

MEK/ERK signaling drives the transdifferentiation of supporting cells into functional hair cells by modulating the Notch pathway

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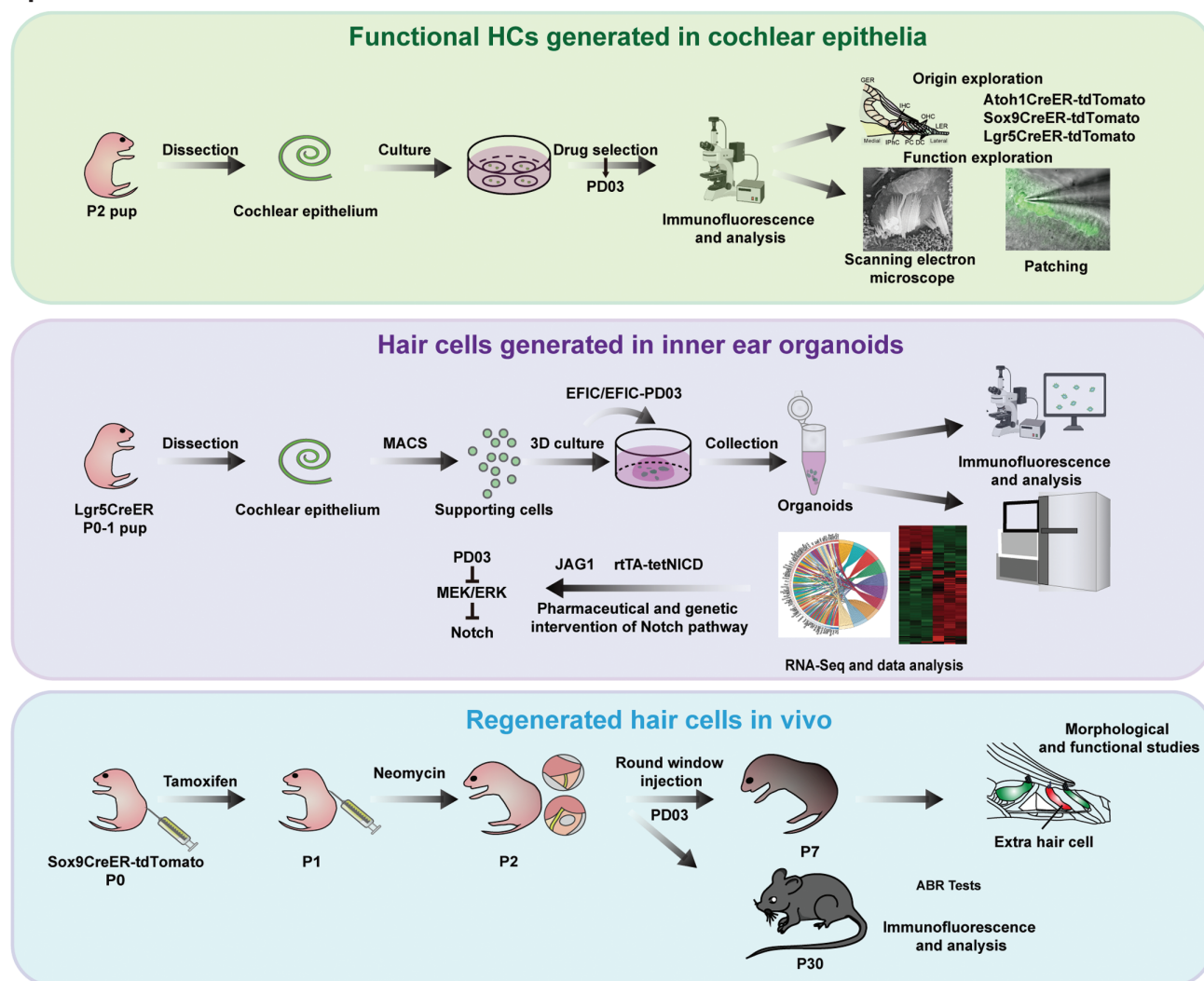
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Abstract

Loss of cochlear hair cells (HCs) leads to permanent hearing loss in mammals, and regenerative medicine is regarded as an ideal strategy for hearing recovery. Limited genetic and pharmaceutical approaches for HC regeneration have been established, and the existing strategies cannot achieve recovery of auditory function. A promising target to promote HC regeneration is MEK/ERK signaling because dynamic shifts in its activity during the critical stages of inner ear development have been observed. Here, we first showed that MEK/ERK signaling is activated specifically in supporting cells (SCs) after aminoglycoside-induced HC injury. We then selected 4 MEK/ERK signaling inhibitors, and PD0325901 (PD03) was found to induce the transdifferentiation of functional supernumerary HCs from SCs in the neonatal mammalian cochlear epithelium. We next found that PD03 facilitated the generation of HCs in inner ear organoids. Through genome-wide high-throughput RNA sequencing and verification, we found that the Notch pathway is the downstream target of MEK/ERK signaling. Importantly, delivery of PD03 into the inner ear induced mild HC regeneration in vivo. Our study thus reveals the importance of MEK/ERK signaling in cell fate determination and suggests that PD03 might serve as a new approach for HC regeneration.

Key words: regeneration; inner ear; hair cells; MEK/ERK signaling; organoids; Notch pathway.

Graphical Abstract



Significance Statement

This study provides a new approach, PD03, for the regeneration of hair cells in vitro and in vivo mediated by the inhibition of the Notch pathway. Moreover, PD03 treatment can significantly promote the differentiation of organoids into HCs. We suggested that PD03 will be a candidate for inclusion in a cocktail of regenerative drugs to achieve the restoration of hearing function.

Introduction

Hearing disorders, either inherited or acquired, are among the most common sensory deficits in humans and affect more than 460 million people around the world.¹ Dysfunction and loss of sensory hair cells (HCs) are the main causes of hearing loss induced by noise, drugs, or aging.² Unlike other vertebrates such as fish and birds, sensorineural hearing loss cannot be restored spontaneously in mammals because HCs do not regenerate in significant numbers once damaged, especially in adulthood.³⁻⁵ HCs and supporting cells (SCs) arise from common progenitors during the inner ear development,⁶ and studies have shown that SCs can give rise to new HCs in the neonatal and adult cochlea under certain conditions.^{7,8} Thus, an ideal strategy for hearing restoration is to regenerate HCs in situ from the residual SCs.

In recent decades, much effort has been into exploring possible strategies for activating mammalian HC regeneration.

Activation of the Wnt pathway promotes SC reentry into the cell cycle, and inhibition of the Notch pathway induces the trans-differentiation of SCs into HCs in the neonatal cochlea, thus resulting in limited HC regeneration.^{7,9-12} In addition, the manipulation of essential genes related to cell cycle and HC fate determination—including *p27kip1* and *Atoh1*—has been shown to reprogram SCs and induce the conversion of SCs into HCs in the cochlea.^{13,14} Although multigene reprogramming can achieve HC regeneration in the mammalian cochlea,^{15,16} no hearing function has been reestablished because the morphological and functional properties of the few regenerated HCs are still far from those of the original HCs. Apart from genetic approaches, some pharmaceutical means of HC regeneration have also been established,¹⁷⁻²⁰ for example, LY411575 (a γ -secretase inhibitor) delivered to the inner ear leads to HC regeneration. In view of the limited strategies available to regenerate HCs, there remains a need to further explore other methods.

MEK/ERK signaling participates in multiple cellular activities including proliferation, differentiation, and stem cell maintenance.^{21,22} In the canonical ERK1/2 pathway, binding of ligands to cell surface receptors activates the small GTPase Ras, leading to the recruitment of the MAPK kinase (MAP3Ks or MEKKs) Raf to the membrane and its activation by phosphorylation. Activated Raf activates the MAPK kinases (MAP2Ks) MEK1/2, which in turn phosphorylate ERK1/2. In mouse embryos, the ERK pathway is detected in the limb bud mesenchyme and neural ectoderm.²³ The regenerative potential of MEK/ERK signaling has been found in several tissues, including the central nervous system, retina, and bone.²⁴⁻²⁶ MEK/ERK signaling is transiently activated during inner ear development, as evidenced by the di-phosphorylated forms of ERK1/2 (dp-ERK) that are observed in the otic placode and otocyst when robust proliferation takes place.^{23,27} In addition, the expression of negative modulators of ERK1/2, including *Dusp6* and *Dusp7*, is detected at embryonic day (E)13.5, the period of time during which essential cell differentiation takes place in the otic sensory epithelium.²⁸ The dynamic activity of MEK/ERK signaling at different stages during inner ear development indicates that MEK/ERK signaling may play an important role in cell fate determination of the cochlear sensory epithelium. However, the role of MEK/ERK signaling in HC fate determination and regeneration has remained unknown.

In this work, we selected 4 MEK/ERK signaling inhibitors and found that PD0325901 (PD03) promotes the direct transdifferentiation of SCs into HCs in the neonatal organ of Corti. Moreover, we confirmed that the regenerated HCs with a typical bundle structure and normal electrophysiological function originate from SCs. Using SC-derived cochlear organoids and RNA sequencing (RNA-seq) analysis, we demonstrated the underlying mechanism behind the HC regeneration induced by inhibition of MEK/ERK signaling and found that the Notch pathway serves as the main downstream target of MEK/ERK signaling to drive the transdifferentiation of SCs into HCs. Finally, we delivered PD03 into the inner ear through the round window membrane, and the inhibition of MEK/ERK signaling also induced mild HC regeneration in vivo.

Material and methods

Mouse model and genotyping

Rosa26^{CAG-loxP-stop-loxP-tdTomato} (tdTomato, Stock#007908),²⁹ Rosa-rtTA (rtTA, Stock#006965), Sox9CreER (Stock#035092), Lgr5EGFP-CreER (Lgr5CreER, Stock#008875),³⁰ and Atoh1CreER (Stock#007684) mice were purchased from the Jackson Laboratory. Tet-on-NICD (tetNICD) mice were provided by Dr. D. Melton (Harvard University, USA), and Atoh1-EGFP mice³¹ were from Jane Johnson (University of Texas Southwestern Medical Center, Dallas).

Transgenic mice were genotyped using genomic DNA extracted from tail tips using the Viagen DirectPCR DNA Extraction System (Viagen). The genotyping primers are listed in [Supplementary Table S1](#).

In vitro cochlear epithelia culture

Cochlear epithelia tissues were dissected from postnatal day (P)1 or P2 mice and attached to coverslips precoated with cell-Tek (Sigma, 1:1 diluted with phosphate-buffered saline [PBS]). The epithelia were then cultured in 100 μ L DMEM/F12 medium supplemented with N2 and B27 (media and

supplements were obtained from Invitrogen). After 4 hours of attachment, 0.5 mM neomycin (Sigma) was added for 6 hours to induce the HC damage model. The cochlear explants were treated with 1, 2, or 4 μ M PD03; 0.25, 0.5, or 1 μ M Selumetinib; 5, 10, or 20 μ M PD98059; or 5, 10, or 20 μ M U0126. The culture media were changed every day.

Magnetic cell sorting and culture of inner ear organoids

Cochlear epithelia were dissected from P0 to P1 Lgr5CreER mice in PBS (Hyclone). The isolated sensory epithelia were treated with 0.125% trypsin (Thermo Fisher Scientific) for 15 minutes at 37 °C, and the digestion was stopped with an equivalent volume of trypsin neutralizer solution (Thermo Fisher Scientific). The samples were triturated with a plastic pipette and then passed through a 40- μ m cell strainer (Corning) to obtain single cells. The cell solution was centrifuged at 800 $\times$ g for 10 minutes at 4 °C, and the cell pellet was resuspended in magnetic cell sorting (MACS) buffer containing 2 mM EDTA and 0.5% bovine serum albumin in PBS (all from Gibco/Invitrogen).

For MACS, cells were incubated with rabbit anti-Lgr5 antibody (1:100 diluted with PBS, Abcam, #ab75850) for 45 minutes at room temperature. After being washed with cold MACS buffer and centrifuged, the cell pellet was resuspended in MACS buffer and incubated for 15 minutes at 4 °C with anti-rabbit IgG microbeads (1:10 dilution, Miltenyi Biotec, #130048602). The Lgr5+ cells were sorted on a MACS magnetic column (Miltenyi Biotec) in a magnetic field according to the manufacturer's instructions.

The single-cell suspension was cultured in low-attachment 24-well plates (Corning). The culture medium was composed of DMEM/F12 Glutamax supplemented with penicillin/streptomycin, N2, B27, EGF (20 ng/mL), bFGF (10 ng/mL), and IGF (50 ng/mL), all from Thermo Fisher Scientific. For proliferation studies, organoids were treated with CHIR99021 (3 μ M, Sigma) with or without PD0325901 (0.25, 0.5, or 1 μ M, Sigma) for 8 days, with media changes every other day. For differentiation studies, organoids first underwent an 8-day treatment with CHIR99021 (3 μ M, Sigma) to establish proliferation, followed by a 15-day period with CHIR99021 and PD0325901 for differentiation.

Immunohistochemistry analysis

Cochlear explants were fixed with 4% paraformaldehyde (Sigma) for 20 minutes at room temperature in the dark, washed 3 times with 0.1 M PBS, and blocked with 1% PBST containing 10% goat serum for 2 hours at room temperature. Primary antibodies were then added overnight at 4 °C, and after washing in 0.1 M PBS 3 times the secondary antibodies were incubated overnight at 4 °C. Antibodies were all diluted with 1% PBST. The primary antibodies applied in this study were goat anti-SOX2 (1:500 dilution, #AF2018, R&D Systems), rabbit anti-MYO7A (1:800 dilution, #25-6790, Proteus BioSciences), and chicken anti-GFP (1:1000 dilution, #ab13970, Abcam). The appropriate Alexa Fluor-conjugated secondary antibodies were chosen. DAPI was used to label nuclei (1:1000 dilution, #D9542, Sigma), and phalloidin was used to label HC bundles (1:200 dilution, #A12380, Life Technologies).

Protein extraction and Western blot

Cultured cochlea or organoids were washed twice with PBS and lysed in RIPA Lysis Buffer presupplemented with PMSF

for 30 minutes. Lysed samples were then boiled at 95–100 °C for 10 minutes and electrophoresed in 10% bis-tris protein gels. After being transferred to PVDF membranes (GE Healthcare Life Sciences, #10600023), the blots were blocked with 5% nonfat milk (Cell Signaling Technology, #9999S) and treated with the following primary antibodies diluted with TBST at 4 °C for 12 hours: rabbit anti-ERK1/2 (1:1000 dilution, #9102, Cell Signaling Technology), rabbit anti-p-ERK1/2 (1:1000 dilution, #4370, Cell Signaling Technology), rabbit anti-MEK1/2 (1:1000 dilution, #ab178876, Abcam), rabbit anti-p-MEK1/2 (1:1000 dilution, #9154, Cell Signaling Technology), rabbit anti-NICD (also named Cleaved Notch1, 1:1000 dilution, #4147, Cell Signaling Technology), rabbit anti-JAG1 (1:1000 dilution, #2620, Cell Signaling Technology), rabbit anti-HEY2 (1:500 dilution, #10597-1-AP, Proteintech), rabbit anti-HES5 (1:1000 dilution, #ab194111, Abcam), and rabbit anti-GAPDH (1:500 dilution, #AF1186, Beyotime Biotechnology). After washing 3 times in TBST for 10 minutes each time, the membranes were incubated with HRP-conjugated secondary antibodies for 60 minutes at room temperature. Signals were detected and screened with super sensitive ECL luminescence reagent (Meilunbio, #MA0186).

Electrophysiology

To assess the functions of the newly regenerated HCs after PD03 treatment, we performed patch-clamp recordings. The extracellular solution contained 135 mM NaCl, 5.8 mM KCl, 1.3 mM CaCl₂, 0.9 mM MgCl₂, 0.7 mM NaH₂PO₄·H₂O, 10 mM HEPES, 5.6 mM D-glucose, and 2 mM Na-pyruvate (310 mOsm, pH was adjusted to 7.40 with NaOH). Atoh1-EGFP mice were used to search for new HCs. Random regenerated HCs in the pillar cell region of the apical turn, which have high expression of EGFP, were selected for recording.

An EPC10/2 amplifier (HEKA Electronics, Lambrecht Pfalz, Germany) with Patchmaster software (HEKA Electronics) was used for all measurements. Patch pipettes were 3–5 MΩ and were pulled from borosilicate glass capillaries (Sutter). The intracellular solution contained 110 mM K-methane sulfonate, 20 mM KCl, 10 mM HEPES, 2 mM ethylene glycol tetraacetic acid, 10 mM Na-phosphocreatine, 3 mM Mg-ATP, and 0.5 mM Na-GTP (285 mOsm, pH was adjusted to 7.40 with KOH). The specimens were observed on an upright microscope (Olympus) with Nomarski differential interference contrast optics (60× water-immersion objectives).

Cells were held at –80 mV for voltage clamp, and for total K⁺ current recording, we used voltage steps from –120 mV to +50 mV at 10 mV per step. For induced action potentials recording, we used current steps of 500 ms with an amplitude of 10–100 pA under the current-clamp mode. All experiments were carried out at room temperature, and all voltages were corrected for a measured liquid junction potential of 10 mV (K internal). Electrophysiology data were analyzed with GraphPad Prism6 and Igor software (Pro 6.22). For K⁺ current analysis, data were accepted if the series resistance (R_s) was no greater than 20 MΩ. The data are reported as means ± SEM. For K⁺ current analysis, we converted current into conductance and fitted the activation of IK with a Boltzmann equation: $G/G_{\max} = 1/[1 + \exp[(V_m - V_{1/2})/K]]$.

Microinjection of PD03 into the inner ear

We used Sox9CreER-tdTomato mice to trace the original SCs, and CreER was activated by injection of tamoxifen at

P0. P2 mice were placed on ice to anesthetize them with low-temperature exposure. We made a skin incision behind the right ear to expose the otic bulla and the stapedia artery. For the in vivo experiment, PD03 was injected into the scala media of the cochlea through the round window with a total volume of 1 μL per cochlea. Glass micropipettes (WPI, Sarasota, FL) held by a Nanoliter Microinjection System (WPI) were used to deliver PD03, and the speed of injection was controlled by a micromanipulator at 5 nL/s. After injection, the incision was closed. The P2 pups were placed on a 37 °C heating pad for recovery and returned to their mother after recovering. The cochleae were dissected and fixed for immunohistochemical staining 5 days after injection (P7) and on P30.

RNA sequencing and analyses

To investigate the impact of PD03 treatment on gene expression, we conducted 3 replicates of RNA-seq on organoids treated with PD03 or an equal volume of DMSO for 10 days during the differentiation period. RNA-Seq libraries of organoids were prepared with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing and the Illumina Stranded mRNA Prep kit (Ligation, Illumina). Libraries were size selected for cDNA target fragments of 300 bp on 2% Low Range Ultra Agarose followed by PCR amplification using Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantification using Qubit 4.0, the paired-end RNA-seq sequencing library was sequenced with a NovaSeq 6000 sequencer (2 × 150 bp read length). Fastq files were trimmed with Trimmomatic. Clean reads were mapped to the mouse reference genome (mm10) using HISAT2, followed by transcript assembly using HTSeq and differential gene expression analysis using DESeq2. Genes with adjusted *P*-values < .05 and fold change >1.5 were marked as significantly differentially expressed. This analysis used the software Goatools for GO enrichment analysis using Fisher's exact test. In order to control for the calculated false positive rate, 4 multiple-test methods (Bonferroni, Holm, Sidak, and false discovery rate) were used to correct the *P*-value. Generally, when the corrected *P*-value (*p*_{fdr}) is ≤.05, the GO function is considered to be significantly enriched. Protein-protein interaction network analysis of differentially expressed genes in the GO terms (including regulation of cell cycle, HC differentiation, etc.) was performed using STRING software.

Scanning electron microscopy

Cochlear explants were fixed in 2.5% glutaraldehyde at 4 °C for 6 h. Specimens were then rinsed in 0.1 M PB for 15 minutes 3 times and post-fixed in 1% osmium tetroxide for 2 hours at 4 °C. Samples were rinsed in 0.1 M PB for 15 minutes 3 times and dehydrated through a graded ethanol series (50%, 70%, 90%, and 100%) for 15 minutes at each step. Samples were then run through a graded mixture of ethanol and *N*-pentyl acetate (ethanol:*N*-pentyl acetate [2:1], ethanol:*N*-pentyl acetate [1:2], and *N*-pentyl acetate) for 15 minutes at each step. The specimens were critical point dried (HCP-2, Hitachi) and sputter coated with 10 nm of platinum (IB-3, EIKO). Samples were observed with a field emission scanning electron microscope (SU8010, Hitachi).

ABR test

The ABR tests were recorded using the BioSigRZ system (Tucker-Davis Technologies, Alachua, FL, USA). According to our previously published methods,^{32,33} briefly, mice were

anesthetized (100 mg/kg ketamine and 25 mg/kg xylazine sodium, i.p.) before recording and kept warm at 38 °C during the ABR recordings. Stimuli were generated by digital input/output cards (PXI-4461 National Instruments) in a PXI-1042Q chassis, amplified by a SA-1 speaker driver (Tucker-Davis Technologies, Inc.), and delivered to the studied ear by 2 electrostatic drivers (CUI Miniature Dynamics). The hearing threshold was assessed at 5 frequencies (4, 8, 16, 24, and 32 kHz) on a TDT System III apparatus (Tucker Davies Technologies, Gainesville, FL, USA).

Statistical analysis

For statistical comparisons, GraphPad Prism software (Vision 7.0) was used to conduct ANOVA and 2-tailed Student's *t* tests. Images were carefully selected to show the average effect for each experimental treatment. All descriptive statistics are presented as the mean $\pm$ SD. For statistical analysis, each cochlear explant or organoid was considered independent. For all statistical tests, *P*-values < .05 were considered statistically significant. All error bars show the SEM (the mean of the SD).

Results

Activation of MEK/ERK signaling in the cochlear SCs following HC loss

In neonatal mammals, SCs in the sensory epithelia have some proliferative capacity and have limited ability to regenerate HCs after HC damage.^{34,35} Thus, exploring the gene expression changes in an injury model is an ideal starting point for exploring regeneration. To explore the potential of MEK/ERK signaling in HC regeneration, we measured the profile of MEK/ERK in the HC damage model. We dissected the cochlear epithelia from P2 mice and adhered the epithelia to the dish for further culture (Figure 1A). To explore the HC damage-evoked changes of related gene expression in the cochlear epithelia, neomycin was used to ablate HCs in the cultured mammalian cochlear epithelium. After treatment with 1 μ M neomycin for 6 hours and recovery for 24 hours (Figure 1B), the number of HCs showed 13.1%, 43.2%, and 75.9% decreases in the apical, middle, and basal turns, respectively (Figure 1C, 1D; Supplementary Figure S1A, S1B).

We further measured the expression pattern of MEK/ERK signaling in the cochlear epithelium at different time points. Western blot assays indicated no substantial change in MEK/ERK activity in the vehicle-treated group during culture as indicated by phosphorylated MEK1/2 (p-MEK1/2) and phosphorylated ERK1/2 (p-ERK1/2; Figure 1E). Remarkably, significantly increased expression of p-MEK1/2 and p-ERK1/2 was observed after 6 hours recovery, and the activation of MEK/ERK signaling persisted for the following 18 hours. To further determine which cell type contributes to the activity change in MEK/ERK signaling induced by HC damage, we performed immunofluorescence to detect the cellular localization of p-ERK. There is no evidence of MYO7A/SOX2 double-positive cells, indicating an absence of newly regenerated cells in both the control and neomycin groups, and crucially, no p-ERK1/2 expression was detected in MYO7A-positive HCs, even after neomycin treatment (Figure 1F). Notably, p-ERK1/2 expression in SOX2-positive SCs was significantly altered, with HC loss prompting an elevated p-ERK response (Figure 1G, 1H). Consistent with the degree of HC damage, p-ERK1/2 showed a gradual increase from the

apical to the basal turn in the neomycin-treated group. These results suggested that MEK/ERK signaling was activated in SCs following HC damage.

Inhibition of MEK/ERK signaling by PD03 induces HC regeneration

Previous studies have shown that SCs have the capacity to dedifferentiate and transdifferentiate in response to different regulatory factors,³⁶ and HC loss is considered to be a factor that promotes endogenous SCs to reenter the cell cycle.³⁷⁻³⁹ Because elevated MEK/ERK activity was detected in SCs followed by HC loss, we hypothesized that inhibited MEK/ERK signaling contributes to the transdifferentiation potential of SCs (Figure 2A). We selected 4 MEK/ERK signaling inhibitors, including PD0325901, U0126, PD98059, and selumetinib, to evaluate the regenerative potential in cochlear epithelia (Figure 2B). After 6 hours of culture, the cochlear explants from P1 mice were treated with the 4 compounds at different concentrations. As indicated by MYO7A/SOX2 double-positive cells, we found that all of these MEK/ERK inhibitors triggered dose-dependent HC regeneration after 5 days incubation, mainly in the apical turn (Figure 2C, 2D). Strikingly, PD03 treatment induced the most significant effect on HC generation among these compounds, and 35.50 ± 2.630 new HCs per 100 μ m were found in the apical turn at the concentration of 1 μ M PD03. Interestingly, although PD98059 and selumetinib inhibit MEK1/2 phosphorylation through a similar mechanism of action as PD03, there were only 3.250 ± 0.4787 (PD98059) and 2.750 ± 0.2500 (selumetinib) regenerated HCs per 100 μ m in the apical turn at the optimal concentration. U0126 blocks the phosphorylation of ERK1/2 by MEK1/2 and induced only 3.000 ± 0.4082 regenerated HCs per 100 μ m in the apical turn at its optimal concentration (Figure 2D).

To further evaluate whether MEK/ERK inhibition can induce HC regeneration in older animals, cochlear explants from P1, P3, P5, and P7 mice were dissected and then cultured in the control medium with or without 1 μ M PD03. We used Atoh1-EGFP transgenic mice to observe HCs in real-time, and the number of Atoh1+ HCs in the PD03-treated group was significantly greater in each turn of the cochlea compared to controls at P1 after 5 days of treatment (Figure 2E, 2F; Supplementary Figure S1C). The number of extra HCs compared to controls gradually increased from the basal turn to the apical turn (base, PD03: 53.40 ± 0.894 vs control: 49.80 ± 1.483 ; middle, PD03: 59.00 ± 2.646 vs control: 51.60 ± 2.074 ; apex, PD03: 86.60 ± 3.647 vs control: 55.00 ± 5.244 ; HCs per 100 μ m), and the regenerated HCs were more prominent in the pillar cell region. As the age of the samples increased from P1 to P7, the number of regenerated HCs gradually decreased in each turn (Figure 2E-2I; Supplementary Figure S1C-S1F). At P7, the regenerated HCs could only be observed in the apex, with 58.00 ± 3.391 HCs after PD03 treatment and 49.20 ± 1.643 in the control group (Figure 2H, 2I; Supplementary Figure S1F). These results show that PD03, as a MEK/ERK signaling inhibitor, enables the regeneration of HCs and that the ability to promote regeneration can continue to about the middle of the neonatal period.

Regenerated functional HCs are derived from the transdifferentiation of SCs induced by PD03

To identify the origin of the regenerated HCs, we used 3 CreER lines (Atoh1CreER, Sox9CreER, and Lgr5CreER) that were

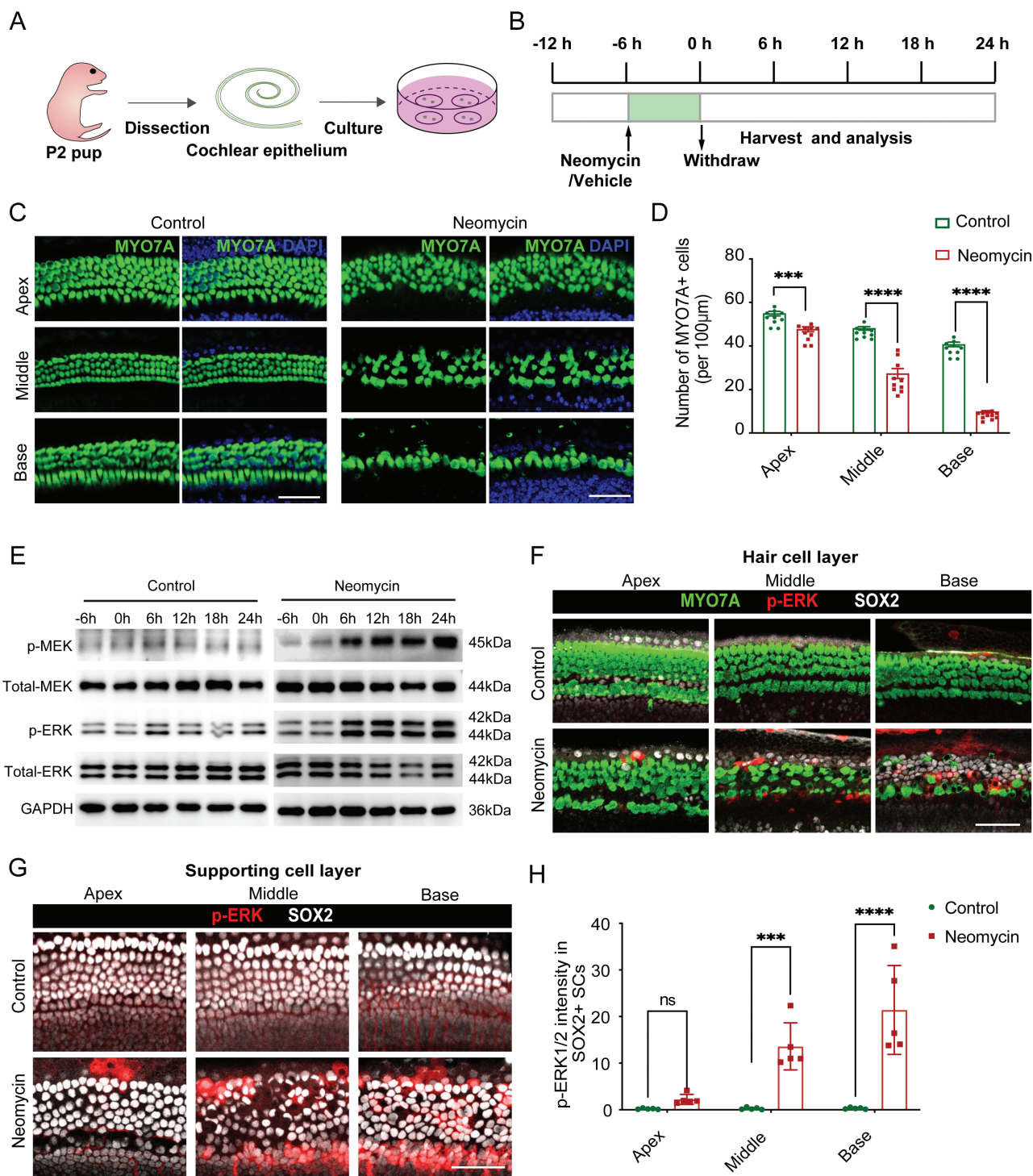


Figure 1. Neomycin treatment leads to the unique activation of the MEK/ERK signaling in SCs. (A) Schematic showing that cochlear epithelia from P1 mice were dissected and attached to coverslips for subsequent processing. (B) Schematic overview of the treatment of cochlear explants. (C) Immunofluorescence staining of MYO7A and DAPI in control and neomycin-treated cochlear explants. Scale bar = 50 μm; $n = 3$ explants for each group. (D) The number of MYO7A+ cells after neomycin-induced damage per 100 μm of the cochlea. Data are presented as means ± SEM ($n = 10$) *** $P < .001$, **** $P < .0001$ (2-way ANOVA followed by Tukey's multiple comparison test). (E) Western blot analysis is shown for the indicated proteins in cochlear explants at 0, 6, 12, 18, and 24 hours after neomycin treatment. GAPDH was used as the loading control. (F) Immunofluorescent staining of MYO7A and p-ERK in the HC layers in control and neomycin-treated cochlear explants. Scale bar = 50 μm; $n = 3$ explants for each group. (G) Immunofluorescent staining of MYO7A and p-ERK in SC layers in the control and neomycin-treated cochlear explants. Scale bar = 50 μm; $n = 3$ explants for each group. (H) p-ERK1/2 intensity in the apical, middle, and basal turns of the cochlear explants in the control group and the neomycin-treated group. The data are presented as the mean ± SEM ($n = 5$), *** $P < .001$, **** $P < .0001$.

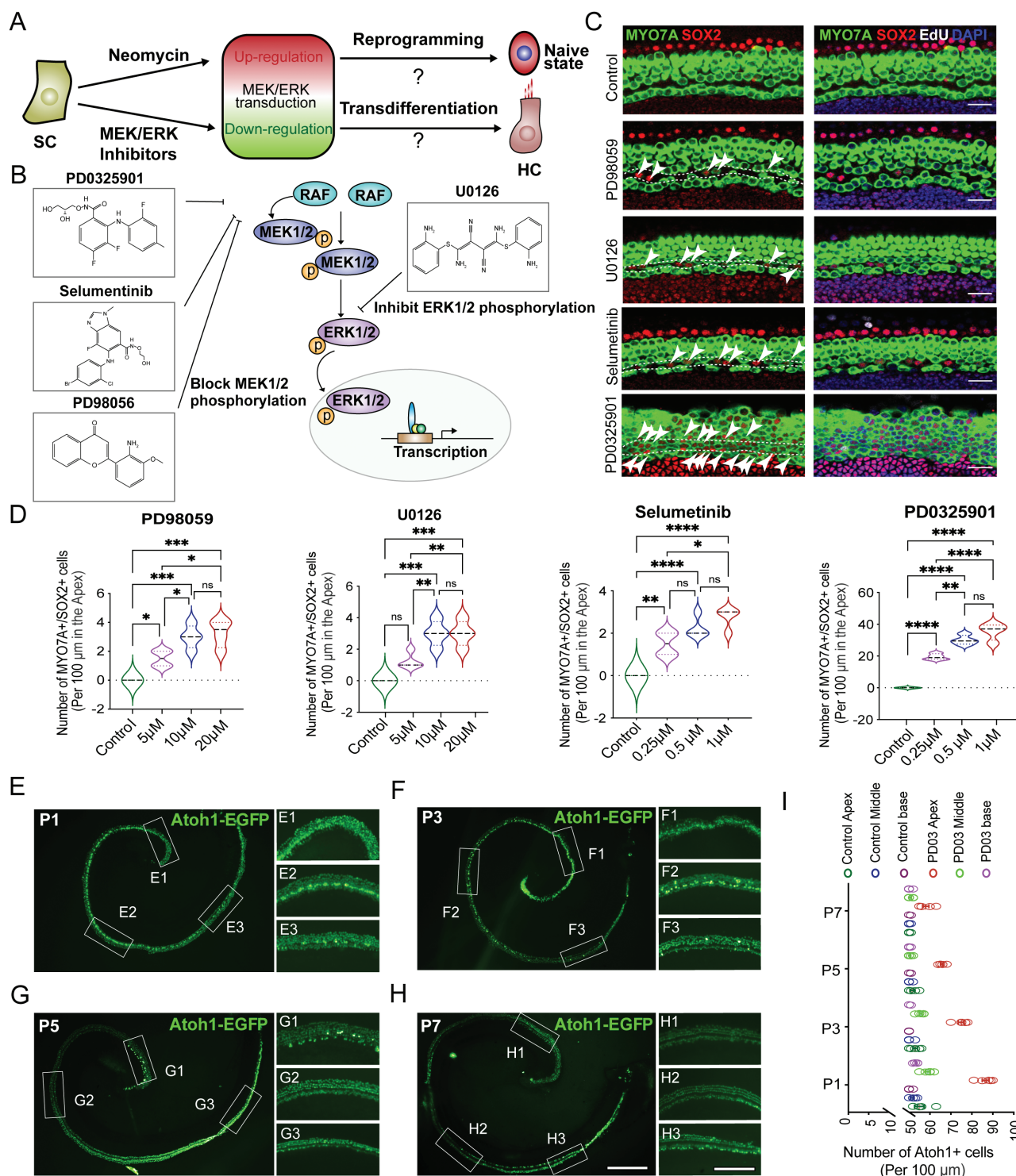


Figure 2. MEK/ERK inhibitors induced HC regeneration in an age-dependent manner. (A) Schematic showing the hypothesis that downregulation of MEK/ERK induces transdifferentiation of SCs. (B) Structure and action mechanism of the MEK/ERK inhibitors used in this study. (C) Immunofluorescence staining of MYO7A, SOX2, EdU, and DAPI in DMSO controls and MEK/ERK inhibitor (10 μ M PD98059, 10 μ M U0126, 0.5 μ M Selumetinib, and 0.5 μ M PD03)-treated cochlear explants. Scale bar = 50 μ m. (D) Number of MYO7A+/SOX2+ cells related to (C). The data are presented as means $\pm$ SEM ($n = 4$). * $P < .05$, *** $P < .001$, **** $P < .0001$ versus DMSO control on the same day (one-way ANOVA followed by Tukey's multiple comparison test). (E-G) The expression of Atoh1-EGFP in P1, P3, P5, and P7 mice after treatment with 1 mM PD03. Scale bar = 200 μ m in the low magnification and 100 μ m in the high magnification. (I) The number of Atoh1+ cells per 100 μ m in the control group and the PD03-treated group.

crossed with the tdTomato reporter strain for fate-mapping of HCs and SCs. Mice of these 3 lines (Atoh1CreER-tdTomato, Sox9CreER-tdTomato, and Lgr5CreER-tdTomato) were injected with tamoxifen (0.5 mg/g, subcutaneously) on P0,

thereby inducing tdTomato expression in the specific Cre+ cell subtypes. The cochleae were then dissected on P1 and cultured in a medium with 1 μ M PD03 or DMSO for 5 days (Figure 3A, 3C, 3E). We labeled all HCs with anti-MYO7A

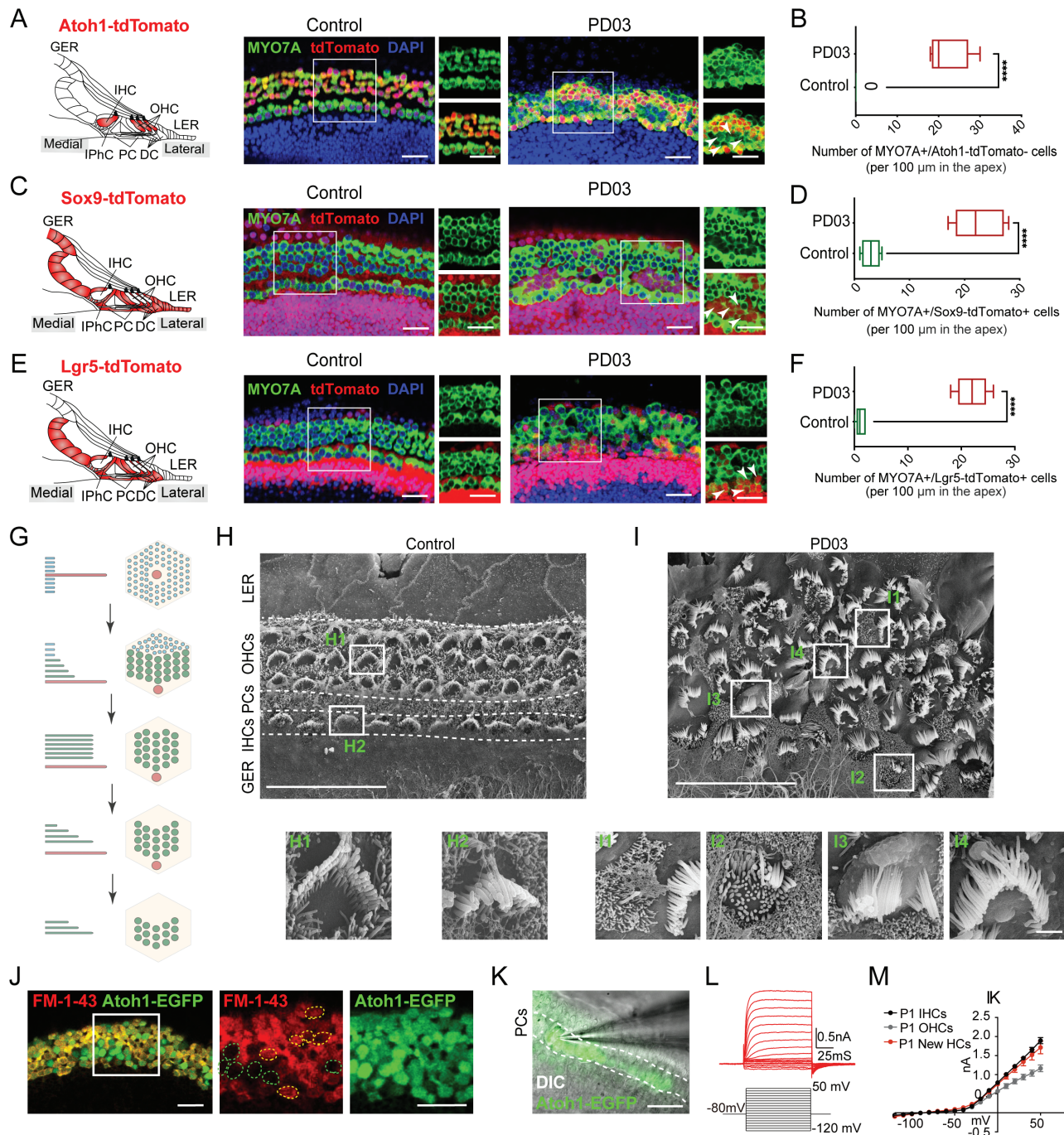


Figure 3. Regenerated HCs are functionally derived from the transdifferentiation of Lgr5+ SCs. (A) Schematic diagram of Atoh1 expression in the cochlea and immunofluorescent staining of MYO7A and DAPI in DMSO control and PD03-treated cochlear explants. Scale bar = 50 μm. (B) Number of MYO7A+/Atoh1-tdTomato- cells related to (A). The data are presented as means ± SEM ($n = 4$), **** $P < .0001$ versus DMSO control on the same day. (C) Schematic diagram of Sox9 expression in the cochlea and immunofluorescent staining of MYO7A and DAPI in DMSO control and PD03-treated cochlear explants. Scale bar = 50 μm. (D) Number of MYO7A+/Sox9-tdTomato+ cells related to (C). (E) Schematic diagram of Lgr5 expression in the cochlea and immunofluorescent staining of MYO7A and DAPI in DMSO control and PD03-treated cochlear explants. Scale bar = 50 μm. (F) Number of MYO7A+/Atoh1-tdTomato- cells related to (E). (G) Schematic diagram of cochlear HC cilia development. (H, I) The representative view of ciliary structures in the organ of Corti in DMSO controls and the PD03-treated group under a scanning electron microscope. The enlarged views of inner HC (IHC) and outer HC (OHC) bundles in the control group (H1, H2) and 4 types of immature HC bundles in the PD03-treated group (I1-I4). Scale bars = 20 μm in the low magnification images (H, I) and 1 μm in the high magnification images (H1-H2, I1-I4). (J) The fluorescence image of FM1-43 and EGFP is shown along the HC plane in the PD03-treated group. Scale bar = 50 μm. (K) Overlay of DIC and EGFP fluorescence images focused at the HC level in the patch-clamp assay. Scale bar = 50 μm. (L) Families of outward K^+ currents of the regenerated HCs, evoked by the voltage protocol are shown above. (M) Mean ± SEM peak current-voltage relations for IHCs ($n = 9$), OHCs ($n = 9$), and the regenerated HCs ($n = 5$).

antibodies and analyzed the specimens by confocal microscopy. In the *Atoh1CreER-tdTomato* line, we observed 22.20 ± 2.200 MYO7A+/tdTomato- cells per 100 μm in the apical turn after PD03 treatment, while no MYO7A+/tdTomato- cells were found after DMSO treatment (Figure 3B). The additional MYO7A+/Atoh1-tdTomato- cells suggested that the newly regenerated HCs triggered by PD03 might be derived from other cells, not native HCs. In the *Sox9CreER-tdTomato* line, we observed 22.60 ± 1.99 MYO7A+/tdTomato+ cells per 100 μm in the apical turn after PD03 treatment, while only 3.000 ± 0.7071 cells were seen after DMSO treatment (Figure 3D). In the *Lgr5CreER-tdTomato* line, we observed 22.00 ± 1.304 MYO7A+/tdTomato+ cells per 100 μm in the apical turn after PD03 treatment, while only 1.200 ± 0.3742 cells were seen after DMSO treatment (Figure 3F). These results indicated that the regenerated HCs transdifferentiated from SCs, particularly the *Lgr5* + SC subtypes, are predominantly localized in the SCs adjacent to the HC region (Supplementary Figure S1G) and not from *Atoh1* + HCs.

To further explore the maturity and functionality of the regenerated HCs, we used a scanning electron microscope to observe the cilia of the regenerated HCs. During inner ear development, the kinocilium and cilium appear in the center of the cuticular plate, followed by the growth of stereocilia. The kinocilium guides the arrangement of stereocilia to form 3 rows of V-shaped cilia and then degenerates after maturation (Figure 3G). In the controls, we observed the normal structure of cilia, namely 3 rows of V-shaped stereocilia with an un-degenerated kinocilium (Figure 3H). After treatment with PD03, regenerated HCs appeared in the original SC regions and their cilia showed 4 different morphologies, namely a kinocilium in the center of the cuticular plate, a kinocilium with growing stereocilia, a kinocilium with rows of stereocilia, and a kinocilium with 3 rows of V-shaped stereocilia (Figure 3I). FM1-43 dye, which enters cells through open transduction channels, was used to determine if the regenerated HCs had functional mechano-transduction activity (Figure 3J). FM1-43 fluorescence was evident in a portion of the *Atoh1-EGFP*+ cells in the pillar cell region, suggesting the presence of functional mechano-transduction channels. All *Atoh1-EGFP*+ cells in the pillar cell region had an outward potassium current like native HCs (Figure 3L, 3M; Supplementary Figure S2A, S2B; IK at 0 mV, IHCs: IK = 0.791 ± 0.016 , $n = 9$, OHCs: IK = 0.569 ± 0.052 , $n = 9$, regenerated HCs: K = 0.751 ± 0.046 , $n = 5$). Then, we converted K current into conductance and fitted the activation of IK with Boltzmann sigmoidal regression and found the characteristics of potassium channels in regenerated HCs are closer to native IHCs (Supplementary Figure S2C, IHCs: $V_{1/2} = -10.20$ mV, $k = 14.86$, OHCs: $V_{1/2} = -15.48$ mV, $k = 12.45$, regenerated HCs: $V_{1/2} = -9.05$ mV, $k = 15.94$). Next, we examined the induced action potentials of regenerated HCs and native HCs (Supplementary Figure S2C). Interestingly, we observed some new HCs have induced action potentials (3 of 6 cells) with a stimulation of 0-100 pA, 10pA a step. However, the morphology of its action potential is not uniform, more similar to OHCs (Supplementary Figure S2D). Thus, the regenerated HCs after PD03 treatment closely resembled the developmental pattern characteristic of original HCs, and the regenerated HCs possessed the electrophysiological characteristics of functional HCs.

PD03 facilitates HC generation in the inner ear organoid system

Due to the complexity of cell types and influencing factors in cochlear epithelia, we used the *Lgr5*+ SC-derived inner ear organoid system to further dissect the potential capacity of PD03 to promote HC formation.⁴⁰ We dissected and digested the epithelia from P0 to P1 *Lgr5-EGFP* reporter mice and used MACS to separate *EGFP*+ cells, and these were then cultured in a Matrigel-encapsulated 3D culture system with a medium containing EGF, FGF, IGF, and CHIR99021 (EFIC medium). After being cultured in EFIC medium or EFIC medium containing PD03 for 8 days, organoids were formed and observed under a confocal fluorescence microscope (Figure 4A). Compared to the organoid cultured in EFIC medium, PD03 treatment led to a decrease in colony-forming efficiency in a dose-dependent manner (Figure 4B). The colony-forming efficiency was 75.33 ± 5.897 , 45.17 ± 1.167 , 29 ± 1.653 , and 18.33 ± 1.909 colonies per 1000 cells in EFIC medium and EFIC medium containing 0.25 μM PD03, 0.5 μM PD03, and 1 μM PD03, respectively (Figure 4C). The diameters of the organoids also decreased with the increasing PD03 concentration (Figure 4C, EFIC medium, 121.1 ± 5.889 ; 0.25 μM PD03, 93.76 ± 4.437 ; 0.5 μM PD03, 62.00 ± 4.271 ; and 1 μM PD03, 36.66 ± 2.235). We further stained the organoids with the proliferation-related markers Ki67 and EdU and found that the cell numbers per organoid, the percentage of Ki67+ cells per organoid, and the percentage of EdU+ cells per organoid all decreased with PD03 treatment (Figure 4D, 4E, Cell numbers per organoid: EFIC medium, 237.5 ± 28.03 ; 0.25 μM PD03, 153.7 ± 20.57 ; 0.5 μM PD03, 113.4 ± 17.22 , and 1 μM PD03, 80.52 ± 8.095 ; percentage of Ki67+ cells per organoid: EFIC medium, 44.15 ± 1.615 ; 0.25 μM PD03, 33.43 ± 2.021 ; 0.5 μM PD03, 23.01 ± 1.833 ; and 1 μM PD03, 18.12 ± 1.126 ; percentage of EdU+ cells per organoid: EFIC medium, 48.74 ± 2.315 ; 0.25 μM PD03, 35.15 ± 2.622 ; 0.5 μM PD03, 20.45 ± 2.006 ; and 1 μM PD03, 12.98 ± 1.279). These results indicated that PD03 inhibits cell proliferation and thus inhibits the expansion of inner ear organoids.

We further explored the role of PD03 in HC formation, and we first cultured the organoids in EFIC medium on days 0-8 followed by a pulse treatment with different concentrations of PD03 on days 8-23 (Figure 4F). On day 23, the organoids were stained for HC markers, and we found that MYO7A+ cells and F-actin+ cells increased with increasing PD03 concentrations (Figure 4G). We also used *Atoh1-EGFP* to identify HCs, and the increased number of *EGFP*+ cells in the organoids confirmed the promotion of HC generation by PD03. The percentage of organoids with MYO7A+ cells and the percentage of MYO7A+ cells per organoid all increased in a dose-dependent manner (Figure 4H, percentage of organoids with MYO7A+ cells: EFIC medium, 10.67 ± 1.856 ; 0.25 μM PD03, 18 ± 1.528 ; 0.5 μM PD03, 23.33 ± 1.764 ; and 1 μM PD03, 30.33 ± 2.728 ; percentage of MYO7A+ cells per organoid: EFIC medium, 3.567 ± 1.468 ; 0.25 μM PD03, 12.17 ± 1.493 ; 0.5 μM PD03, 22.5 ± 1.839 ; and 1 μM PD03, 28.67 ± 3.007). Taken together, these results indicated that PD03 inhibited cell proliferation and triggered HC generation in the inner ear organoid system.

Transcriptional profile of PD03-treated organoids

To determine the mechanism through which PD03 promotes HC generation, we cultured the organoids in 1 μM PD03 or the

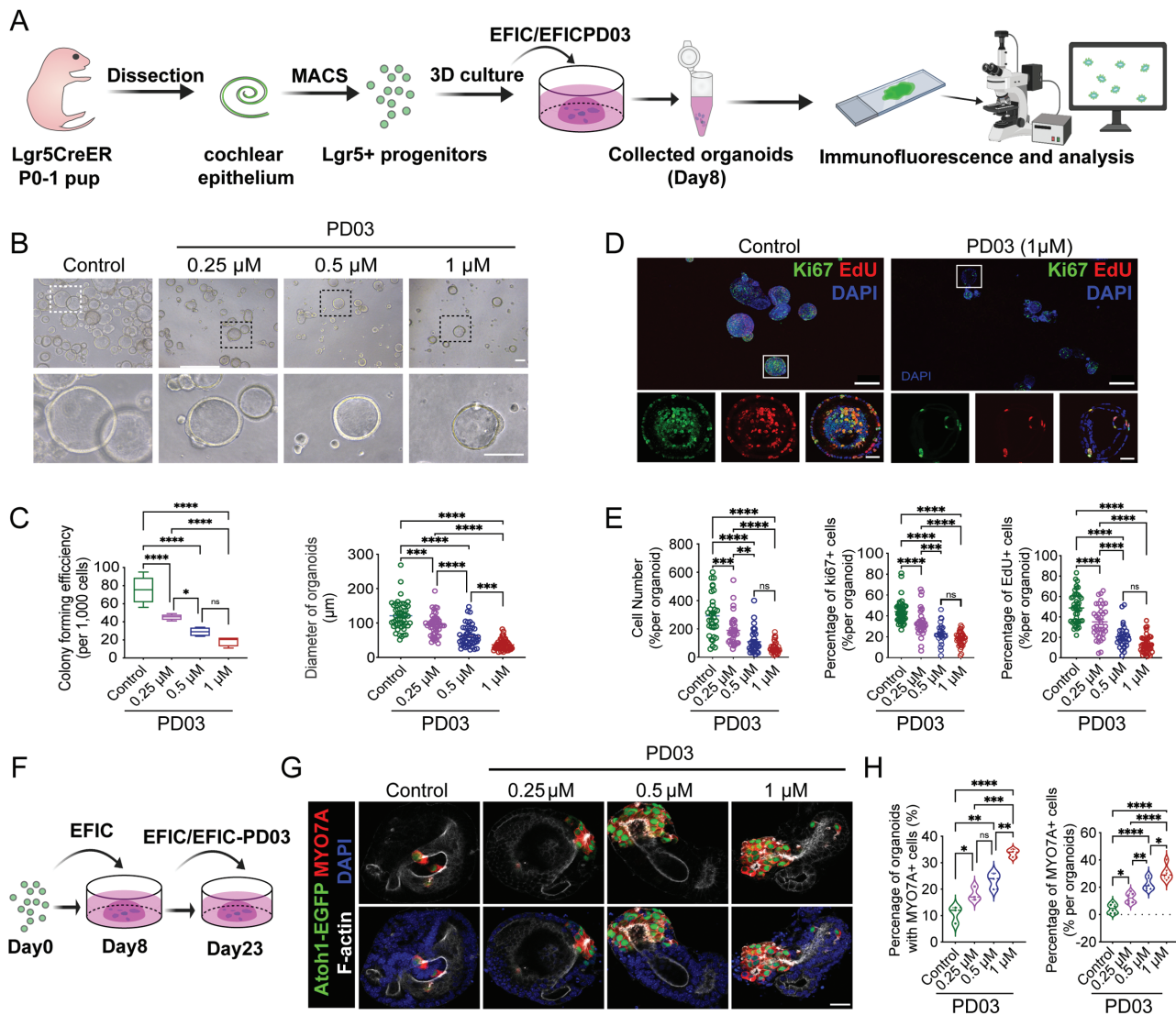


Figure 4. PD03 facilitated HC generation in inner ear organoids. (A) Schematic overview of inner ear organoid culture. (B) At the stage of organoid proliferation, the colony-forming efficiency and the size of the organoid decreased with the increase in PD03 concentration. Scale bar = 50 μ m. (C) Quantification of the colony-forming efficiency and the diameter of organoids from (B). The data are presented as means $\pm$ SEM ($n = 6$ in the colony-forming efficiency figure and $n = 49$ organoids in the organoid diameter figure), $*P < .05$, $***P < .001$, $****P < .0001$ versus DMSO controls (one-way ANOVA followed by Tukey's multiple comparison test). (D) Immunofluorescence staining of Ki67, EdU, and DAPI in the control group and 1 μ M PD03-treated group. Scale bar = 100 μ m in the low magnification and 25 μ m in the high magnification. (E) Quantification of cell number and percentage of Ki67+ cells and EdU+ cells per organoid from (D). The data are presented as means $\pm$ SEM ($n = 30, 27$, and 31 from left to right), $**P < .01$, $***P < .001$, $****P < .0001$ versus DMSO controls (one-way ANOVA followed by Tukey's multiple comparison test). (F) Schematic overview of inner ear organoid differentiation. (G) Fluorescence image of EGFP, MYO7A, F-actin, and DAPI in the control group and PD03-treated group. Scale bar = 25 μ m. (H) Percentage of organoids with MYO7A+ cells and MYO7A+ cells per organoid from (G). The data are presented as means $\pm$ SEM ($n = 3$ and 6 from left to right), $**P < .01$, $***P < .001$, $****P < .0001$ versus DMSO controls (one-way ANOVA followed by Tukey's multiple comparison test).

same volume of DMSO for 10 days, after which we used RNA-seq to establish a database to analyze differentially expressed genes (Figure 5A). PCA analysis showed that the variation between control and PD03 treatment explained 58.58% of the variance, which was revealed by PC1, while the within-group variance only accounted for 11.81%, which was revealed by PC2 (Figure 5B). From the volcano plot analysis, we found that 2339 genes were upregulated and 2654 genes were downregulated after PD03 treatment (P -adjust $< .05$, fold change > 1.5 ; Figure 5C). A heatmap further showed the differentially expressed genes, and we noted that 14 typical HC-related genes were significantly upregulated in the PD03 group, including *Pou4f3*, *Otof*, *Myo15*, *Myo3b*, and *Ptprq* (Figure 5D, 5E).

GO term analysis showed that PD03 treatment affected many important cellular physiological activities and important signal pathways, including epithelial cell differentiation, mitotic cell cycle, inner ear morphogenesis, Notch pathways, and regulation of the ERK1 and ERK2 cascade (Figure 5F). By analyzing the transcripts of organoids after PD03 treatment, protein interaction network analysis indicated that the expression of Notch pathway and Wnt pathway-related genes and ERK1 and ERK2 cascade-related genes varied significantly in the HC differentiation process and engaged in complex interrelationships (Figure 5G). These results indicated that PD03 treatment significantly changes gene expression leading to organoid differentiation into HCs, and

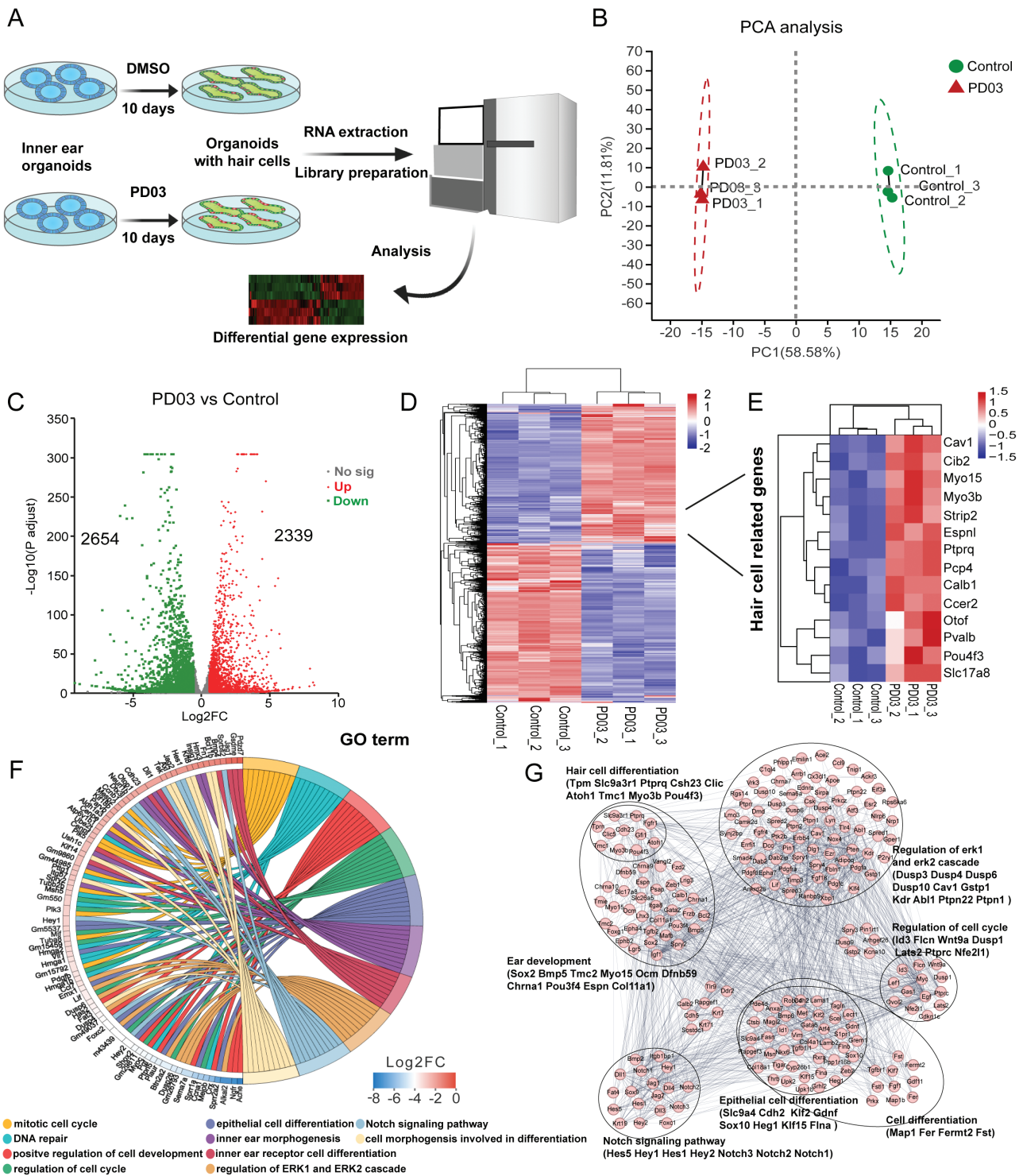


Figure 5. Transcriptomic characteristics of inner ear organoids treated with PD03. (A) Schematic overview of organoid culture and RNA-Seq analysis. (B) Principal component analysis (PCA) from control and PD03-treated organoids demonstrated that the 3 replicates clustered closely with each other (PC1 = 58.58%, PC2 = 11.81%). (C) Volcano plot depicting 4993 significant differentially expressed genes between the control and PD03-treated group, including 2339 upregulated genes and 2654 downregulated genes (P -adjust < .05, fold change > 1.5, n = 3 for each group). (D) Heatmap showing the relative expression levels of the differentially expressed genes after PD03 treatment compared to the control group. (E) Heatmap showing the relative expression levels of HC-related genes among the differentially expressed genes associated with HCs after PD03 treatment. (F) Ten representative GO enrichment terms and their corresponding shared genes. Genes are linked to a certain GO term by the colored bands. (G) Interaction network analysis of representative genes of GO terms in (F) using STRING.

this process was shown to be related to classical signal pathways.

Inhibition of MEK/ERK signaling by regulating the Notch pathway

Because Notch has been shown to promote the differentiation of SCs into HCs, we inferred that the Notch pathway serves as the downstream target of MEK/ERK signaling.^{12,41} Inspired by the RNA-seq data, we further used Western blot to deduce the relationship between the 2 pathways. Western blot showed that PD03 led to the inhibition of the ERK1 and ERK2 cascade along with decreased expression of Notch downstream proteins, including NICD, HES5, JAG1, and HEY2, which indicated that PD03 has a negative regulatory effect on the Notch pathway (Figure 6A).

We further tested whether activated Notch could block the HC differentiation effect of MEK/ERK, and we added JAG1 protein along with PD03 treatment, and this significantly

reduced the effect of PD03 on promoting regeneration (Figure 6B). The number of newly regenerated HCs indicated by MYO7A+/SOX2+ was decreased from 16.17 ± 1.327 to 3.667 ± 0.4944 HCs per 100 μm in the apical turn after JAG1 was added (Figure 6C). In addition, the NICD mouse line was used to activate Notch in a genetic intervention model. We crossed the rtTA mouse line with tetNICD mice, and the cochleae of the offspring were dissected on P2. The cochleae were induced with the tetracycline derivative doxycycline (Dox) to increase NICD activity, and this was followed by culturing in DMSO or PD03 for 5 days (Figure 6D). When the activity of NICD increased, the effect of PD03 on HC regeneration was significantly reduced (Figure 6E). The number of MYO7A+/SOX2+ HCs decreased from 17.17 ± 1.195 to 3.333 ± 0.4944 HCs per 100 μm in the apical turn in response to increased expression of NICD (Figure 6F). These results indicated that the transdifferentiative effect from inhibiting the MEK/ERK pathway was partially through inhibition of the Notch pathway.

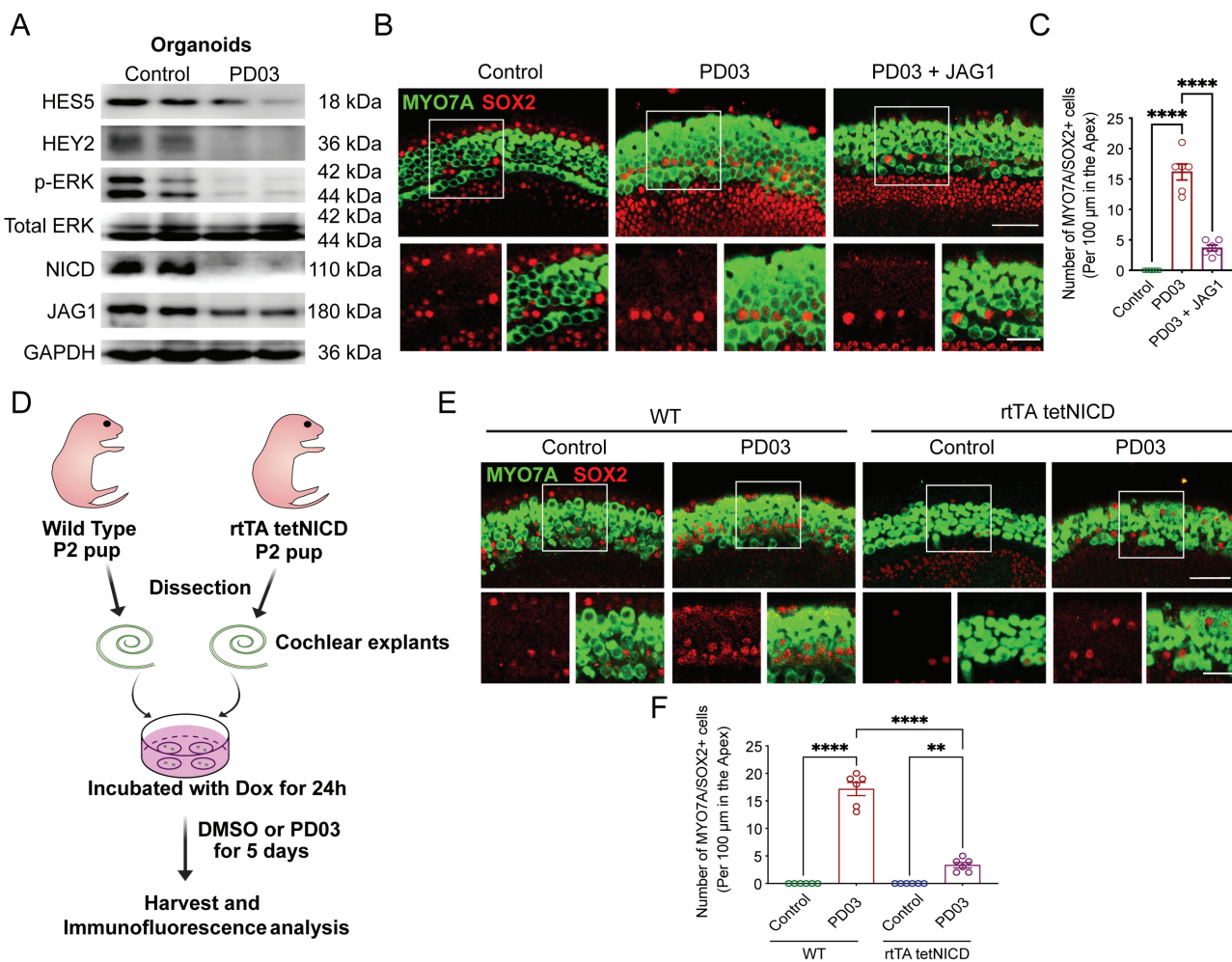


Figure 6. HC regeneration promoted by MEK/ERK inhibition by repressing the Notch pathway. (A) Western blot analysis showing the indicated proteins in PD03-treated organoids compared with the control group. GAPDH was used as the loading control. (B) Immunofluorescent staining of MYO7A and SOX2 in cochlear explants of the control group, PD03-treated group, and PD03 + JAG1-treated group. Scale bar = 50 μm in the low magnification and 25 μm in the high magnification. (C) Quantification of MYO7A+/SOX2+ cells from (B). The data are presented as means $\pm$ SEM ($n = 6$), **** $P < .0001$ versus DMSO controls (one-way ANOVA followed by Tukey's multiple comparison test). (D) Schematic showing PD03 treatment in Dox-treated epithelia from rtTA-tetNICD mice. (E) Immunofluorescent staining of MYO7A and SOX2 in cultured epithelia of the control group, PD03-treated group from WT mice, and rtTA-tetNICD mice. Scale bar = 50 μm in the low magnification and 25 μm in the high magnification. (F) Quantification of MYO7A+/SOX2+ cells from (E). The data are presented as means $\pm$ SEM ($n = 6$), *** $P < .001$, **** $P < .0001$ versus DMSO controls (one-way ANOVA followed by Tukey's multiple comparison test).

PD03 delivery into the inner ear promotes HC generation in vivo

Only a very few studies have observed mammalian cochlear HC regeneration in vivo using pharmaceutical approaches, so we evaluated the effect of PD03 on HC regeneration by inner ear delivery. To trace the original SCs, we injected Sox9CreER-tdTomato mice with tamoxifen (0.5 mg/g, subcutaneously) on P0 and injected 1 μ L PD03 (1 μ M/L) into the scala media of the inner ear through the round window membrane on P2. On P7, we fixed the cochleae and stained them with the HC marker MYO7A (Supplementary Figure S3A). Compared with the DMSO vehicle group, no obvious damage or loss of HCs was observed in the PD03-injected group. Surprisingly, we observed some MYO7A+/tdTomato+ cells in the apical turn after PD03 injection (Supplementary Figure S3B). No MYO7A+/tdTomato+ cells were seen in the control group, while 2.4 ± 0.5099 MYO7A+/tdTomato+ cells were seen per 100 μ m in the apical turn in the PD03-injected group (Supplementary Figure S3C). However, no regenerated HCs were found in the middle and basal turns, and the 2 turns retained the standard structure of 3 rows of outer HCs and one row of inner HCs in the absence of MYO7A+/tdTomato+ cells. Furthermore, in order to explore the role of PD03 after HC injury, we induced neomycin-induced HC injury in mice at P1 and injected 1 μ L PD03 (1 μ M/L) into the scala media of the inner ear through the round window membrane on P2 to observe the effects of PD03 after injuring (Figure 7A). On P7, we fixed the cochleae and stained them with the HC marker MYO7A (Figure 7B). A certain quality of MYO7A+/tdTomato+ cells was observed in the apical to middle turn after neomycin and PD03 treatment (1.833 ± 0.307 cells per 100 μ m), while no MYO7A+/tdTomato+ cells were seen in the neomycin group. Electrophysiological experiments have shown that these regenerated HCs have potassium currents like native HCs, the size of which is between IHCs and OHCs (Figure 7D, 7E). Then, we converted K current into conductance and fitted the activation of IK with Boltzmann sigmoidal regression and found the characteristics of potassium channels in regenerated HCs are closer to native HCs (Figure 7F, IHCs: $V_{1/2} = -7.158$ mV, $k = 17.21$, OHCs: $V_{1/2} = -4.060$ mV, $k = 18.25$, regenerated HCs: $V_{1/2} = -5.562$ mV, $k = 16.69$). Unfortunately, extending the observation time until P30, we did not observe the survival of MYO7A+/tdTomato+ cells or the recovery of hearing after PD03 treatment in adulthood (Figure 7G–7I, MYO7A+ cells per 100 μ m, control: 48 ± 1.065 , $n = 6$, neomycin: 17 ± 1.291 , $n = 6$, neomycin + PD03: 17.5 ± 1.432 , $n = 6$). Therefore, a single dose of PD03 achieved functional regeneration of cochlear HCs in the postnatal mice, but further research and combination of multiple drugs may be necessary for HC regeneration and auditory recovery in adult stages.

Discussion

Using cochlear explants, inner ear organoids, and in vivo delivery, we showed that PD03, a MEK/ERK inhibitor, targets the MEK/ERK pathway to induce SC transdifferentiation into HCs. As far as we know, PD03 is the only other compound in addition to LY411575 that can regenerate HCs in vivo, and we further dissected the transdifferentiative effect of inhibiting the MEK/ERK pathway by modulating the Notch pathway (Figure 7J).

Our results suggest a crucial role for the MEK/ERK pathway in cell fate transition because inhibition of the MEK/ERK pathway reduced cell proliferation and induced the transdifferentiation of SCs into HCs. Cochlear SCs have the potential to be reprogrammed to reenter the cell cycle and convert into HCs, especially after injury.⁴² The MEK/ERK pathway was uniquely activated in the SCs and not in HCs after neomycin treatment, which suggested that activation of the MEK/ERK pathway might participate in the fate determination of cochlear cells. Our results are consistent with the observation that upregulation of MEK/ERK reprograms SCs into an activated state because the pro-proliferative factor IGF induced upregulated p-ERK expression in Hensen's cells and Claudius cells.⁴³ The differential susceptibility of HCs to neomycin damage across the cochlear turns can be linked to the distinct biological characteristics of HCs in these regions.^{2,3} The enhanced regenerative response observed in the apical turn following PD03 treatment may be attributed to the retained stemness of the SCs in this region.⁸ The underlying mechanisms governing the varied activity of the MEK/ERK pathway upon damage, and the differential impact of modulating this pathway in distinct cochlear areas, warrant further investigation. PD03 induced cell-cycle arrest in organoids, which implied that MEK/ERK sustains cell division. However, we have not tested the effect of activation of MEK/ERK by selected activators or by genetic intervention on cell behavior, and further studies should test whether activation of MEK/ERK contributes to mitotic HC regeneration. Interestingly, PD03 induced neonatal SCs to transdifferentiate into HCs in the absence of damage, and such powerful reprogramming capacity can be attributed to the genetic recombination resulting from interventions targeting MEK/ERK signaling. We used 2 types of SC lineage-tracing mice (Lgr5CreER-tdTomato and Sox9CreER-tdTomato), and the findings indicate that PD03-induced HC regeneration predominantly takes place in pillar cells and Deiters' cells. Both mouse models provided comparable results for lineage tracing. Only a few compounds have been found that act on specific targets to regenerate HCs, including LY411575 (a γ -secretase inhibitor),¹⁸ regorafenib (an EGFR inhibitor),¹⁷ Follistatin (an antagonist of activin-type ligands),⁴⁴ and TRULI (an ATP-competitive inhibitor of Lats kinases).⁴⁵ Similar to LY411575, delivering PD03 into the inner ear induced HC regeneration in vivo, which suggests that PD03 has excellent prospects for application in regenerative medicine.

Inhibition of MEK/ERK alone induced SC transdifferentiation into HCs only in postnatal mice, and the reprogramming effects gradually decreased with the time after birth. However, new HCs could be generated after treatment with LY411575 in the adult cochlea, which resulted in some hearing recovery after noise-induced hearing loss.¹⁸ PD03 has been reported to induce cellular regeneration and differentiation in several systems, including the heart (where it induces vascular smooth muscle cell regeneration from pericytes after myocardial infarction),⁴⁶ the central nervous system (where it induces oligodendrocyte precursor cells to differentiate into oligodendrocytes),²⁴ and multiclass cancers (where it induces stem-like cancer cells to differentiate).^{47,48} The limited HC regeneration induced by PD03 in P7 cochlear epithelium dampened our further attempts at regeneration in the adult cochlea, and the optimal strategy seems to be to formulate a cocktail of compounds to activate SCs to reenter the cell cycle and then promote their transformation into HCs. Recent studies on compound cocktail systems have successfully

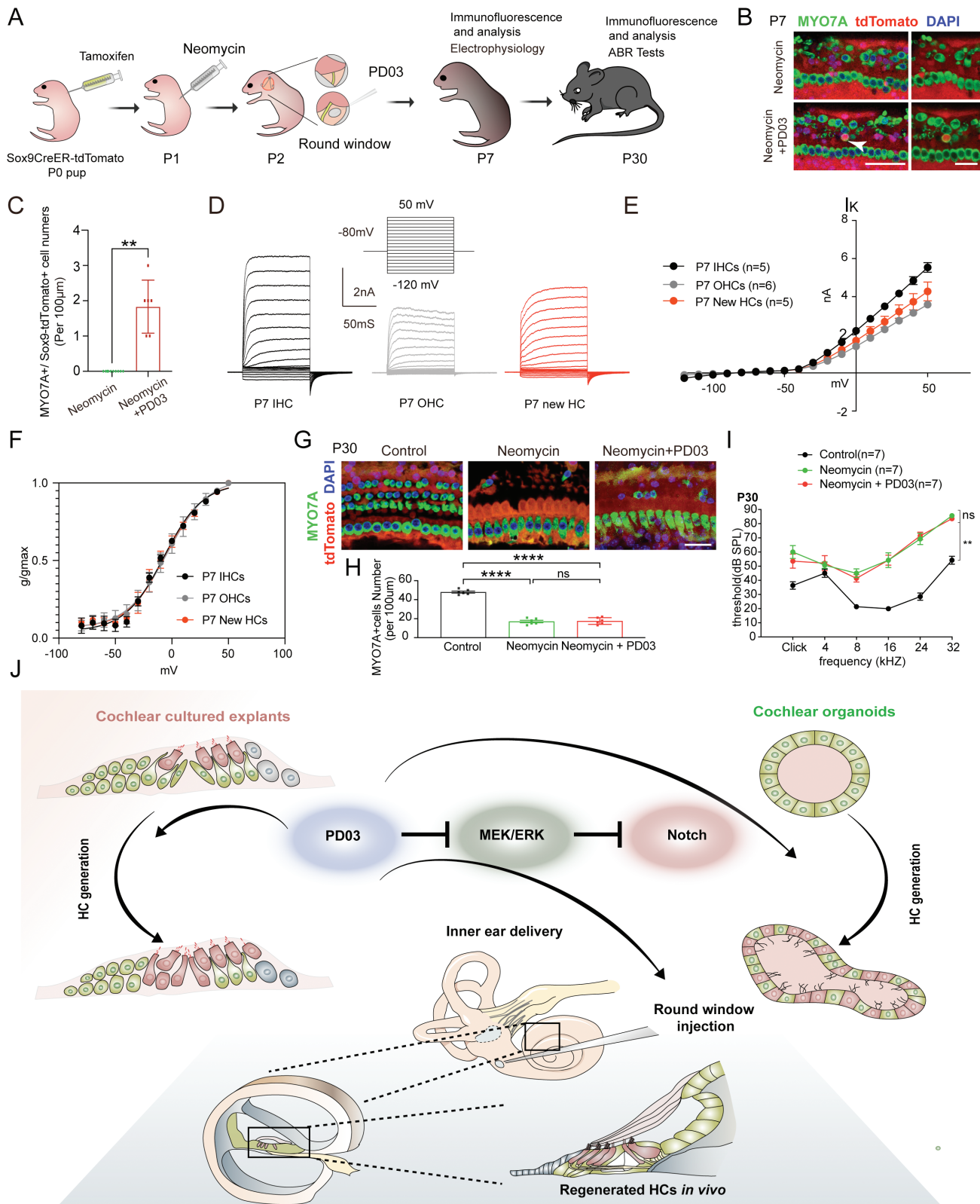


Figure 7. PD03-induced HC regeneration in vivo through round window delivery. (A) Schematic showing that tdTomato tracing was activated in Sox9CreER-tdTomato mice followed by neomycin treatment and PD03 delivery. (B) Immunofluorescence staining of MYO7A and SOX9 in P7 cochlear epithelia of the neomycin-treated and neomycin + PD03-treated groups from apical to middle turn. Scale bar = 50 μ m in the low magnification and 25 μ m in the high magnification. (C) Quantification of MYO7A+/SOX9+ cells from (B). The data are presented as means $\pm$ SEM ($n=6$), ** P < .01. (D) Families of outward K^+ currents of P7 IHCs, P7 OHCs, and the regenerated HCs, evoked by the voltage protocol are shown above. (E) Mean $\pm$ SEM peak current-voltage relations for IHCs ($n=5$), OHCs ($n=6$), and the regenerated HCs ($n=5$). (F) Normalized GV relations for outward potassium currents recorded from IHCs ($n=5$), OHCs ($n=6$), and regenerated HCs ($n=5$). Error bars show SEM, and lines show single Boltzmann distribution fits. (G) Immunofluorescence staining of MYO7A and SOX9 in P30 cochlear epithelia of control, the neomycin-treated and neomycin + PD03-treated groups from midturn. Scale bar = 50 μ m. (H) Quantification of MYO7A+/SOX9+ cells from (G). The data are presented as means $\pm$ SEM ($n=6$), **** P < .0001. (I) ABR thresholds for control, neomycin-treated, and neomycin + PD03-treated groups on P30. (J) Summary of the role and mechanism of PD03 in promoting HC regeneration.

achieved in situ regeneration after injury in heart and muscle tissue in vivo,^{49,50} which suggests the great prospects of cocktails in regeneration research. We anticipate that PD03 will be an integral component of such cocktails for the efficient induction of HC regeneration in adult mammals.

One exciting finding in our work was that PD03 alone promoted HC generation in inner ear organoids. Most previous studies have focused on the role of PD03 in downregulating MEK/ERK in inhibiting proliferation, and it has been used as a tumor treatment in clinical trials.^{51,52} However, in stem cell and organoid research PD03 has been found to have complex functions. On the one hand, PD03 is one of the important molecules in the production of human induced pluripotent stem cells⁵³ and it is also beneficial for maintaining a naïve pluripotent state along with CHIR99021 or vitamin C in naïve pluripotent embryonic stem cells and embryonic germ cells.^{54,55} On the other hand, PD03 combined with BMP4 has been shown to be an efficient method for generating functional human differentiated gastric cells from human pluripotent stem cells in stomach organoids^{56,57} and has been shown to promote the differentiation of adult stem cells in the heart.⁴⁶ However, inducing and maintaining a pluripotent state and promoting differentiation seem to be 2 mutually exclusive processes, which may be attributed to MEK/ERK playing different roles in different tissue cells at different stages. Our results suggest that PD03 can nearly double the differentiation efficiency in organoids, but we have not studied the combined effect of PD03 with other organoid differentiation factors, including LY411575, CHIR99021, RA, or regorafenib. More efforts are, therefore, needed to clarify the interaction between PD03 and other differentiation factors in order to establish an efficient differentiation system. Because organoids have great potential in disease modeling and therapeutic schedule screening, the increased proportion of HCs induced by PD03 in the inner ear organoid system will lead to improvements in the construction of organoids.

We further revealed the effect of HC regeneration by inhibition of MEK/ERK signaling together with repressing the Notch pathway. Guided by the RNA-seq results, we expressed the JAG1 protein in NICD transgenic mice and showed that the differentiation-promoting effect of PD03 is related to the inhibition of the Notch pathway, and our results showed that inhibition of MEK/ERK transduction by PD03 can downregulate the core factors of the Notch pathway, including *Hey1*,⁵⁸ *Hey2*,⁵⁹ *Hes1*,⁶⁰ and *Hes5*,⁶¹ which are reported to be crucial for HC differentiation. The MEK/ERK signal pathway has been confirmed to affect the Notch pathway in oral cancer (through JAG1),⁶² in breast cancer (through JAG1),⁶³ and in thyroid cancer (through HES1/HEY1).⁶⁴ Previous studies have also shown that downregulation of Notch leads to the downregulation of MEK/ERK in myeloid leukemias (through DUSP1).⁶⁵ In addition, in stem cell research MEK/ERK inhibitors and Notch inhibitors have similar effects and can be substituted for each other.⁶⁶ Taken together, these studies show that MEK/ERK and Notch interact with each other and engage in complex and close crosstalk, and further studies should analyze the specific interaction points between MEK/ERK and the Notch pathway and should investigate whether there is a positive feedback relationship between the 2 pathways.

Although we observed regenerated HCs with the typical electrophysiological characteristics of primary HCs, the formation of functional HCs remains a major problem in hearing restoration. Existing HC regeneration strategies, including

Notching inhibition and Atoh1 overexpression, have failed to successfully obtain HCs that are functionally similar to HCs in vivo.¹⁶ Recently, Chen et al. improved the functional maturity of regenerative HCs by co-regulation of *Gfi1*, *Pou4f3*, and *Atoh1* in the mouse cochlea,⁶⁷ but this strategy still did not achieve hearing recovery. The results of FM1-43 staining, electrophysiological experiments, and scanning electron microscopy presented here show that PD03 could induce HCs with similar structure and function as HCs in vivo. However, in terms of the maturity of their ciliary structure and the strength and type of their electrophysiological signals, there was a noticeable difference between the induced HCs and the original HCs. Apart from considering the characteristics of HCs themselves, we should also confirm the long-term survival of the regenerated HCs, the functional connection between regenerated HCs and the spiral ganglion neurons so as to realize the transmission of auditory signals.

Conclusions

In summary, our research identifies PD0325901 as a compound that promotes the differentiation of SCs into HCs with partial functional and morphological properties. Additionally, this differentiation-promoting effect is mediated by the inhibition of the Notch pathway. We suggest that PD0325901 is a candidate for inclusion in a cocktail of regenerative drugs in order to achieve the restoration of hearing function.

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Author contributions

Jiaoyao Ma: conducted the experiments, acquired and analyzed the data, and manuscript writing. Mingyu Xia: conception and design, conducted experiments, acquired and analyzed the data, and manuscript writing. Shan Sun: conception and design, financial support, manuscript writing, and final approval of manuscript. Bing Chen: conception and design, financial support, and final approval of manuscript. Jin Guo: conducted the experiments. Wen Li: technical support.

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Conflicts of interest

The authors declare no potential conflict of interests.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

The RNA-seq analysis data have been deposited in the GEO public database repository (GEO: GSE261682).

Supplementary material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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