Notch inhibition induces mitotically generated hair cells in mammalian cochleae via activating the Wnt pathway

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The activation of cochlear progenitor cells is a promising approach for hair cell (HC) regeneration and hearing recovery. The mechanisms underlying the initiation of proliferation of postnatal cochlear progenitor cells and their transdifferentiation to HCs remain to be determined. We show that Notch inhibition initiates proliferation of supporting cells (SCs) and mitotic regeneration of HCs in neonatal mouse cochlea in vivo and in vitro. Through lineage tracing, we identify that a majority of the proliferating SCs and mitotic-generated HCs induced by Notch inhibition are derived from the Wnt-responsive leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5$^+$) progenitor cells. We demonstrate that Notch inhibition removes the brakes on the canonical Wnt signaling and promotes Lgr5$^+$ progenitor cells to mitotically generate new HCs. Our study reveals a new function of Notch signaling in limiting proliferation and regeneration potential of postnatal cochlear progenitor cells, and provides a new route to regenerate HCs from progenitor cells by interrupting the interaction between the Notch and Wnt pathways.

Notch1 | β-catenin | proliferation | hair cells | inner ear

Sensory hair cell (HC) loss is the major cause of hearing loss and balance disorder. In nonmammalian vertebrates, HCs are regenerated in both auditory and vestibular systems after HC loss, leading to functional recovery of hearing and balance function (1–3). In mammals, limited spontaneous HC regeneration occurs in the vestibular system (4–8). In the adult mammalian vestibular sensory epithelium, inner ear stem cells were isolated with the capacity to differentiate into HCs and other inner ear cell types (9). In contrast, only neonatal mammalian cochleae have limited HC regeneration capacity in vivo, and harbor stem cells or progenitor cells that could proliferate and regenerate new HCs (10–12); however, no spontaneous HC regeneration has been observed in the mature cochlea (13).

Recent studies reported that in neonatal mouse cochlea, Wnt-responsive leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5$^+$) cells are the progenitors with the capacity to regenerate HCs under certain condition (11, 12, 14–17). However, endogenous Lgr5$^+$ progenitors maintain mitotic quiescence in neonatal mouse cochlea, suggesting the existence of negative regulators that inhibit the proliferation of those progenitors. Overexpressing β-catenin in the Lgr5$^+$ or Sox2$^+$ [SRY (sex determining region Y)-box 2] cells initiates proliferation by forming BrdU$^+$ foci adjacent to HCs (14, 16), serving as a potential approach to overcome the nonproliferative barrier of progenitors in the cochlear sensory epithelium. Alternatively, the identification and removal of the negative regulators could provide a new route to activate cochlear progenitor proliferation to achieve HC generation.

Inner ear sensory epithelium consists of a mosaic of HCs and supporting cells (SCs), generated from the same precursor pool in the prosensory domain during development (18, 19). The formation of the mosaic HC and SC pattern is mediated by lateral inhibition through the Notch signaling pathway (20, 21). Evidence from birds and mice suggests that Notch signaling negatively regulates the formation of HCs and the loss of Notch signaling generates supernumerary ectopic HCs at the expense of SCs (22–26). During early embryonic development, it has been shown that inhibition of Notch/JAG2 and DLL1 may prolong the proliferation process of the prosensory cells in the inner ear (27, 28). Notch signaling may play an important role maintaining the homeostasis of cochlear sensory epithelium on cell number and structures.

Although Wnt and Notch signaling are two fundamental pathways that regulate progenitor cell proliferation and determine the cell fate in the inner ear, their relationship remains largely unclear in the postnatal mouse cochlea. Here, by inhibiting the Notch signaling using Notch1 conditional knockout (KO) mice in vivo and by γ-secretase inhibitor IX (DAPT) treatment in vitro, we found that both inhibitions led to the proliferation of SCs and mitotic generation of HCs in the postnatal cochlear sensory epithelium. Lineage tracing demonstrated that a majority of the proliferating SCs and mitotically generated HCs were of Lgr5$^+$ lineage. In addition, we showed that Notch inhibition resulted in

Significance

Notch signaling is known as a fundamental pathway that regulates the cell-fate determination in the inner ear. In present study, we show that Notch signaling also acts as a negative regulator that inhibits the proliferation of Lgr5$^+$ progenitors and maintains the homeostasis of cochlear sensory epithelium on cell numbers. More importantly, to our knowledge we provide the first piece of evidence illustrating the interaction between Notch and Wnt in the postal mouse cochlea: Notch inhibition activates the canonical Wnt pathway in the progenitor cells, which leads to mitotic generation of hair cells; but Notch inhibition induced direct supporting cell-to-hair cell transdifferentiation that is Wnt-independent. Our findings may be useful in dissecting the mechanisms regulating mammalian inner ear proliferation and hair cell generation.

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β-catenin up-regulation in the Sox2$^+$ SCs, whereas inhibition of Wnt signaling significantly decreased SCs proliferation and mitotic generation of HCs induced by Notch inhibition.

Results

Inhibition of Notch in Sox2$^+$ SCs Initiates Proliferation of SCs in Vivo. We studied whether Notch inhibition could promote proliferation of Sox2$^+$ SCs in vivo by generating the Sox2-CreEr, flox-P flanked Notch1 transgenic (TG) mice (Sox2-CreEr/Notch1$^{f/f}$), in which tamoxifen exposure results in Notch1 deletion in Sox2$^+$ SCs. In addition to tamoxifen administration to postnatal days 0 and 1 (P0 and P1) to label Sox2$^+$ SCs, thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) was administered twice per day from P0–P3/P0–P7 to label proliferating cells. In control mice lacking of the Sox2-CreEr allele, no EdU$^+$/Sox2$^+$ SC was observed at P3 or P7 ($n = 8$), confirming previous reports that postnatal SCs are mitotically quiescent (29) (Fig. 1*A*). In Sox2-CreEr/Notch1$^{f/f}$ mice, EdU$^+$/Sox2$^+$ SCs started to appear at P3 with a gradient from apex to base (Fig. 1 *B* and *F*), and significantly more EdU$^+$/Sox2$^+$ SCs were observed at P7 (Fig. 1 *C* and *F*). Moreover, EdU$^+$/Sox2$^+$ SCs in different stages of mitosis were observed (Fig. 1*D*). Confocal microscopy showed that a majority of EdU$^+$/Sox2$^+$ SCs was located in the Pillar cell region (Fig. 1 *B–D*). From the sections of cochleae, we found significantly more cells in the Pillar cell region in the Sox2-CreEr/Notch1$^{f/f}$ mice (Fig. 1*G*), suggesting that these proliferating SCs may be derived from Lgr5$^+$ cells.

In mouse cochlear SCs, function of cyclin-dependent kinase inhibitor 1B (P27^{kip1}), a cell-cycling inhibitor, is essential to maintain quiescence status. In control Notch1$^{f/f}$ mice, P27^{kip1} was expressed in SCs at P7 (Fig. 1*H*), consistent with the previous report (10). In the Sox2-CreEr/Notch1$^{f/f}$ mice P27^{kip1} was significantly down-regulated in the EdU$^+$ SCs at P7 (Fig. 1*I*). When we compared the ratios of low-expression P27^{kip1} SCs to Pillar and Deiter's cells between Notch1$^{f/f}$ (13.20 ± 8.03%) and Sox2-CreEr/Notch1$^{f/f}$ (40.33 ± 7.20%) groups, more low expression P27^{Kip1} SCs were identified after Notch1 deletion ($P < 0.01$, $n = 3$), supporting that Notch1 deletion down-regulated P27^{kip1}, thereby promoting SC proliferation in vivo.

Inhibition of Notch in Sox2$^+$ SCs Initiates Mitotic Generation of HCs in Vivo. In Notch1$^{f/f}$ control mice administrated with tamoxifen at P0 and P1 and EdU from P0–P3/P0–P7, no EdU$^+$/Myo7a$^+$ HCs were observed at P3/P7 ($n = 8$), consistent with previous reports that there was no spontaneous postnatal mitotic generation of HCs (29) (Fig. 2*A*). In Sox2-CreEr/Notch1$^{f/f}$ mice receiving the same drug regimen, EdU$^+$/Myo7a$^+$ HCs, an indication of mitotic HC generation became visible at P3 in the apex only (2.60 ± 0.51 per cochlea, $n = 4$) (Fig. 2 *B* and *F*). By P7 significantly more EdU$^+$/Myo7a$^+$ HCs were observed (18.50 ± 1.56 per cochlea, $P < 0.01$, $n = 4$) (Fig. 2 *C*, *D*, and *F*). Furthermore, all of the EdU$^+$/Myo7a$^+$ HCs also had Sox2$^+$ nuclei ($n = 74$ cells) (Fig. 2 *B–D*). Sox2 is transiently expressed in immature HCs in the embryonic cochlea (30–32) and becomes restricted to SCs after birth (13), and thus can be considered as an immature HC marker. This result suggested that the EdU$^+$/Myo7a$^+$/Sox2$^+$ HCs were immature mitotic-generated HCs and may be derived from Sox2$^+$ SCs.

In Sox2-CreEr/Notch1$^{f/f}$ mice treated with tamoxifen, significantly more Myo7a$^+$ HCs were observed at P3 and P7 than in controls (Fig. 2*E*) and many of them also expressed Sox2 (Fig. 2 *B–D*). The mitotic generated EdU$^+$/Myo7a$^+$ HCs only constitute a small portion of the overall increase of Myo7a$^+$ HCs at P3 and P7, suggesting that direct transdifferentiation is the main mechanism for Notch inhibition induced HC generation in vivo (22–25). In summary, we demonstrated that Notch inhibition in the Sox2$^+$ SCs generates ectopic HCs via both mitotic generation and direct transdifferentiation in vivo.

Inhibition of Notch Initiates Proliferation of SCs in Vitro. To further investigate whether Notch inhibition can initiate the proliferation of SCs in vitro, cochlear sensory epithelium from P0–P1 WT mice were dissected and cultured in the presence of DAPT (5 μM; EMD) from day 0 to day 3. DMSO treatment was served as control. BrdU or EdU was present throughout the experiment. In control cochleae, no BrdU$^+$/Sox2$^+$ SC was observed in the sensory epithelial region 3 d after culture ($n = 4$), indicating that SCs are mitotic quiescent in vitro (Fig. 3*A*). In the DAPT-treated cochleae, a large number of BrdU$^+$/Sox2$^+$ SCs were observed, concentrating in the Pillar cell region with a gradient from apex to base (Fig. 3 *B* and *H* and Fig. S1 *D* and *E*), demonstrating that Notch inhibition initiates proliferation of SCs in vitro. EdU labeling confirmed the results (Fig. 3 *C* and *D* and Fig. S1 *B* and *C*).

To further study SC proliferation, we labeled the cochlea with G1-S phase marker, CyclinD1 (Ccnd1), and M-phase marker,

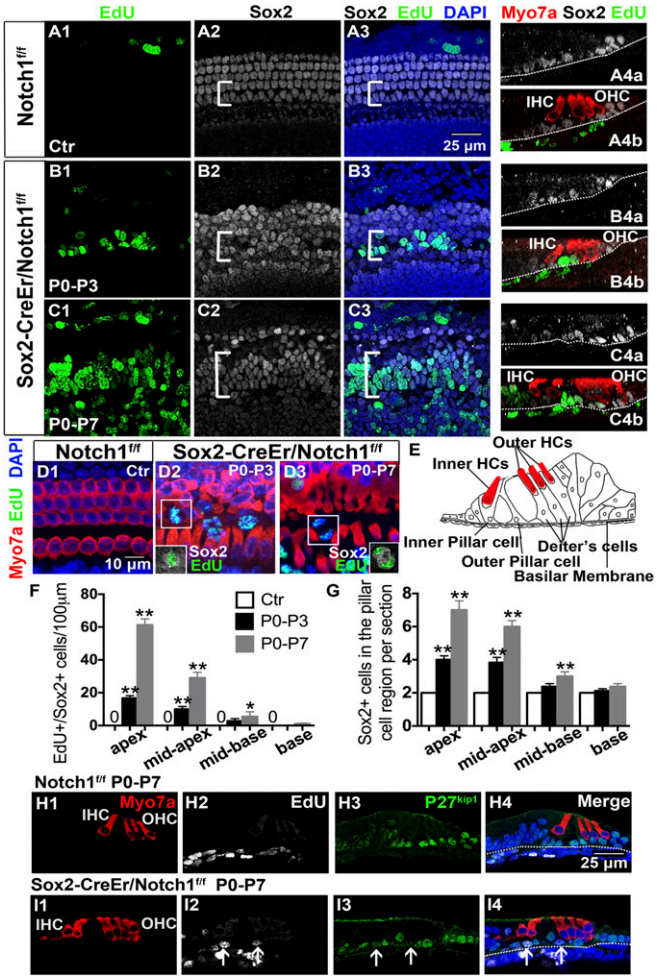

Fig. 1. Notch inhibition in Sox2$^+$ SCs initiates proliferation of SCs in vivo. (*A*) In Notch1$^{f/f}$ control cochlea, no EdU$^+$/Sox2$^+$ SC was detected at P3/P7. *A1–A3* show the expression of EdU, Sox2, and merged pictures at the SC layer in the sensory epithelium of the inner ear, and *A4* shows the cross-section of the organ of Corti. (*B*) In contrast, in the Sox2-CreEr/Notch1$^{f/f}$ cochlea, numerous EdU$^+$/Sox2$^+$ SCs were detected in the Pillar cell region (bracket) at P3. (*C*) A large number of EdU$^+$/Sox2$^+$ SCs were seen in the P7 Sox2-CreEr/Notch1$^{f/f}$ cochlea. (*D*) Representative images of EdU$^+$/Sox2$^+$ SCs at different stages of mitosis. (*E*) A diagram of the organ of Corti. (*F*) Quantification and comparison of the number of EdU$^+$/Sox2$^+$ SCs in the apex, midapex, midbase, and base of Notch1$^{f/f}$ and Sox2-CreEr/Notch1$^{f/f}$ cochlea. (*G*) The Sox2-CreEr/Notch1$^{f/f}$ cochlea had significantly more Sox2$^+$ SCs in the Pillar cell region at P3 and P7. (*H* and *I*) P27^{kip1} was expressed in SCs in Notch1$^{f/f}$ control mice and down-regulated in the proliferating SCs in Sox2-CreEr/Notch1$^{f/f}$ mice at P7; arrowhead indicates the proliferated SCs. *$P < 0.05$, **$P < 0.01$, $n = 4$ mice in *F* and *G*. The bracket indicates the Pillar cell region and the dotted line indicates the basilar membrane.

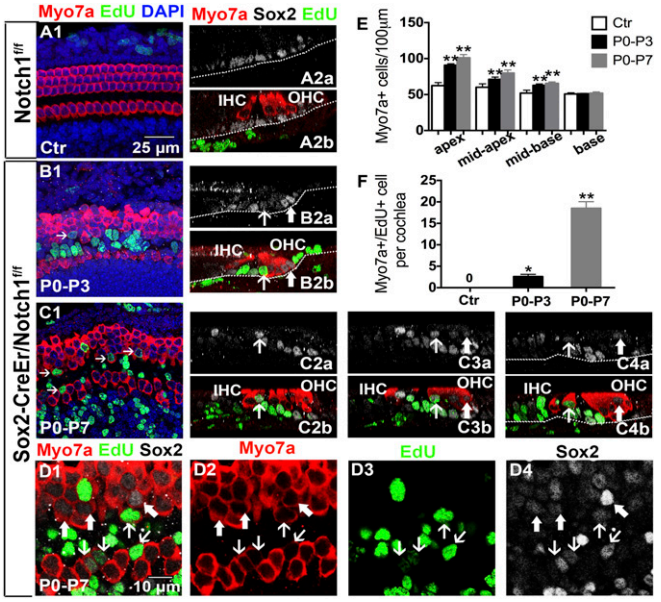

Fig. 2. Notch inhibition in Sox2$^+$ SCs initiates mitotic HC generation in vivo. (*A*) Cochlea in Notch1$^{f/f}$ control mice had no EdU$^+$/Myo7a$^+$ HC at P3/P7. (*B*). In contrast, cochlea in the Sox2-CreEr/Notch1$^{f/f}$ mice had several EdU$^+$/Myo7a$^+$ HCs (↑) and Sox2$^+$/Myo7a$^+$/EdU$^-$ HCs (⇑) at P3. (*C* and *D*) P7 cochlea in Sox2-CreEr/Notch1$^{f/f}$ mice had significantly more EdU$^+$/Myo7a$^+$ HCs than P3, and all EdU$^+$/Myo7a$^+$ HCs were also Sox2$^+$ (↑), Sox2$^+$/Myo7a$^+$/EdU- HCs (⇑) were also identified. (*E*) Numbers of Myo7a$^+$ HCs in the apex, midapex, midbase, and base of Sox2-CreEr/Notch1$^{f/f}$ mice and control littermates. (*F*) Compared with control littermates, Sox2-CreEr/Notch1$^{f/f}$ cochlea had more EdU$^+$/Myo7a$^+$ HCs at P3 and P7. *$P < 0.05$, **$P < 0.01$, $n = 4$ mice in *E* and *F*.

phospho-histone 3 (pH3). We found Ccnd1$^+$/EdU$^+$ SCs in the Pillar cell region only after DAPT treatment (Fig 3 *E* and *F*). We also observed SCs colabeled with the M-phase marker pH3 (23.53 ± 3.42 per cochlea, $n = 3$) (Fig. 3*G*). Because pH3 only labels dividing cells in the M phase, which lasts around 1 h (33), the pH3 labeling was likely to underestimate the number of dividing SCs after Notch inhibition. Taken together, the results demonstrated that Notch inhibition promotes SC proliferation in vitro with the highest activity in the apex, which decreased toward the base.

Inhibition of Notch Initiates Mitotic Generation of HCs in Vitro. In the DMSO-treated control group, no BrdU$^+$/Myo7a$^+$ HC were observed 3 d in culture (Fig. 3*A*). In the DAPT-treated cochlea, mitotic HCs were observed 3 d in culture in a gradient from more BrdU$^+$/Myo7a$^+$ in the apex to fewer in the base (Fig. 3 *B* and *I* and Fig. S1 *D* and *E*). Furthermore, significantly more HCs (Myo7a$^+$) were seen in all three turns after DAPT treatment (Fig. 3*J*). Overall, 15% (14.96 ± 0.02%) of new HCs were from dividing SCs, consistent with the evidence that Notch inhibition primarily promotes SC to HC transdifferentiation without proliferation in vitro (22–25).

We performed quantitative PCR (qPCR) and showed that compared to the DMSO control, the DAPT treatment significantly reduced the mRNA expression level of Notch target genes, *Hes1* and *Hes5* (Fig. S1 *F* and *G*). *Atoh1*, a HC transcription factor, was significantly increased, whereas *P27^{kip1}*, a cell-cycle inhibitor, was decreased (Fig. S1 *H* and *I*), correlating with increased HC generation and SC proliferation.

We further performed study using cultured Atoh1-nGFP cochlea, in which all HCs were GFP$^+$ because of Atoh1 promoter activity (34). We observed an increase in the number of EdU$^+$/Atoh1-nGFP$^+$ HCs after DAPT treatment, compared to DMSO-treated controls (Fig. 4 *A* and *B*). To determine whether mitotic regenerated HCs have active mechanotransduction channels (MET), we performed an FM1-43 dye (5 μM) uptake experiment. HCs with active MET rapidly take up FM1-43 dye, which is then trapped in the cytoplasm (35, 36). After being in culture for 3 d, the sensory epithelium was exposed to FM1-43 dye for 30 s. FM1-43 labeling was found in the EdU$^+$/Myo7a$^+$ HCs in the DAPT-treated samples; however, in the controls FM1-43 was only found in Myo7a$^+$ HCs (Fig. 4 *C*–*E*). This result suggested that mitotic regenerated HCs were likely to possess functional MET based on rapid uptake of FM1-43 dye.

Majority of Proliferating SCs and Mitotic-Generated HCs Induced by Notch Inhibition Derive from the Wnt-Responsive Lgr5 Lineage. Lgr5 is a Wnt downstream target gene and recent studies have reported that mitotic regenerated HCs originated from the Wnt-responsive Lgr5$^+$ SCs by overexpression of β-catenin or ablation of the HCs in neonatal mice cochlea (12, 16). In the Sox2-CreEr/Notch1$^{f/f}$ mice treated with tamoxifen with EdU exposure, we observed that a majority of the EdU$^+$/Sox2$^+$ SCs were located in the Pillar cell region, suggesting proliferating SCs may derive from the Lgr5+ cells (Figs. 1 *B*–*D* and 2 *B*–*D*). To determine the origin of proliferating SCs and mitosis-derived HCs by Notch inhibition, we generated an Lgr5-CreEr; ROSA26-tdTomato TG mouse line to lineage trace the Lgr5$^+$ cells. With this model, we injected tamoxifen (P0 and P1), which marks the Lgr5$^+$ SCs tdTomato. P2 cochleae were then cultured and treated with DAPT (P2–P5) in the presence of EdU. In the DMSO-treated control, tdTomato expression was observed at day 5 in the SC subtypes, as previously reported (37). Neither EdU$^+$/tdTomato$^+$/Sox2$^+$ SC nor EdU$^+$/tdTomato+/Myo7a$^+$ HC was observed in all three turns ($n = 3$) (Fig. 5*A*). In the cochlea treated with DAPT, the number of tdTomato$^+$ cells was increased 1.8-fold (Fig. 5 *A*–*C*), corresponding to proliferation in the lineage-traced Lgr5$^+$ SCs by Notch inhibition. Significantly more Lgr5$^+$ SCs (tdTomato$^+$/Sox2$^+$) than Lgr5$^-$ SCs (tdTomato$^-$/Sox2$^+$) were EdU-labeled

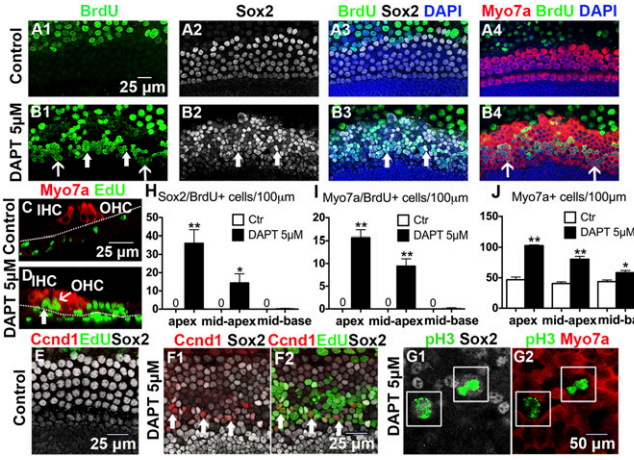

Fig. 3. Notch inhibition initiates SC proliferation and mitotic HCs generation in vitro. (*A*) No BrdU$^+$/Sox2$^+$ SCs or BrdU$^+$/Myo7a$^+$ HCs were detected in cultured control cochlea. (*B*) A significant increase in BrdU$^+$/Sox2$^+$ SCs (⇑) and BrdU$^+$/Myo7a$^+$ HCs (↑), in the Pillar cell region, were detected after DAPT treatment. (*C* and *D*) With EdU labeling, proliferation was detected in Sox2$^+$ SCs (⇑) and Myo7a$^+$ HCs (↑) only after DAPT (*D*) but not in DMSO (*C*) treatment groups. (*E* and *F*) Ccnd1$^+$ was detected in the SCs after DAPT treatment (Ccnd1$^+$/Sox2$^+$: 17.76 ± 1.39, per 100-μm cochlear length) (*F1* and *F2*) but not in control (*E*). Of the Ccnd1$^+$ cells, 96% (96.55 ± 1.49%) were EdU$^+$ (Ccnd1$^+$/EdU$^+$/Sox2$^+$: 17.16 ± 1.49, per 100-μm cochlear length). Among EdU$^+$/Sox2$^+$ SCs, 41.67 ± 4.10% cells are Ccnd1$^+$. (*G*) pH3$^+$/Sox2$^+$ SCs were detected in the Pillar cell region after DAPT treatment. (*H*) DAPT-treated cochlea had significantly more BrdU$^+$/Sox2$^+$ SCs, with a gradient from high in the apex to low in the base. (*I* and *J*) In the DAPT-treated cochlea, significantly more BrdU$^+$/Myo7a$^+$ HCs and Myo7a$^+$ HCs were detected, and both displayed a gradient from higher number in the apex to lower number in the base. *$P < 0.05$, **$P < 0.01$, $n = 4$ in *H*–*J*.

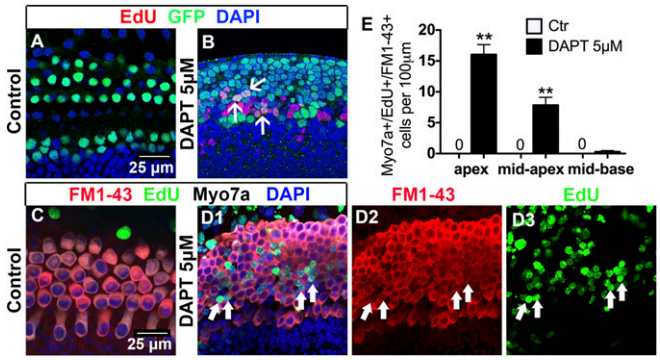

Fig. 4. Mitotically generated HCs induced by Notch inhibition. (*A*) No EdU$^+$/GFP$^+$ HCs were detected in the DMSO-treated Atoh1-nGFP control cochlea. (*B*) Detection of EdU$^+$/GFP$^+$ HCs (↑) in the Atoh1-nGFP cochlea treated with DAPT. (*C*) In the DMSO-treated control cochlea, no EdU$^+$/FM1-43$^+$ HC were detected. (*D*) In the DAPT-treated cochlea, EdU$^+$/FM1-43$^+$ HCs (⇑) were seen in the sensory epithelium. (*E*) EdU$^+$/FM1-43$^+$ HCs were quantified from apex to midbase regions in the DMSO and the DAPT-treated cochleae, $n = 5$. $^{**}P < 0.01$.

(48.44 ± 4.18% vs. 10.73 ± 1.83%, $P < 0.01$, $n = 3$) (Fig. 5 *C* and *D1*), indicating that Lgr5$^+$ SCs were more proliferative under Notch inhibition. In fact, a majority (73.09 ± 1.87%) of proliferating SCs (EdU$^+$/Sox2$^+$, 40.41 ± 6.340 EdU$^+$/Sox2$^+$ cells per 100-μm cochlear length, $n = 3$) were tdTomato$^+$ (EdU$^+$/Sox2$^+$/tdTomato$^+$, 29.68 ± 5.10) (Fig. 5 *C*, *D2*, and *D3*), again supporting that proliferating SCs by Notch inhibition were from Wnt-responsive Lgr5$^+$ SCs. We observed EdU$^+$/Myo7a$^+$ HCs (9.90 ± 1.12 EdU$^+$/Myo7a$^+$ HCs per 100-μm cochlear length, $n = 3$), of which 77.00 ± 1.23% were of Lgr5$^+$ origin (7.59 ± 0.75, EdU$^+$/Myo7a$^+$/tdTomato$^+$) (Fig. 5 *B*, *D2*, and *D3*). Thus, Notch inhibition preferentially promotes proliferation of Wnt-responsive Lgr5$^+$ SCs and their transdifferentiation to HCs, indicating that the Notch inhibition-induced mitotic HC generation is likely to be Wnt-dependent.

To further confirm these findings, we generated an Lgr5-CreEr/Notch1$^{f/f}$ TG mouse model in which Notch1 can be conditionally deleted in the Lgr5$^+$ SCs. In cultured P0 cochlea treated with 4OH-tamoxifen for 4 d, we identified EdU$^+$/Myo7a$^+$ HCs in the Pillar cell region of the Lgr5-CreEr/Notch1$^{f/f}$ but not control Notch1$^{f/f}$ cochlea (Fig. 5 *E*–*G*), supporting that Notch inhibition promotes the Wnt-responsive Lgr5$^+$ SCs to generate HCs mitotically. In summary, our results showed that Notch inhibition initiates proliferation of the Wnt-responsive Lgr5$^+$ SCs and induces their transdifferentiation to HCs. Our work illustrates potential interaction between Notch and Wnt signaling pathways, suggesting Notch inhibition may activate the Wnt signaling in the postnatal mice cochlea, enabling the Wnt-responsive Lgr5$^+$ SCs to divide and to generate HCs.

Inhibition of Notch Activates the Canonical Wnt Signaling Pathway. To investigate the interaction between Notch and Wnt signaling pathways, we inhibited the Notch signaling and examined the expression of β-catenin, a key factor in the canonical Wnt pathway. We performed labeling of phosphorylated-β-catenin (p-β-catenin), the active form of β-catenin (38), in cultured P0/P1 cochlea treated with DAPT or DMSO. In control, few p-β-catenin$^+$/Sox2$^+$ SCs, none of which was EdU$^+$, were seen in the lateral region of cochlea (2.90 ± 0.17 per 100-μm cochlear length, $n = 3$) (Fig. S2 *A* and *C*). In the DAPT-treated cochlea, significantly more p-β-catenin$^+$/Sox2$^+$ SCs were detected (32.05 ± 4.55, per 100-μm cochlear length, $n = 3$) (Fig. S2 *B* and *C*), demonstrating that Notch inhibition significantly activated the canonical Wnt signaling and up-regulated the expression of p-β-catenin in SCs in vitro. In addition, a majority of the p-β-catenin$^+$/Sox2$^+$ SCs was EdU$^+$ (90.65 ± 2.51%, $n = 3$) (Fig. S2*D*).

Inhibition of Wnt Decreases the Proliferation of SCs and Mitotic Generation of HCs Induced by Notch Inhibition. To determine if activation of canonical Wnt signaling is necessary for the Notch inhibition-induced SC proliferation and mitotic generation of HCs, we blocked the Wnt signaling concurrently with the Notch inhibition. Cultured P0/P1 WT cochleae were treated DAPT together with a small-molecule Wnt inhibitor, IWP-2, which blocks secretion of Wnt (39). In the DMSO control, neither EdU$^+$/Sox2$^+$ SCs nor EdU$^+$/Myo7a$^+$ HCs were observed after 3 d in culture ($n = 4$) (Fig. S3*A*). In the DAPT-treated cochlea, a large number of EdU$^+$/Sox2$^+$ SCs and EdU$^+$/Myo7a$^+$ HCs were observed after 3 d in culture (35.64 ± 2.63 for EdU$^+$/Sox2$^+$ SCs and 15.34 ± 2.56 for EdU$^+$/Myo7a$^+$ HCs, per 100-μm cochlear length). In the DAPT and IWP-2 cotreated cochlea, the number of EdU$^+$/Sox2$^+$ SCs and EdU$^+$/Myo7a$^+$ HCs were significantly decreased compared with the DAPT-treated cochlea (16.71 ± 1.31 for EdU$^+$/Sox2$^+$ SCs and 7.45 ± 0.59 for EdU$^+$/Myo7a$^+$ HCs, per 100-μm cochlear length, $P < 0.01$, $n = 4$) (Fig. S3 *B*–*E*), demonstrating that Wnt inhibition significantly decreased the proliferation of SCs and reduced mitotic generation of HCs induced by Notch inhibition in vitro. Thus, Wnt signaling is necessary for mitotic HC generation induced by Notch inhibition. Interestingly the number of EdU$^-$/Myo7a$^+$ HCs was not significantly different between DAPT and DAPT/IWP-2 groups (64.28 ± 4.92 vs. 68.54 ± 5.33 per 100-μm length for DAPT and DAPT/IWP-2 treatment, respectively, $P = 0.57$, $n = 4$) (Fig. S3*F*), strongly supporting that Wnt signaling is not required for nonmitotic direct transdifferentiation of SCs to HCs induced by Notch inhibition.

We further checked the effects of IWP-2, DAPT, and DAPT with IWP-2 on the Wnt signaling pathway in explanted cochleae

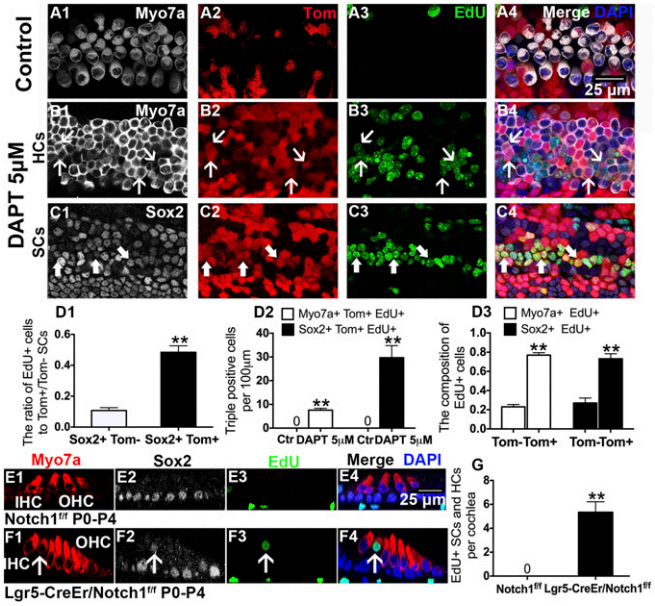

Fig. 5. Lineage tracing of Lgr5$^+$ SCs in vitro. (*A*) Using Lgr5-CreER; ROSA26-tdTomato mice, DMSO-treated control cochlea had no EdU$^+$ in tdTomato cells, which included third Deiter's cells, inner pillar cells, inner phalangeal/border cells, and GER cells. (*B* and *C*) DAPT-treated cochlea showed EdU$^+$/tdTomato$^+$/Myo7a$^+$ (↑) HCs (*B*) and EdU$^+$/tdTomato$^+$/Sox2$^+$(⇑) SCs (*C*) in the sensory epithelium. (*D1*) DAPT treatment resulted in significantly more EdU$^+$/tdTomato$^+$/Sox2$^+$ SCs than EdU$^+$/tdTomato$^-$/Sox2$^+$ SCs. (*D2*) DAPT-treated cochlea had significantly more EdU$^+$/tdTomato$^+$/Myo7a$^+$ HCs and EdU$^+$/tdTomato$^+$/Sox2$^+$ SCs than control cochlea. (*D3*) A majority of EdU$^+$/Sox2$^+$ SCs and EdU$^+$/Myo7a$^+$ HCs were also tdTomato$^+$. (*E* and *F*) EdU$^+$/Myo7a$^+$ HCs were detected in the pillar cell region in the Lgr5-CreEr/Notch1$^{f/f}$ cochlea but not in the Notch1$^{f/f}$ control cochlea. (*G*) Overall, 5.33 ± 0.88 EdU$^+$ HCs and SCs were identified per Lgr5-CreEr/Notch1$^{f/f}$ cochlea, but no EdU$^+$ HCs or SCs were observed in the Notch1$^{f/f}$ control cochlea. $^{**}P < 0.01$, $n = 3$ in *D1*–*D3* and *G*.

by investigating the expression of a key factor of Wnt signaling pathway, *β-catenin*, and the Wnt downstream target gene, *Sp5*. We found that IWP-2 down-regulated the expression of *Sp5* and *β-catenin*, an indication that it is an effective inhibitor of the Wnt signaling pathway in the cultured neonatal cochlea. Activation of the Wnt signaling pathway by DAPT treatment was further supported by the up-regulation of *Sp5* and *β-catenin*, whereas the IWP-2 treatment suppressed the Wnt signaling pathway induced by Notch inhibition (Fig. S4). Taken together, our results demonstrate two functions of Notch inhibition in the context of proliferation and HC regeneration: the induction of proliferation of SCs and mitotic generation of HCs that is mediated by active canonical Wnt signaling; and SC to HC direct transdifferentiation that is Wnt-independent.

Discussion

In mammals, limited mitotic regeneration of HCs has been detected in the vestibular sensory epithelia and cochlea of newborn mice (4–8, 12), Atoh1 acts as one of the most important factors in the fate decision of HCs (40, 41), and Notch inhibition is known to promote SCs to produce nonmitotic generation of HCs by suppressing lateral inhibition and up-regulating Atoh1 in SCs. However, the role of Notch signaling in regulating SC proliferation and the mitotic generation of HC is unknown. Although Wnt activation leads to HC regeneration, the interaction between the Notch and Wnt signaling pathways in proliferation and HC regeneration remains to be determined in the postnatal mice cochlea. In this study, we unveil a new role of Notch signaling in regulating the proliferation of SCs and mitotic generation of HCs in postnatal cochlear sensory epithelia in vivo and in vitro. We further demonstrate that Notch signaling conveys through the Wnt pathway that targets primarily the $Lgr5^+$ SCs for proliferation and mitotic generation of HCs.

Loss of Notch Signaling in $Sox2^+$ Cells Initiates SCs Proliferation in Sensory Epithelia of Postnatal Cochlea. Notch is essential in defining the prosensory domain in the otocyst and is instrumental in establishing lateral inhibition that determines HC vs. SC fate in the cochlear sensory epithelia (22, 42, 43). Loss of Notch signaling produces ectopic HCs through direct transdifferentiation of SCs during late development and postnatal cochlea (22–25), yet the role of Notch in cell proliferation under those conditions remains to be determined. A recent study in zebrafish showed that Notch inhibition is accompanied with cell cycle reentry immediately after HC damage in the lateral line neuromasts (44). In this study, we demonstrate that SC proliferation was induced by Notch inhibition in vivo and in vitro. We show in the cochlear sensory epithelia, EdU and BrdU labeled SCs after conditional KO Notch1 in $Sox2^+$ cells in vivo and after DAPT treatment in vitro. Upon Notch inhibition, pH3-labeled SCs and mitotic figures are detected in the cochlear sensory epithelia, coinciding with up-regulation of cyclinD1 and down-regulation of $P27^{kip1}$. It is interesting to note that despite numerous studies of Notch in vivo and in vitro, SC proliferation induced by Notch inhibition was not reported. The identification of such proliferation in our study may be in part a result of the use of the Cre-Loxp system and Sox2-CreEr/$Notch1^{f/f}$ mice for specific Notch1 deletion in $Sox2^+$ SCs in vivo, and the use of the Sox2-CreEr mice with higher Cre activity (17). We also used P0/P1 mice with higher proliferation potential than older mice. Finally, frequent injections of EdU (twice a day) for 3 and 7 d in vivo or continuous presence of EdU in the culture media may increase the chance of identifying dividing cells.

Notch Inhibition Initiates the Proliferation of Wnt-Responsive $Lgr5^+$ SCs and Promotes Their Transdifferentiation to HCs. Activating Wnt signaling in the $Lgr5^+$ SCs leads to proliferation and mitotic generation of HCs in the postnatal mouse cochlea in vivo (14–16). The pattern of SC proliferation in our Notch inhibition model mirrors the Wnt activation in $Sox2^+$ SCs. Our lineage-tracing data showed that mitotic generation of HCs were derived from the Wnt-responsive $Lgr5^+$ SCs, suggesting that the loss of Notch signaling releases proliferative competent $Lgr5^+$ progenitor cells to reenter the cell cycle and transdifferentiate into HCs.

FACS-isolated progenitor cells by Lgr5-GFP or purified SCs by different SC markers ($P27^{kip1}$ or $CD326^+/CD146^{low}/CD271^{low}$) have the capacity to proliferate and transdifferentiate to HCs in vitro (10, 14, 45). However, these proliferative competent cells remain mitotic quiescent in the cochlear sensory epithelia of newborn mice in vivo. Our study strongly suggests that the quiescence of Wnt-responsive $Lgr5^+$ progenitor cells is likely maintained by active Notch signaling.

Notch Inhibition Initiates the Proliferation of SCs and Mitotic Generation of HCs via Activating Canonical Wnt Signaling. Interaction of Notch and the Wnt pathway plays a pivotal role in regulating proliferation and differentiation of progenitors in different cellular and biological contexts. When Notch1 is constitutively activated in stromal cell line ST-2, the effect of Wnt3a and the Wnt-dependent gene expression are decreased, leading to inhibition of the osteoblastogenesis (46). During skin development, Notch inhibition displays a hyperplastic epidermis by accumulation of nuclear β-catenin (47); and in squamous cell carcinoma, Notch inhibition by dominant-negative Mastermind Like 1, a pan-Notch inhibitor, leads to enhanced accumulation of nuclear β-catenin and cyclinD1 in suprabasilar keratinocytes (48). Consistent with these studies, we found Notch inhibition induces overexpression of β-catenin and activation of Wnt downstream target genes in the SCs of postnatal mice cochlea.

Overexpression of β-catenin in $Sox2^+$ or $Lgr5^+$ cells of cochlear epithelium results in proliferation and HC generation (14, 16). Our work supports that Notch inhibition leads to up-regulation of β-catenin and activation of Wnt signaling in the same cells. We show two mechanisms underlying Notch inhibition-induced HC regeneration: direct transdifferentiation of SCs that is mitosis-independent and proliferative HCs regeneration that is mitosis-dependent. Only the mitosis-derived HCs are Wnt-dependent, because inhibition of Wnt signaling significantly decreased SC proliferation and mitotic generation of HCs initiated by Notch inhibition, but did not affect the number of regenerated HCs by direct transdifferentiation. Given the evidence, it is highly likely that multilayered control is in place in maintaining the quiescent status of $Lgr5^+$ SCs by the balanced interaction between Notch and Wnt signaling. This Notch and Wnt signaling interaction may also partially explain why proliferative competent $Lgr5^+$ progenitor cells stay mitotic quiescent in situ but start to proliferate and form spheres when they are isolated. We hypothesize that when the progenitor cells are isolated and sorted to a single cell, the cell–cell interaction (lateral inhibition) disappears and then cell proliferation erupts to form spheres.

In summary, we demonstrate that Notch inhibition induces proliferation of SCs and mitotic regeneration of HCs, in addition to direct HC transdifferentiation in the cochlear sensory epithelia of postnatal mice. Notch inhibition primarily acts on the Wnt-responsive $Lgr5^+$ SCs with the effect of proliferation and HC regeneration, which is mediated by up-regulation of β-catenin and activation of the canonical Wnt pathway. Our findings may be useful in dissecting the mechanisms regulating mammalian inner ear proliferation and HC generation, and provide a new avenue to stimulate HC regeneration after HC loss.

Materials and Methods

Animal Models. Sox2-CreEr was a gift from Konrad Hochedlinger, Harvard University, Cambridge, MA (49). Lgr5-CreEr (Stock No. 008875), Notch1-flox (exon1) (Stock No. 007181), and CAG-tdTomato (Stock No.007914) mice were obtained from the Jackson Laboratories. Atoh1-nGFP was provided by Jane Johnson, University of Texas Southwestern Medical Center, Dallas, TX. Primers used for genotyping are shown in Table S1. For Cre activation, 80 μL tamoxifen dissolved in corn oil (50 mg/mL; Sigma) was intraperitoneally injected to mothers of TG litters and transferred to the pups via the milk at P0 and P1. Ten microliters (Click-iT imaging kit, Invitrogen) was injected

(5 mg/mL, s.c.) on the back twice per day at P0–P3/P0–P7 to the pups. All animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University.

Organotypic Culture of Neonatal Mice Cochlea. The mice were killed at P0 (for culture) or P2 (for lineage tracing) and the organ of Corti were isolated and seeded intact on a glass coverslip coated with Matrigel (1:10; Invitrogen). The explanted cochlea were treated with 5 μM DAPT (γ-secretase inhibitor IX, EMD), 5 μM IWP-2 (Stemgent), or DAPT (5 μM) and IWP-2 (5 μM), and 10 μM BrdU/EdU was added to the culture medium for the whole culture period.

Cell Proliferation Assay. The BrdU incorporation was detected by immunocytochemistry. The EdU signals were detected with the Click-iT cell proliferation kit (Invitrogen) followed by antibody labeling according to the immunofluorescent staining protocol described below.

Immunohistochemistry. All cultured cochleae were fixed with 0.1 M phosphate-buffered 4% (wt/vol) paraformaldehyde for 30 min and followed by a standard procedure. The primary antibodies used in our experiments were rabbit anti-myo7a (1:500; *Proteus* Biosciences), goat anti-Sox2 (1:200; Santa Cruz Biotechnology), mouse antiphospho-Histone H3 (1:200; Cell Signaling Technology), CyclinD1 (1:200; Fisher Thermo-Scientific), mouse antiphospho-489-β-catenin (1:200; Developmental Studies Hybridoma Bank), and rat anti-BrdU (1:200; AbD serotech) that had been diluted in the blocking solution at 4 °C overnight.

To detect functional mechanoeletrical transduction channels, 5 μM FM1-43 was incubated with the cultured organ of Corti for 30 s, followed by rinsing with PBS for three times before fixation.

Further details about qRT-PCR (primer sequences are listed in Table S2), image acquisition, cell quantification, and statistics are in *SI Materials and Methods*.

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