pathways selectively in different tissues and cell types. For example, in chondrocytes, FGFR3 signals through both the MAPK and STAT1 pathways.^{15,16} In the lung epithelium, FGFR2 and FGFR3 differentially signal through MAPK and PI3K, respectively.¹⁷ In early embryonic development, FGFR1 differentially activates MAPK, PI3K, and PLC γ pathways.¹⁸⁻²⁰

The best characterized intracellular pathway downstream of FGFR1 during inner ear development is the MAPK pathway. At initial stages of inner ear development, FGFR signals through the MAPK pathway during otic placode induction.²¹ At slightly later stages, ERK1/2 signaling is active in unspecified cochlear epithelial progenitors at E10.5 and E11.5 and is reduced in cochleae with reduced FGFR1 signaling.¹⁰ During sensory progenitor differentiation, *Etv1*, *Etv4* (*Pea3*) and *Etv5* (*Erm*), known downstream effectors of FGF signaling, have decreased expression in the prosensory domain of *Fgf20*^{-/-} mice at E14.5.^{4,12,22} Expression of *Dusp6*, a negative feedback regulator of ERK signaling,²³ is also decreased in *Fgf20*^{-/-} cochlea.²² Similarly, *MEKK4* (*Map3k4*), a MAP2K kinase expressed throughout the floor of the cochlea at E13 that becomes restricted to HCs and SCs by E16.5, is required for HC and SC development. Mutants of *MEKK4* produced lower levels of dpERK, the activated form of ERK, resulting in only two rows of OHCs and Deiters' cells. This phenotype is attributed to perturbed differentiation that is similar to but less severe than that seen in *Fgf20*^{-/-} cochlea.^{6,11,24} Furthermore, MEKK4 protein expression was decreased in *Fgf20*^{-/-} and *Foxg1*^{Cre}; *Fgfr1*^{fl/fl} cochlea.²⁴ Collectively, these results suggest that while expression of MAPK pathway components are important for HC and SC differentiation and are regulated by FGF20-FGFR1 signaling, they may not account for the complete differentiation program regulated by FGF20-FGFR1 signaling. Interestingly, Ono et al. concluded that FGFR1 regulation of dpERK is not important at E14.5.^{10,24} As such,

contradictory evidence exists regarding dpERK signaling downstream of FGF20-FGFR1 during HC and SC differentiation.

The importance of PI3K-AKT signaling is also implicated in organ of Corti development. Changes in AKT phosphorylation were not observed at E12.5 in *Fgfr1* conditional knockouts or by treatment of E13 cochlear explants with an FGFR inhibitor.^{10,24} However, PI3K inhibition of explants starting from E13.5 did produce a dose dependent decrease in the number of cochlear HCs, which was attributed to the loss of IGF1 signaling.²⁵

To systematically interrogate the activity of signaling pathways that could function downstream of FGF20 and FGFR1 during prosensory cell differentiation, we used a cochlear explant model coupled with small molecule pathway-specific inhibitors. This showed that both PI3K and ERK1/2 signaling are important for sensory HC differentiation. Furthermore, inhibition of Phosphatase and Tensin Homolog (PTEN) to potentiate PI3K signaling in *Fgf20*^{-/-} mice resulted in a partial rescue of the HC phenotype and treatment of *Fgf20*^{-/-} cochleae with FGF9 resulted in higher levels of dpERK. These results suggest that both PI3K and MAPK may be part of the FGF20-FGFR1 signaling pathway during HC differentiation.

RESULTS

MEK1/2 and PI3K, but not PLC γ and p38, are important for cochlear HC development

To determine which pathways downstream of FGFR1 could potentially regulate sensory progenitor cell differentiation, we used an inhibitor approach on cochlea explant cultures targeting each pathway individually (U0126 for MEK1/2, LY294002 for PI3K, U73122 for PLC γ , and SB203580 for p38).²⁵⁻³² Explant cultures were set up from dissected wild-type E14.5 cochleae and treated with an inhibitor or equivalent concentration of DMSO (vehicle control) for two days and harvested on day four in vitro (DIV4; the equivalent of E18.5) (Fig. 1A). Explants were fixed and immunostained for myosin VI (MYO6, a marker of differentiated HCs) to allow quantification of total HC numbers for each cochlea explant.³³

Using concentrations derived from the literature for each inhibitor,²⁵⁻²⁸ we found that explants treated with 10 μ M U73122 to inhibit PLC γ showed no difference in final HC number compared to vehicle-treated controls (vehicle: 1943 \pm 257, n=3; U73122: 1943 \pm 141, n=3, p=1.0) (Fig. 1B, C, G). Similarly, there was no difference in HC numbers between vehicle controls (image not shown) and explants treated with 10 μ M

SB203580 to inhibit p38 (vehicle: 1632 ± 98 , $n=3$; SB203580: 1573 ± 264 , $n=3$, $p=0.73$) (Fig. 1D and H). After 4 DIV, cochlea explants from both inhibitor treatments showed normal phenotypes consisting of one row of IHCs and multiple rows of OHCs.

Inhibition of PI3K produced a decrease in HC number. Explants treated with $40 \mu\text{M}$ LY294002 showed a 59% decrease in HCs compared to vehicle controls (vehicle: 1937 ± 166 , $n=5$; LY294002: 780 ± 207 , $n=5$, $p<0.005$) (Fig. 1E and I). Decreasing the dose to $20 \mu\text{M}$ LY294002 resulted in a 57% reduction in HC number compared to vehicle-treated controls (vehicle: 2063 ± 219 , $n=4$; LY294002: 888 ± 108 , $n=4$, $p<0.05$) (Fig. 1I). Inhibition of MEK1/2 with $50 \mu\text{M}$ U0126 resulted in a 41% decrease in HC number compared to vehicle controls (vehicle: 1782 ± 201 , $n=6$; U0126: 1047 ± 278 , $n=6$, $p<0.005$) (Fig. 1F and J). Inhibition of MEK1/2 appeared to be dose dependent, as treatment of explants with $25 \mu\text{M}$ U0126 resulted in a 19% reduction in HC number compared to vehicle-treated controls to inhibitor treated explants (vehicle: 2035 ± 172 , $n=4$; U0126: 1657 ± 186 , $n=4$, $p=0.04$) (Fig. 1J). Together, these data show that PI3K and MEK1/2 are both necessary for the production of HCs.

AKT is necessary for HC development but its activation is not sufficient to rescue the *Fgf20*-null phenotype

PI3K has been shown to activate a number of different downstream pathways including AKT (Protein Kinase B, PKB).^{34,35} To determine if canonical PI3K-AKT signaling is necessary for HC development, E14.5 wildtype cochlea explants were treated with 10 μ M ARQ092, a highly specificity inhibitor targeting the activity of all three AKT isoforms, using the treatment plan shown in Fig. 1A.³⁶ We found a 36% decrease in total HCs in 10 μ M ARQ092 treated explants compared to vehicle-treated controls (vehicle: 2007 \pm 377, n=6; ARQ092: 1273 \pm 267, n=6, p<0.01) (Fig. 2A, B). 5 μ M ARQ092 produced a similar but smaller effect with a 25% reduction in total HCs (vehicle: 1965 \pm 301, n=6; ARQ092: 1480 \pm 230, n=6, p=0.017) (Fig. 2A, B), showing pAKT inhibition to be dose dependent.

To determine whether AKT activation is sufficient to rescue the *Fgf20*^{-/-} (*Fgf20*^{Cre/ β gal}) phenotype, we overactivated AKT signaling using the AKT agonist SC-79 in E14.5 *Fgf20*^{-/-} cochlea explants treated for two days and harvested on DIV4. SC-79 binds directly to the PH domain of AKT, inhibiting translocation to the membrane but altering its conformation to facilitate its phosphorylation by cytosolic, upstream kinases.³⁷ We treated cochlea explants with 30 μ M SC-79, which based on previous studies effectively increases the levels of pAKT.^{38,39} We found no significant differences in HC number

between inhibitor treated explants and vehicle controls (vehicle: 750 ± 249 , $n=3$; SC-79: 704 ± 83 , $n=3$, $p=0.76$) (Fig. 2C, D). These results suggest that while AKT is necessary, it is not sufficient to support normal HC differentiation.

PTEN inhibition partially rescues HC loss in *Fgf20*-null cochleae

Next, we asked whether PI3K functions downstream of FGF20. PI3K phosphorylates PIP2 (phosphatidylinositol-(4,5)-bisphosphate) to PIP3 (phosphatidylinositol-(3,4,5)-triphosphate). Molecules with Pleckstrin Homology (PH) domains are capable of binding PIP3 and are recruited to the cell membrane. Both 3-phosphoinositide-dependent protein kinase-1 (PDK1) and its target of phosphorylation, AKT (PKB), contain PH domains and upon binding PIP3 translocate to the cell membrane resulting in AKT activation. PTEN negatively regulates PI3K signaling by dephosphorylating PIP3 to PIP2, and inactivation of PTEN leads to overactivation of PI3K signaling.³⁴ Additionally, in the cochleae, PTEN is expressed in the prosensory domain at E14.5, suggesting that it has a potential role during HC and SC differentiation.⁴⁰ To investigate whether PI3K functions downstream of FGF20, we overactivated the PI3K pathway in *Fgf20*^{-/-} cochlea explants using BpV(HOpic), an inhibitor of PTEN known to be effective in mouse cochleae.⁴¹ Maintaining similar explant culture procedures as before (Fig. 1A), E14.5 *Fgf20*^{-/-} cochleae explants treated with 5 μ M BpV(HOpic) produced an increase of 31% in total HCs compared to *Fgf20*^{-/-} explants treated with vehicle (vehicle: 769 ± 131 , $n=7$;

BpV(HOpic) 1010 ± 144 , $n=7$, $p<0.001$) (Fig. 3A, B). This is similar to the results of rescuing *Fgf20*^{-/-} explants with FGF9 at E14.5.¹¹

PTEN inactivation has been suggested to produce supernumerary HCs through inhibition of p27^{Kip1} expression in *Pax2*^{Cre}; *Pten*^{fllox/fllox} conditional knockout mice.⁴² However, *Pax2*^{Cre} is active in the otic vesicle starting as early as E9.5, much earlier than the start of HC and SC differentiation.^{42,43} To determine if FGF20-PI3K signaling is limiting for HC differentiation under wild-type conditions, we treated wild-type E14.5 cochleae with 5 μ M BpV(HOpic) as before (Fig. 1A). We found no significant difference in total HCs in BpV(HOpic) treated cochlea explants compared to vehicle-treated controls (vehicle: 1659 ± 323 , $n=5$; BpV(HOpic): 1809 ± 203 , $n=4$, $p=0.45$) (Fig. 3A, B). These results suggest that PI3K functions downstream of FGF20 and that in wild-type cochleae the level of PI3K signaling during initial stages of differentiation is not the sole limiting factor for HC production.

FGF9 treatment of *Fgf20*-null cochlear explants activates dpERK signaling

To determine whether the ERK signaling pathway lies directly downstream of FGF20-FGFR1 activation, we examined levels of dpERK in cochlea explants in response to FGF signaling. E14.5 *Fgf20*^{-/-} cochlea explants were treated with recombinant FGF9 plus Heparin or vehicle (media with Heparin) for 15 min. FGF9 is biochemically similar

to FGF20 and has been shown to rescue differentiation in *Fgf20*^{-/-} mice in vivo and in vitro.^{4,11} Following treatment with FGF9, the floor of the cochlear epithelium was isolated from the mesenchyme using dispase/collagenase. Western blot analysis showed a significant increase in levels of dpERK in FGF9-treated explants compared to vehicle-treated controls ($p < 0.01$) (Fig. 4A, B). This shows that FGF signaling can lead to downstream ERK phosphorylation at this stage during cochlea development.

DISCUSSION

Previous studies have shown the importance of FGF20-FGFR1 signaling for cochlear HC differentiation.^{4,7,10,11,24} However, the relative requirement of different signaling pathways downstream of FGF20-FGFR1 remain unknown. In this study, we found that PI3K and MEK1/2 signaling are independently necessary for HC development. We also provide evidence that these intracellular signaling pathways function downstream of FGF20-FGFR1.

The main method we used to interrogate the necessity of various signal mediators was through the use of inhibitor-treated cochlear explant cultures. It should be noted that while inhibitors can be very specific, they may also have off-target effects or have other toxic nonspecific effects. We generally tested multiple concentrations that were in range of previously published effective and target-specific concentrations.

FGF20 signals through MAPK and PI3K to regulate HC development

Previous studies suggested that PI3K is important for HC differentiation. PI3K was found to be necessary for HC development when E13.5 cochleae treated with LY294002 (PI3K inhibitor) showed a decrease in HC number.²⁵ In this previous study, PI3K was linked to the IGF1 signaling pathway and the timing of treatment suggested that the effects could be partially due to inhibition of progenitor cell proliferation. Other studies have shown that conditional knockout of PTEN produces supernumerary HCs and suppresses p27^{Kip1} expression, further suggesting that PI3K has a role in sensory progenitor proliferation.⁴² By contrast, our experiments were initiated at E14.5 after progenitor cell cycle exit,⁴ demonstrating an additional role for PI3K in HC differentiation. In addition, treating E13.5 wild-type cochleae with 40 μ M LY294002 in the previous study resulted in fewer HCs compared to our treatments starting on E14.5,⁴⁴ further supporting a role for PI3K in both differentiation and progenitor cell proliferation. The results presented here demonstrate an interaction between PI3K and FGF20, potentially placing PI3K signaling downstream of FGF20.

Previous studies have suggested a relationship among FGF20, MEKK4, and dpERK. Mutations in MEKK4 results in fewer cochlear HCs and a decrease in the levels of dpERK, suggesting a role for dpERK in HC development.²⁴ Here, we treated explants

directly with a MEK1/2 inhibitor. In addition, by treating *Fgf20*^{-/-} cochleae with FGF9 as a surrogate for FGF20, we showed that FGF signaling is sufficient for ERK activation. Together, these results suggest that FGF20 signaling regulates HC differentiation at least partly through ERK signaling.

Furthermore, our data confirms results from other papers with respect to PLC γ . Ono et al. found that the *FGFR1*^(Y766F/Y766F) mutant cochleae, which carry a mutation thought to prevent activation of PLC γ ,⁴⁵ did not show a decrease in the number of HCs.¹⁰ Our results showing no change in HC number in PLC γ inhibited cochlea explants provide additional evidence that PLC γ is not necessary for HC development.

Pathways downstream of PI3K in addition to pAKT are important for HC differentiation

PTEN inhibition resulted in a partial rescue of HC differentiation in *Fgf20*^{-/-} cochleae explants but did not affect HC number in wild-type explants. AKT inhibition decreased the number of HCs while the AKT agonist did not rescue *Fgf20*^{-/-} cochleae. This suggests that a pathway downstream of PI3K other than pAKT may be required for normal cochlear development. It is possible that dpERK functions downstream of PI3K as previous studies showed that PI3K could regulate levels of dpERK through PDK1.^{35,46} However, other pathways downstream of PI3K may also be important such

as AGC kinases, BTK/Tec Kinase, or p70S6 kinase downstream of PtdIns(3,4,5)P3 or TAPP1/2 downstream of PtdIns(3,4)P2.^{34,35,47} Previous studies showed that rescuing *Fgf20*^{-/-} cochleae with FGF9 resulted in roughly a 70% increase in the number of cochlear HCs while we found that PTEN inhibition only increased HC numbers in *Fgf20*^{-/-} cochleae by 31%. These results suggest that a pathway independent of PI3K may be necessary for the full rescue, which is consistent with a role for ERK in parallel to PI3K.

PI3K and ERK pathways may be activated by separate ligands

Inhibition of either PI3K or dpERK resulted in roughly a 50% decrease in HCs, similar to the phenotype found in *Fgf20*^{-/-} cochleae. Both conditional deletion of *Fgfr1* and FGFR1 inhibition during differentiation produce a more severe phenotype compared to deletion of *Fgf20* alone.^{7,11} It has been posited that there is another ligand in addition to FGF20 that also regulates HC differentiation that could account for these differences.⁴ One caveat of our study is that while FGF9 overactivation of dpERK and partial rescue through PTEN inhibition suggest that FGF20 may signal through these mediators, it does not say whether FGF20 is responsible for regulating the level of either phosphoprotein. As such, dpERK and PI3K could be activated downstream of FGFR1 by different ligands.⁴ Furthermore, activation of a single FGFR by different ligands can produce different downstream signals and outcomes.⁴⁸ Additional studies will be needed

to identify other FGFs that function with FGF20 to regulate sensory progenitor differentiation.

EXPERIMENTAL PROCEDURES

Animals

Mice were group housed by sex or maintained in breeding harems (1 male to 2 females) with food and water provided ad libitum. Mice were maintained on a mixed C57BL/6J x 129x1/SvJ background. *Fgf20^{Cre}* mice contained an allele encoding a GFP-Cre fusion protein replacing exon 1 of *Fgf20* resulting in a null mutation.⁹ *Fgf20^{βgal}* mice contained a knockin sequence encoding β-galactosidase (βgal) replacing exon 1 of *Fgf20* resulting in a null mutation.¹¹ *Fgf20* knockout mice (*Fgf20^{-/-}*) were either *Fgf20^{Cre/Cre}* or *Fgf20^{Cre/βgal}*. Studies performed were in accordance with the Washington University in St. Louis Institutional Animal Care and Use Committee (protocol #20190110 and #20170258).

Cochlear Organ Cultures

Organ Cultures treated with Inhibitors and Activator: Explant cultures followed published methods with minor changes.⁷ In brief, wild-type or *Fgf20^{Cre/βgal}* E14.5 embryos were dissected to expose the sensory epithelium which was subsequently transferred to

Matrigel (Corning Inc., #356231) coated MatTek dishes (MatTek Corporation, P35GC-0-14-C) with the epithelium facing upward. Explants were cultured in Dolbecco's Modified Eagle Medium (Thermofisher Scientific, #11965-084) with N2 supplement (Thermofisher Scientific, #17502-048) and pen/strep, containing small molecule inhibitors/activator or DMSO vehicle control for the first two days (E14.5-E16.5) and then cultured in regular media (no DMSO or inhibitors/activators) until E18.5 when they were fixed in paraformaldehyde (Electron Microscopy Sciences, 15714-S) (4%). Heat inactivated FBS (5%) (Gibco/Thermofisher Scientific, 10437-028) was included in culture medium starting on the second day of culture. Culture media was fully replaced each day except for the third day (E17.5) on which half the media was replaced.

The following small molecule inhibitors and activator were used:

1. Phosphatidylinositol-3-Kinase (PI3K) inhibition: 20 μ M (0.04% DMSO) and 40 μ M (0.08% DMSO) 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002, Cell Signaling Technology, #9901).
2. p38 inhibition: 10 μ M (0.1% DMSO) 4-(4'-Fluorophenyl)-2-(4'-methylsulfinylphenyl)-5-(4'-pyridyl)-imidazole (SB203580, Cell Signaling Technology, #5633) with 0.5% DMSO vehicle control.
3. Phospholipase C Gamma (PLC γ) inhibition: 10 μ M (0.2% DMSO) 1-[6-[[[(17 β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122, Tocris Bioscience, #1268).

4. Mitogen-Activated Protein Kinase Kinase 1 & 2 (MAP2K1/2, a.k.a. MEK1/2) inhibition: 25 μ M (0.03% DMSO) and 50 μ M (0.5% DMSO) 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126, Cell Signaling Technology, #9903).
5. Phosphatase and Tensin Homolog (PTEN) inhibition: 5 μ M (0.25% DMSO) and 10 μ M (0.5% DMSO) Dipotassium bisperoxo (5-hydroxypyridine-2-carboxyl) oxovanadate (V) (BpV(HOpic), Santa Cruz Biotechnology, #SC-221377).
6. AKT (Protein Kinase B) inhibition: 5 μ M (0.1% DMSO) and 10 μ M (0.2% DMSO) 3-[3-[4-(1-aminocyclobutyl)phenyl]-5-phenyl-3H-imidazo[4,5-b]pyridin-2-yl]-2-pyridinamine (Miransertib, ARQ092, Selleckchem.com, #S8339).
7. AKT (Protein Kinase B) activation: 10 μ M (0.1% DMSO) and 30 μ M (0.3% DMSO) 2-Amino-6-chloro- α -cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester (SC-79, Tocris Bioscience, #4635).

Organ culture for immunoblot protein quantification: Cochleae from E14.5 *Fgf20^{Cre/βgal}* were dissected to reveal the prosensory epithelium and allowed to equilibrate in DMEM (with N2 supplement and Pen/Strep but no FBS) for 2 h at 37°C. Cochleae were then treated for 15 min in media containing 1 μ g/ml FGF9 (Peprotech Inc., #100-23) with 1.13 mg/ml heparin (MilliporeSigma, #H3393) or only heparin vehicle controls. Cochleae were then incubated in 0.1% Dispase (Corning Inc., #354235) and 0.1% Collagenase (Worthington, #LS004177) in Hanks Buffered Salt Solution (Gibco/Thermo Fisher Scientific, #14025-076) for 10 min at room temperature to facilitate removal of mesenchyme. Isolated cochleae epithelium was then washed briefly in PBS to remove

excess dispase and collagenase, centrifuged at 800 rcf for 4 min at 4°C to facilitate removal of excess PBS, flash frozen in liquid nitrogen, and then stored at -80°C.

Immunoblot

Both cochleae from each pup were lysed together as one sample using mortar and pestle (Thermo Fisher, K7495211590) in RIPA Lysis Buffer (MilliporeSigma, #R0278) with ProBlock Mammalian Protease Inhibitor Cocktail (Gold Biotechnology, #GB-331-1) and Simple Stop 2 Phosphatase Inhibitor Cocktail (Gold Biotechnology, #GB-451). Samples were then placed on ice for 30 min followed by centrifugation at 13,200 rcf at 4°C after which supernatant was collected. BCA assay (Thermo Fisher Scientific, #23227) was used to quantify protein before denaturation in LDS Sample Buffer (Life Technologies Corporation, #NP0007) and Sample Reducing Buffer (Life Technologies Corporation, #NP0009) at 70°C for 10mins. Samples were then stored at -80°C.

Samples were run at 160V for 1.5 h on ice at 4°C using NuPage Bis-Tris 10% gel (Thermo Fisher Scientific, #NP0302BOX) in MES buffer (Thermo Fisher Scientific, #NP000202). iBlot System (Thermo Fisher Scientific, #IB1001) was used to transfer protein onto PVDF membrane, according to manufacturer protocol. The membrane was then incubated in TBS-Tween 0.05% (TBS-T) for 10 min before blocking for 1hr at room temperature using TBS-T with 5% (w/v) non-fat milk (TBS-TM). Membranes were then

incubated overnight in the following antibodies in TBS-TM: dpERK (Cell Signaling Technology, #4370 S), GAPDH (Cell Signaling Technology, #97166). Following wash (3x10 min TBS-T), blots were incubated for 1 h at room temperature in Goat anti-Rabbit HRP secondary antibody (Santa Cruz Biotechnology, #SC-2301), washed 3x10 min TBS-T, then incubated in ECL (Bio-Rad Laboratories, #1705061) followed by imaging.

Western blot membranes were imaged and quantified using Biorad Chemidoc (Bio-Rad Laboratories) and Image Lab Software (Bio-Rad Laboratories). Densitometry readings were generated by drawing rectangles surrounding each band and both bands for dpERK1/2 using the same sized rectangle on all bands for each protein. “Adjusted values” from excel exported files of densitometry readings were then used in statistical analysis.

Sample Preparation and Immunostaining

Cochlear organ cultures treated with inhibitors or activator were fixed in 500 μ l of 4% PFA for 20 min at room temperature and washed in PBS (3 x 10 min). Cochleae placed in MatTek culture dishes were incubated in PBS with 0.5% Tween-20 (PBSTw) for 1 h to permeabilize and then blocked using PBSTw with 5% donkey serum (DS) for 1 h. Cochleae were then incubated in PBSTw with 1% DS and Rabbit anti-MYO6 (1:100, Santa Cruz Biotechnology, #C1210) primary antibody overnight at 4°C. Following a

wash step (PBS x 3), cochlea were incubated in PBS with 1% Tween20 with Alexa Fluor 488 Donkey anti-Rabbit secondary antibodies (1:500; Thermo-Fisher Scientific, #A-21206) at room temperature and finally washed (PBS x3) and mounted in VectaShield antifade mounting medium with DAPI (Vector Labs, Burlingame, #H-1200).

Zeiss Axio Imager Z1 with Apotome 2 (Zeiss Microscopy) was used for all fluorescence imaging. Images taken were converted using Zen imaging software and then converted to JPEG files using FIJI ImageJ (Imagej.nih.gov). Images of portions of the cochlea were stitched together using GNU Image Manipulation Software (gimp.org).

Hair Cell Quantification and Statistical Analyses

Hair cells from whole cochleae were counted by hand using the FIJI cell counter plug in (<https://www.janelia.org/open-science/fiji-cell-counter>). All figures were made in Canvas X (ACD systems) image processing software. Graphs and statistical analysis were performed using GraphPad Prism Edition 5 software. Comparisons of two means were performed using two-tailed, un-paired Student's t-test and multiple comparisons were performed using one-way ANOVA. For significant ANOVA results at $\alpha = 0.05$, Tukey's HSD was performed for post-hoc pair-wise analysis. In all cases, $p < 0.05$ was considered statistically significant. Each point represents one cochlea.

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Author Contributions

Conceptualization: Y.S, L.M.Y., D.M.O.; Methodology: Y.S., L.M.Y. and D.M.O.; Formal Analysis: Y.S., D.M.O; Investigation: Y.S; Resources: D.M.O.; Writing – Original Draft: Y.S.; Writing – Review & Editing: Y.S., L.M.Y., D.M.O.; Supervision: D.M.O.; Funding Acquisition: D.M.O.

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Figure Legends

Figure 1. MEK1/2 and PI3K signaling pathways, but not PLC γ and p38, are necessary for cochlear development. **A**, Schematic representation of inhibitor or vehicle treatment of cochlea explants. Cochleae were explanted on E14.5 and were treated for 2d in media containing DMSO (**B**) or inhibitor for PLC γ (U73122, **C**), p38 (SB203580, **D**), MEK1/2 (U0126, **E**), or PI3K (LY294002, **F**) before continuing culture in regular media. Full media changes were given on the first three days of culture and a half media change given on the third day. **B-F**, Whole mount images of vehicle and inhibitor treated cochlea explants immunostained for Myo6 with 20X magnifications of mid base regions shown in **B'-F'**. **G-J**, Quantification of total HC number in explants treated with 10 μ M U73122 (**G**) or 10 μ M SB203580 (**H**), 20 and 40 μ M LY294002 (**I**), and 25 and 50 μ M U0126 (**J**). Graphs show mean \pm SD, with each point representing a single cochlea. Student's t-test, * $p < 0.05$. ** $p < 0.005$. Scale bars: 200 μ m (whole), 100 μ m (magnifications).

Figure 2. AKT is necessary but not sufficient for HC development. **(A)** Wildtype, whole mount of cochleae treated with vehicle control ARQ092 (5 μ M and 10 μ M) to inhibit AKT activity for 2DIV starting on E14.5 and harvested after 4DIV with 20X magnifications shown below. **(B)** Quantifications of whole cochleae HCs shown in **(A)**. **(C)** E14.5 *Fgf20^{cre/ β gal}* whole mount of cochleae treated for 2DIV with vehicle control or AKT

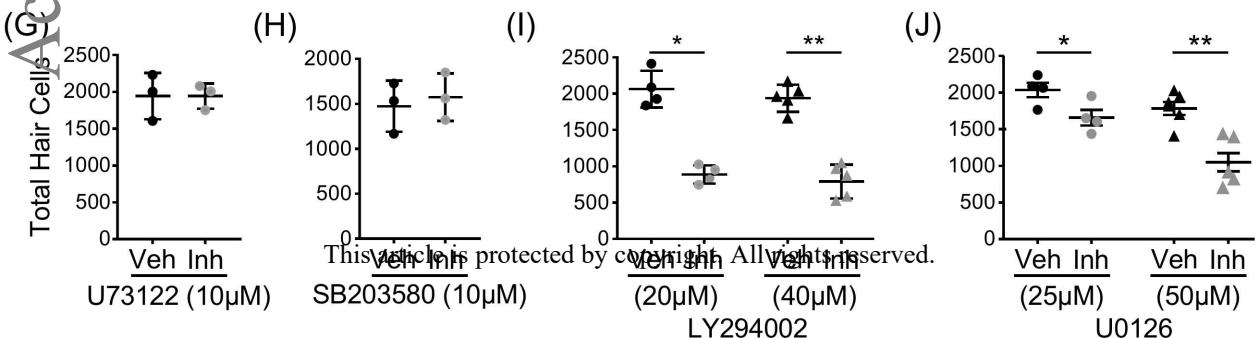
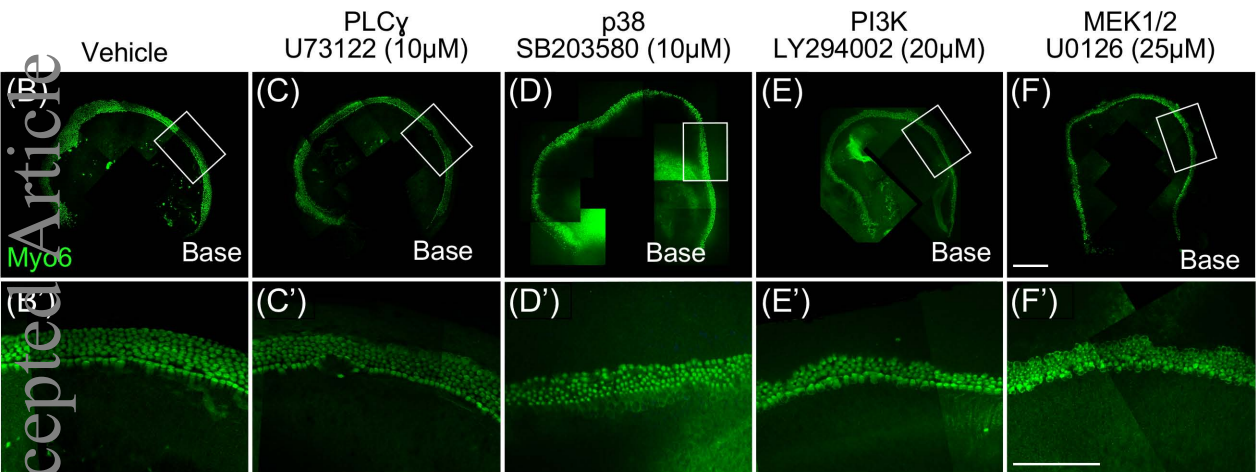
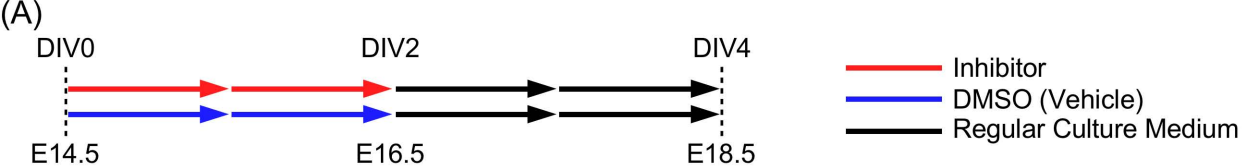
agonist, SC-79, at 30 μ M and harvested at 4DIV with 20X magnifications shown below. (D) Quantifications of whole cochleae HCs shown in (C). Graphs show mean \pm SD, with each point representing a single cochlea. Student's t-test, * p <0.05. Scale bars: 200 μ m (whole), 100 μ m (magnifications).

Figure 3. PI3K signaling pathway is shown to be downstream of FGF20 signaling and important for HC differentiation. (A) Whole mount cochlea explants of *Fgf20^{cre/βgal}* and wild-type cochleae treated with vehicle control or 5 μ M BpV(HOpic) PTEN inhibitor. 20X magnifications are shown below whole cochleae. (B) Quantifications of whole cochleae HCs shown in (A). Graphs show mean \pm SD, with each point representing a single cochlea. ANOVA, ns, not significant, * p <0.05. Scale bars: 200 μ m (whole), 100 μ m (magnifications).

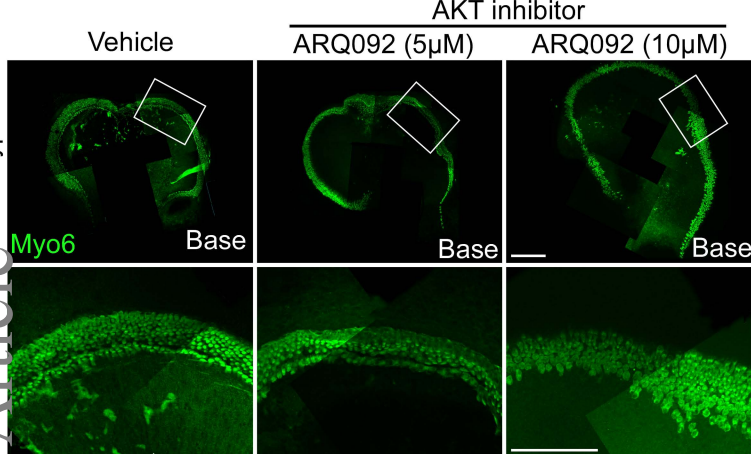
Figure 4. FGF9 increased dpERK in *Fgf20^{cre/βgal}* cochleae. (A) Western blot of cochlea immunostained for dpERK or GAPDH as a loading control. Each image was created from different sections of the same blot. E14.5 *Fgf20^{cre/βgal}* cochleae were treated with FGF9 plus Heparin (+) or vehicle (Heparin only) (-) for 15 min before harvest. Two replicates are shown. (B) Western blot densitometry analysis of dpERK protein levels normalized to GAPDH, with or without FGF9 treatment (n=8 for each treatment). Graphs

show mean \pm SD, with each point representing a single treated sample (1 cochlea).

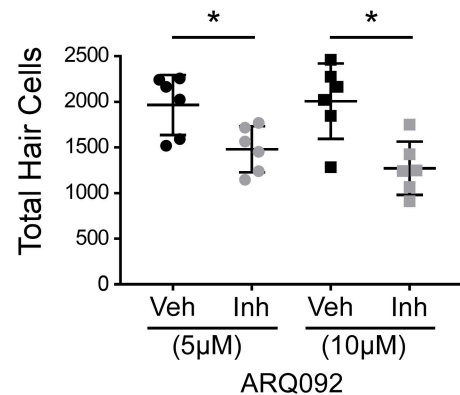
Student's t-test, ** $p < 0.01$.



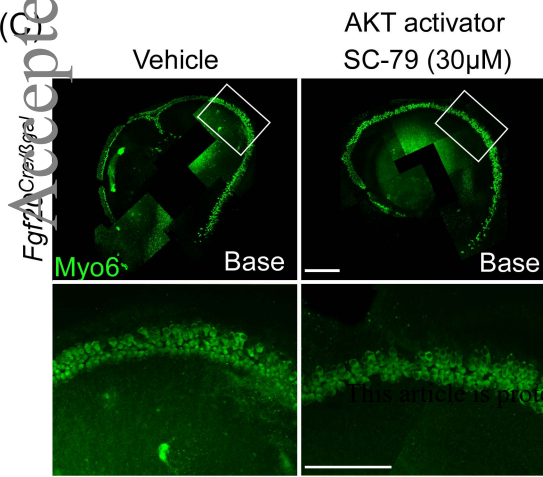
(A)



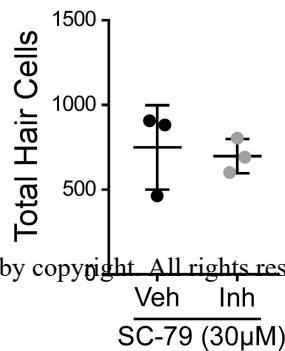
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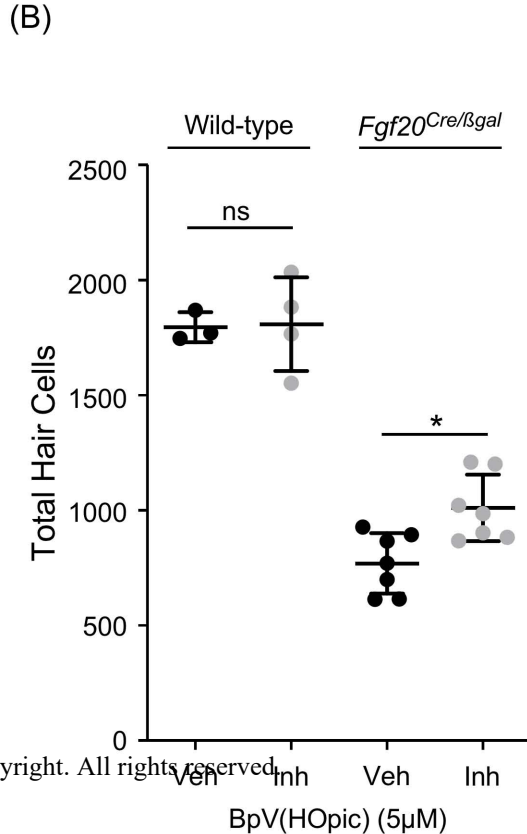
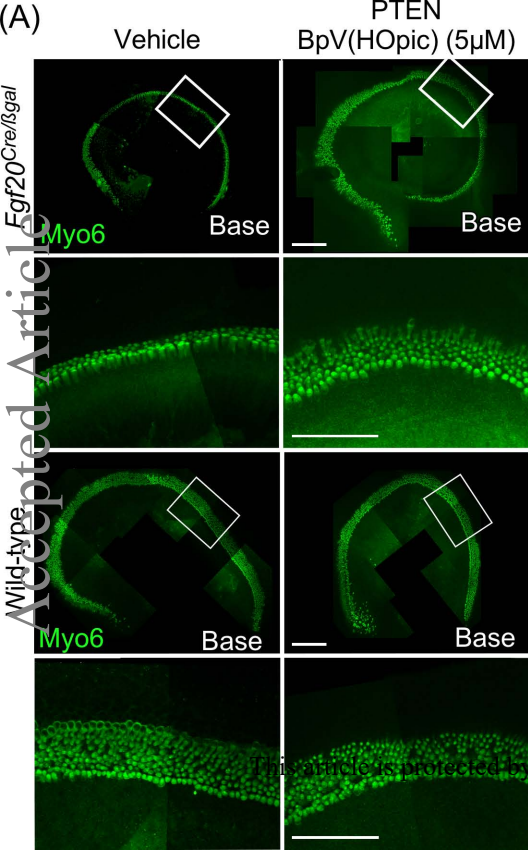
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Fgf2^{Cre/Cre}

(D)

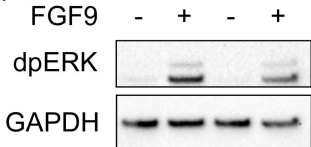


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