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RESEARCH ARTICLE

Unidirectional and stage-dependent roles of Notch1 in Wnt-responsive Lgr5$^+$ cells during mouse inner ear development

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Abstract **Wnt and Notch signaling play crucial roles in the determination of the prosensory domain and in the differentiation of hair cells (HCs) and supporting cells during mouse inner ear development; however, the relationship between the two signaling pathways in the mouse cochlea remains largely unknown. Here, we investigated the interactions between Notch and Wnt signaling on the basis of the bidirectional regulation of Notch1 specifically in Wnt-responsive Lgr5$^+$ progenitors during different cochlear development stages. We found that the downregulation of Notch1 in Lgr5$^+$ cells from embryonic day (E) 14.5 to E18.5 can drive the quiescent Lgr5$^+$ cells to re-enter the cell cycle and differentiate into extra HCs, whereas the upregulation of Notch1 expression did not affect the proliferation or differentiation of otic progenitor cells. No effect was observed on the upregulation or downregulation of Notch1 in Lgr5$^+$ cells from E10.5 to E14.5. We concluded that the roles of Notch1 in Wnt-responsive Lgr5$^+$ cells are unidirectional and stage dependent and Notch1 serves as a negative regulator for Lgr5$^+$ progenitor activation during cochlear differentiation. Our findings improved the understanding of the interactions between Notch and Wnt signaling in cochlear development.**

Keywords inner ear; cochlear; Wnt; Notch; Lgr5; auditory system

Introduction

The mammalian inner ear is a highly complex sensory organ. Almost all the cell types of the mouse inner ear are derived from the otic placode and are formed on embryonic day (E) 8.5 [1]. The primordia of the mouse auditory and vestibular organs are formed in the ventral and dorsal parts of the otic vesicle, respectively, at E10.5 [1,2]. At E12.5, the progenitor cells in the cochlear prosensory epithelium begin to exit the cell cycle sequentially from the apical to the basal turn along with the expression of P27kip1 [3]. Hair cell (HC) differentiation begins in the midbasal part of the cochlear turn at E13.5 and spreads to the basal and the apical regions along with HC marker expression [2]. During inner ear development, the proliferation and differentiation of the progenitor cells are precisely regulated by several signaling pathways and transcription factors, including the Notch and Wnt signaling pathways [4].

Wnt signaling participates in maintaining the pluripotency of otic progenitors in inner ear development [5–7]. *Lgr5*, the most-studied downstream gene of the Wnt signaling pathway, is expressed in embryonic (E15.5–E18.5) and neonatal cochlear progenitors [8,9]. Lgr5$^+$ cells can act as progenitors of HCs and supporting cells (SCs) in the embryonic and postnatal stages because of their ability to self-renew, proliferate, and differentiate [10–13]. Chai *et al*. showed that the proliferation and differentiation of Lgr5$^+$ cells are regulated by Wnt inhibitors and enhancers, suggesting that Lgr5$^+$ cells serve as Wnt-responsive progenitor cells [13]. Wnt agonist treatment in the neonatal cochlea enhances Lgr5$^+$ progenitor cell proliferation and increases HC formation [5,6].

Notch signaling is known as a fundamental pathway that regulates the cell-fate determination in the inner ear. The Notch signaling receptors (Notch1–4), ligands (Jag1 and Delta1), and effectors (Hes1, Hes5) were first identified in the otic placode and are consistently active throughout the

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entire process of cochlear development [14–16]. During the early stage, Jag1-mediated Notch signaling is required for establishing the prosensory regions of inner ear [17], whereas during the later stage, Notch signaling serves as a negative regulator for HC formation in the auditory sensory epithelium by "lateral inhibition," and the loss of Notch signaling generates supernumerary ectopic HCs at the expense of SCs [18–22]. During HC regeneration, Notch signaling was upregulated after HC damage in mature avian auditory sensory epithelium, which prevents SCs from regenerating excessive HCs [23].

In previous studies [24,25], we explored the roles of Notch signaling in postnatal mouse cochlea by specifically deleting Notch1 in Sox2$^+$ SCs, which resulted in the upregulation of Wnt signaling, followed by SC proliferation and HC generation in cochlea. To further dissect the interaction between Notch and Wnt signaling during early mouse cochlea development, we specifically down and upregulated Notch signaling in Wnt-responsive Lgr5$^+$ cells during the developmental stage at which the prosensory region is determined (E10.5–E14.5) and during the developmental stage at which HCs and SCs undergo differentiation in the cochlea (E14.5–E18.5). The prosensory region of the cochlea was evaluated by staining P27Kip1 and Jag1, and the HCs and SCs were labeled with Myo7a and Sox2, respectively, to investigate cell differentiation in the organ of Corti. We found that Notch1 deletion in Lgr5$^+$ cells starting from E10.5 showed no evident effect on prosensory region formation or on progenitor cell proliferation, whereas Notch1 deletion in Lgr5$^+$ cells from E14.5 onwards drove the quiescent progenitor cells to re-enter the cell cycle and differentiate into extra HCs. By contrast, the overexpression of Notch1 in embryonic Lgr5$^+$ cells showed no evident effects on the cochlear prosensory epithelia formation or HC differentiation. These results suggest that Notch signaling plays an important role in maintaining the homeostasis of the cochlear sensory epithelium in terms of cell numbers and structures, and the manipulation of the Notch signaling pathway may provide a new route for HC regeneration in the mammalian cochlea.

Materials and methods

Lgr5-EGFP-IRES-CreERT2 (Lgr5$^{CreERT2/+}$, Stock No. 008875), Notch1$^{flox/flox}$ (Notch1$^{f/f}$, Stock No. 007181), and Rosa^{Notch1} (Notch1$^{OE/+}$, Stock No. 008159) mice were purchased from the Jackson Laboratory. To generate Lgr5$^{CreERT2/+}$;Notch1$^{flox/flox}$ embryonic mice, male Lgr5^{CreERT2};Notch1$^{flox/flox}$ mice were mated with female Notch1$^{flox/flox}$ mice overnight, and the mice were separated the next day, and this was considered E0.5. Lgr5$^{CreERT2/+}$; Notch1$^{OE/+}$ mice were generated by mating male Lgr5$^{CreERT2/+}$ mice with female Notch1$^{OE/+}$ mice. Mice of both sexes were used in the study. Approximately 80 μL of 40 mg/mL tamoxifen (Sigma-Aldrich) was injected intraperitoneally into the pregnant mice at E10.5 or E14.5. Exactly 5 mg/mL of 5′-ethynyl-2′-deoxyuridine (EdU, Invitrogen) was injected into the mice once daily during E10.5 – E14.5 or E14.5 – E18.5.

Embryos were harvested from timed pregnant females. Whole heads were fixed in 4% PFA (Sigma-Aldrich) for 1 h at room temperature and immersed in a graded series of sucrose solutions. After washing with 10 mmol/L PBS, 14 μm-thick cross-sections were prepared with a cryostat microtome (Leica). Whole-mount specimens were prepared by delicate dissection, incubated for 1 h in a solution of 10% donkey serum, 1 mmol/L PBS, and 1% Triton X-100, and incubated with primary antibody overnight at 4 °C. The primary antibodies were goat anti-Sox2 (1:500 dilution; Santa Cruz Biotechnology), chicken anti-EGFP (1:800 dilution; Abcam), rabbit anti-Jag1 (1:500 dilution; Santa Cruz Biotechnology), mouse anti-P27kip1(1:200 dilution; BD), and rabbit anti-Myo7a (1:800, Proteus Bioscience). Corresponding donkey anti-rabbit, anti-goat, anti-chicken, or anti-mouse secondary antibodies conjugated with Alexa Fluor® 488, Alexa Fluor® 568, or Alexa Fluor® 647 (ThermoFisher Scientific, 1:500 dilution) were used. The appropriate secondary Alexa Fluor-conjugated antibodies were incubated for 1 h at room temperature. EdU was detected with Alexa Flour azide using the Click-iT EdU Imaging Kit (Invitrogen) in accordance with the manufacturer's protocol. Fluorescent images were acquired using a Leica SP8 confocal microscope.

Results

Lgr5 was expressed in the embryonic cochlear epithelium

Lgr5 expression has been observed in the embryonic (E15.5–E18.5) cochlear duct [9,14]. To confirm that Wnt-responsive cells exist in the prosensory epithelium, we initially investigated Lgr5 protein expression in the developing cochlea using Lgr5-EGFP-IRES-CreERT2 mice. Sox2 and Jag1 were used as markers of the prosensory epithelium that produces the organ of Corti [7,26]. At E13.5, Lgr5 protein was expressed in the ventral part of the mouse cochlear duct (Fig. 1), including the Sox2$^+$ and Jag1$^+$ sensory epithelium, suggesting that Wnt signaling is active in the ventral cochlear duct and may play roles in cochlear sensory epithelium formation.

Notch1 deletion in Lgr5$^+$ cells in the otic vesicle showed no remarkable effect on prosensory region formation

To explore the role of Notch inhibition in Wnt-responsive otic progenitor cells, we generated Lgr5$^{CreERT2/+}$;

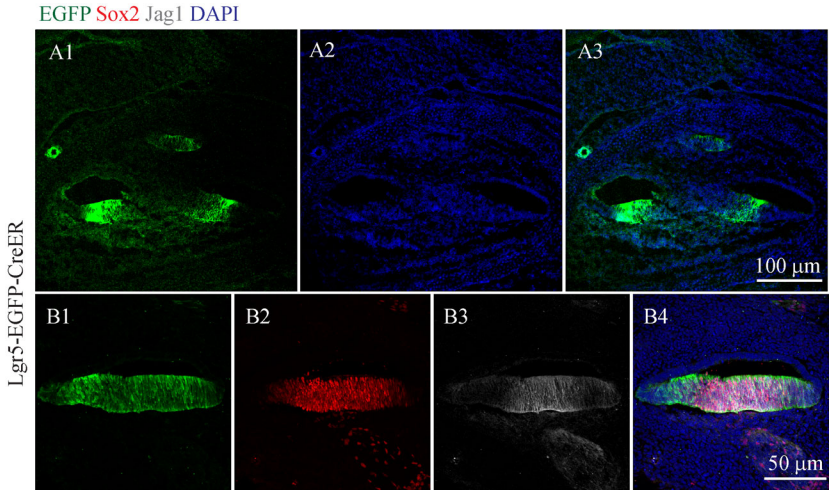

Fig. 1 EGFP immunofluorescence in Lgr5-EGFP-CreER mice showed that Lgr5 was expressed in the cochlear duct at E13.5. Sox2 and Jag1 were used as markers of cochlear prosensory epithelium. The images of the lower magnification (A) and the higher magnification of apical turn (B) are shown. Scale bar: (A) 100 μm; (B) 50 μm.

Notch1$^{flox/flox}$ mice, in which constitutive deletion of Notch1 in Lgr5^{+} cells was achieved by tamoxifen administration at E10.5. Lgr5$^{CreERT2/+}$ mice served as control. Embryos were harvested at E14.5. During auditory sensory epithelium development, the progenitor cells exit the cell cycle sequentially from the apical to the basal regions along with the expression of P27kip1 [3]. We found that the expression pattern of P27kip1 was similar to the expression patterns of P27kip1 in the control and Lgr5$^{CreERT2/+}$;Notch1$^{flox/flox}$ mice (Fig. 2). In addition, the location of Jag1^{+} cells was comparable between the two groups (Fig. 2), indicating that Notch1 deletion in Lgr5^{+} cells of the otic vesicle exhibited no considerable effect on prosensory epithelium formation.

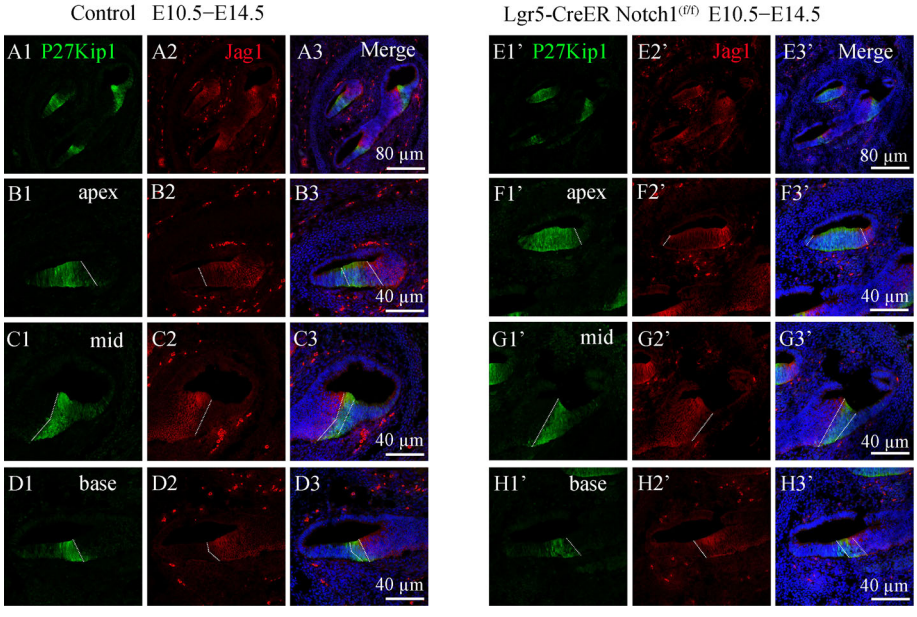

Fig. 2 Notch inhibition at E10.5 in Lgr5^{+} cells showed no evident effect on the expression pattern of P27kip1. (A–D) Typical immunofluorescence images of control cochleae at E14.5. (E–H) Typical immunofluorescence images of Lgr5$^{CreERT2/+}$;Notch1$^{flox/flox}$ cochleae at E14.5. Tamoxifen was administered at E10.5, and specimens were harvested at E14.5. Scale bars: (A, E) 80 μm; (B–D, F–H) 40 μm.

Notch1 deletion in Lgr5^{+} cells of the prosensory epithelium drove the quiescent progenitors to re-enter the cell cycle and give birth to new HCs

To explore the role of Notch inhibition in the cochlear prosensory epithelium, we injected tamoxifen into pregnant Lgr5$^{CreERT2/+}$;Notch1$^{flox/flox}$ mice at E14.5. EdU was injected once daily for the labeling of the proliferating cells, and embryos were harvested at E18.5. Lgr5$^{CreERT2/+}$ mice served as control. Myo7a and Sox2 were used as the markers of HCs and SCs, respectively. In the control group, one row of inner HCs and three rows of outer HCs were organized neatly in the organ of Corti at E18.5, and no EdU^{+}/Myo7a^{+} cells were observed (Fig. 3). In the Lgr5$^{CreERT2/+}$;Notch1$^{flox/flox}$ mice, several extra EdU^{+}/Myo7a^{+} and EdU^{+}/Myo7a^{+}/Sox2^{+} cells were observed in the pillar cell region, which is the region between the inner and outer HCs, of the cochlear apical and middle turn. Many EdU^{+}/Sox2^{+} cells were observed in the SC layer in Notch1-deficient cochleae (Fig. 3). In the control group, no EdU^{+}/Myo7a^{+} cells were found in the whole cochlear epithelium (n = 4). In the Lgr5$^{CreERT2/+}$;Notch1$^{flox/flox}$ group, the number of EdU^{+}/Myo7a^{+} cells was remarkably increased to 12.42 ± 3.11/cochlea (n = 5). These results indicated that Notch1 inhibition drove the Lgr5^{+} cells in the pillar cell region to re-enter the cell cycle, proliferate, and differentiate into new HCs.

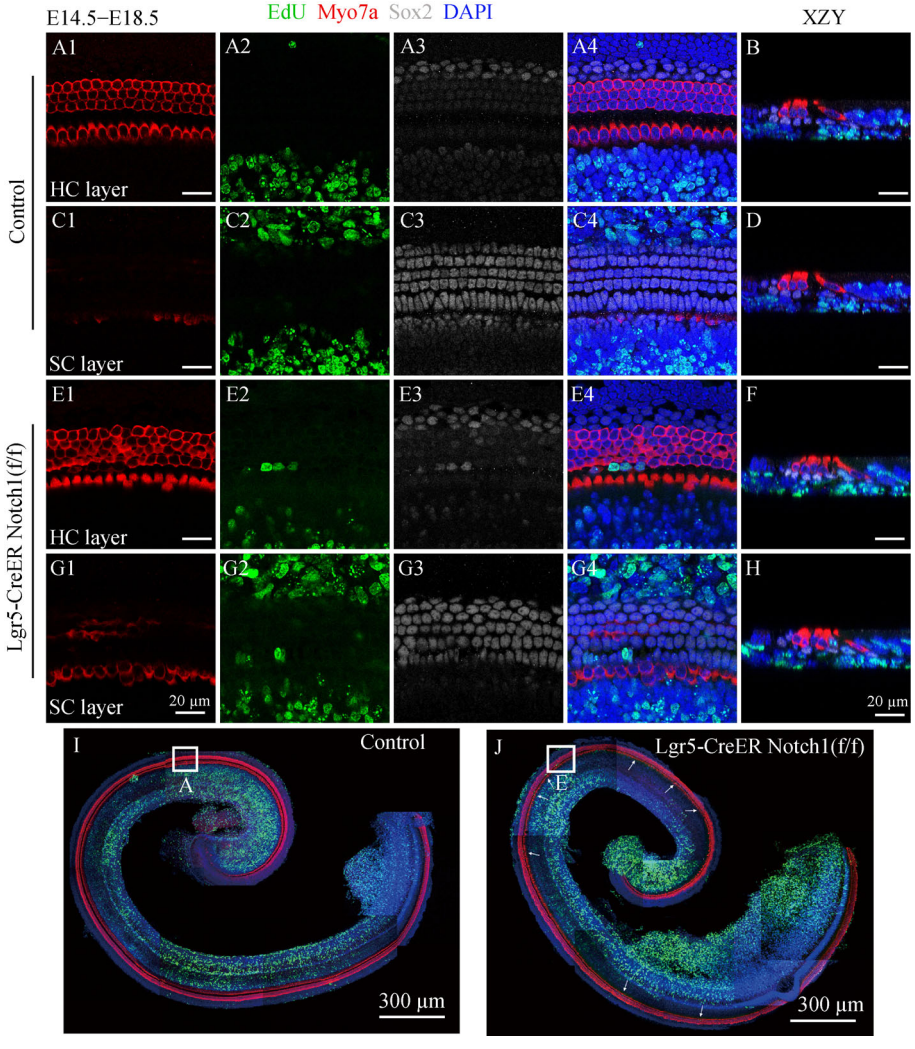

Fig. 3 Notch1 inhibition at E14.5 drove the Lgr5^{+} cells in the pillar cell region to re-enter the cell cycle and differentiate into new HCs. Tamoxifen was administered at E14.5, and specimens were harvested at E18.5. Typical XYZ (A, C, E, and G) and XZY (B, D, F, and H) images of the apical and middle turn in the cochlear epithelium from the control and Lgr5$^{CreERT2/+}$;Notch1$^{flox/flox}$ mice are shown. The typical images of whole cochleae in each group are shown in I and J. The white box indicated the position of A in I and E in J. The arrows indicated the extra new hair cells (HCs). HC layer: hair cell layer. SC layer: supporting cell layer. Scale bars: (A–H) 20 μm; (I, J) 300 μm.

The overexpression of Notch1 in Lgr5^{+} cells showed no evident effects on cochlear sensory epithelium development

To explore the role of Notch1 overexpression in Wnt-responsive Lgr5^{+} cells, we generated Lgr5$^{CreERT2/+}$; Notch1$^{OE/+}$ mice in which the constitutive overexpression of Notch1 in Lgr5^{+} progenitor cells was achieved by tamoxifen administration at E10.5 or E14.5, and embryos were harvested at E14.5 or E18.5. Lgr5$^{CreERT2/+}$ mice served as control. We found no differences between the two groups regarding the size of the cochlear sensory epithelium, the expression pattern of Sox2 and Jag1 at E14.5 (Fig. 4A and 4B), or the number of HCs and SCs at E18.5 (Fig. 4C and 4D). This result suggested that Notch1 overexpression exhibited no evident effect on cochlear prosensory epithelia formation or HC differentiation.

Discussion

Cochlear sensory epithelium formation during embryonic development in mammals requires the spatial and temporal orchestration of multiple signaling pathways. Notch and Wnt are two fundamental and conserved pathways that are active throughout the entire developmental process of the inner ear [4]. Notch signaling acts as a promoter for the determination of the prosensory domain through the "lateral induction" effect during the early development of the inner ear and is involved in the pattern formation of the specialized mosaic structure of the organ of Corti through the "lateral inhibition" effect during HC and SC differentiation [14,15,26,27]. Wnt/β-catenin signaling has been implicated in maintaining the pluripotency of progenitors during inner ear development, and Wnt-responsive Lgr5^{+} cells are progenitor cells in the inner ear that can be activated to proliferate and differentiate into HCs under

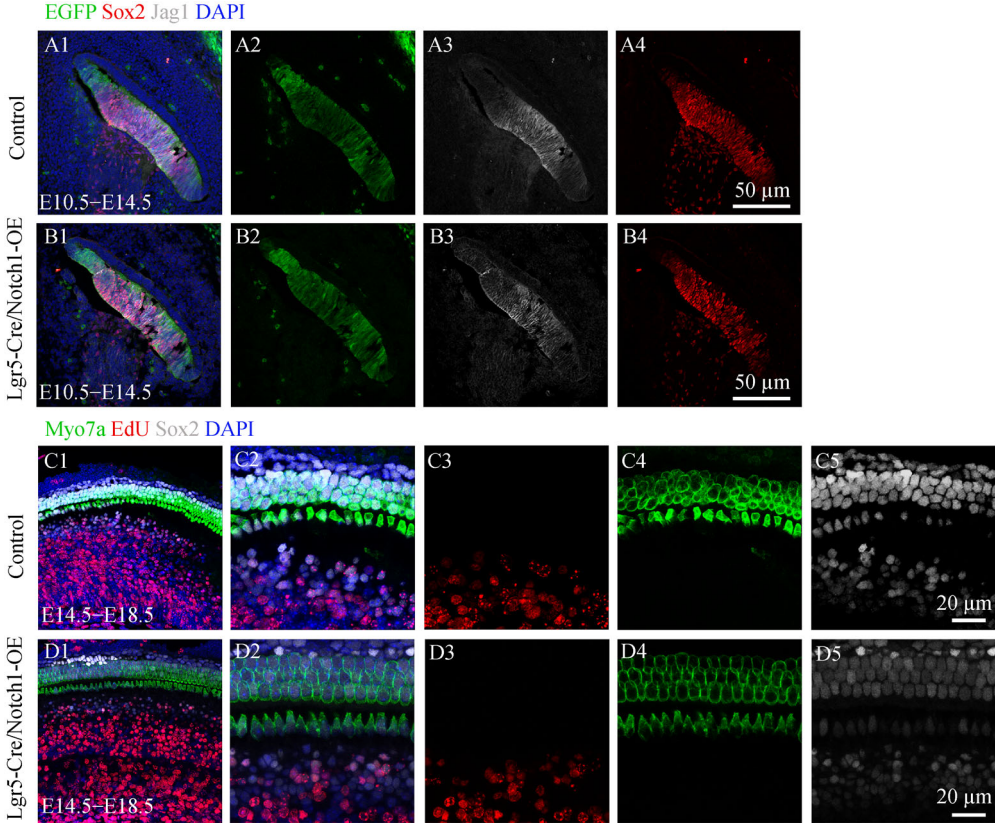

Fig. 4 Notch1 overexpression in embryonic Lgr5^{+} cells showed no evident effect on cochlear sensory epithelium formation or cell differentiation. (A, B) Typical immunofluorescence images of the apical turn in the cochlear epithelium from control and Lgr5$^{CreERT2/+}$; Notch1$^{OE/+}$ cochleae at E14.5. Tamoxifen was administered at E10.5, and specimens were harvested at E14.5. (C, D) Typical immunofluorescence images of the apical and middle turn in the cochlear epithelium from control cochleae and Lgr5$^{CreERT2/+}$; Notch1$^{OE/+}$ cochleae at E18.5. Tamoxifen was administered at E14.5, and specimens were harvested at E18.5. Scale bars: (A, B) 50 μm; (C, D) 20 μm.

certain conditions [6,11,12]. In our previous study, we showed that Notch signaling acts as a negative regulator of the proliferation of Wnt-responsive Lgr5$^+$ progenitors in the mouse cochlea during the neonatal period [24]. However, the interactions between these two complex pathways during inner ear development must be further dissected.

Notch signaling activation in all otic epithelial cells in the mouse inner ear induces ectopic prosensory region formation, and this event is followed by HC and SC differentiation [26–28]. Notch1 overexpression in the otic vesicle in Col2a1-Cre mice with a Tet-On/Cre induction system causes ectopic sensory HCs and SCs in the non-sensory regions of the cochlea [27], and Notch1 overexpression in the ectoderm in Pax2-cre mice causes otic placode expansion [14]. The lateral induction of the prosensory region and the ectopic HCs induced by the overexpression of Notch1 occur in an age-dependent manner, and no ectopic HCs are generated in the cochlea after E13.5 [26,29]. In the current study, we specifically regulated Notch signaling in Lgr5$^+$ cells at different developmental stages. According to the GFP staining of Lgr5$^+$ transgenic mice (Fig. 1), the Lgr5$^+$ cells were mainly located in the ventral part of the cochlear duct that generates the prosensory region of the cochlea at E10.5–E14.5. The overexpression of Notch1 in Lgr5$^+$ cells during this stage resulted in no considerable difference in the range of the prosensory region labeled either by Jag1 or by P27Kip1 when compared with controls, and no ectopic HCs were found in the prosensory region (Fig. 4). Furthermore, Notch signaling overexpression in Lgr5$^+$ cells at E14.5–E18.5 showed no effect on the differentiation of HCs and SCs. The lack of effect after the upregulation of Notch1 in Lgr5$^+$ cells may be due to endogenous activation and functional saturation of Notch signaling in the prosensory and sensory regions during inner ear development [16,30].

When we conditionally deleted Notch1 in Lgr5$^+$ cells starting from E10.5, the markers for the prosensory domain still appeared at the proper time and location at E14.5 (Fig. 2), and progenitor cell proliferation was not affected. This finding may be explained by gene redundancy in the Notch signaling pathway, and the orchestration of the lateral induction and inhibition of Notch signaling in the developing inner ear occurs in a ligand-dependent manner [31], and the visualization of Notch1 proteolysis showed that Notch1 is nearly undetectable during the lateral induction of the prosensory domain [32]. Our results further demonstrated that Notch1 does not play a core role in Lgr5$^+$ cells during the early mammalian inner ear development stages.

In a previous study, we showed that the deletion of Notch1 in Sox2$^+$ cells promotes extensive re-entry into the cell-cycle by Lgr5$^+$ progenitor cells in the neonatal mouse cochlea [24]. However, given that the transgenic mice used in our previous study cannot completely eliminate the increased cochlear regeneration through Sox2 haploinsufficiency effects, which was observed in a later study [33], we further investigated the effects of Notch1 deletion specifically in the Lgr5$^+$ cells during E14.5–E18.5 in the current study. Notch1 activity considerably increases during the lateral inhibition process during inner ear development [32]. As expected, we observed the proliferation of SCs, followed by the differentiation of HCs, in the cochlear sensory epithelium (Fig. 3), especially in the inner pillar cell region. However, Angelika *et al.* reported that Notch signaling is not necessary for the differentiation and maintenance of pillar cell fate, which are distinguished by Hey2 expression [19]; whereas the quantitative high-resolution cellular map of the organ of Corti showed that other Notch effector genes, *Hes5*, *Hey1*, and *Lfng*, are expressed in the inner and outer pillar cells [34], which suggests that the Notch signaling plays important roles in pillar cells. In current study, we found that the pillar cells displayed great regenerative potential and generated EdU$^+$/Myo7a$^+$ HCs after Notch1 deletion in Lgr5$^+$ cells (Fig. 3), suggesting that Notch signaling served as a negative regulator for the proliferation of Lgr5$^+$ progenitors in mouse cochlea development, which is consistent with the results in our previous paper [24].

We concluded that the roles of Notch1 in Wnt-responsive Lgr5$^+$ cells are unidirectional and stage dependent during mouse inner ear development. Specifically upregulating Notch signaling in Lgr5$^+$ cells exhibited no effect on the prosensory region determination or cell differentiation in the organ of Corti. Specifically downregulating Notch1 in Lgr5$^+$ cells during E14.5–E18.5 caused cell cycle re-entry and mitotic HC generation in the sensory region of the cochlea, which suggests that Notch signaling serves as a negative regulator in Lgr5$^+$ progenitor activation. No effect was observed from upregulation or downregulation of Notch1 in Lgr5$^+$ cells during E10.5–E14.5, which might be caused by the endogenous Notch signaling activation or gene redundancy in Notch signaling during cochlear prosensory domain determination. Thus, our findings improved the understanding of the interactions between Notch and Wnt signaling.

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Compliance with ethics guidelines

Hui Jiang, Shan Zeng, Wenli Ni, Yan Chen, and Wenyan Li declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed. This study was carried out in strict accordance with the Guiding Directive for Humane Treatment of Laboratory Animals' issued by the Chinese National Ministry of Science and Technology on September, 2006. All experiments were approved by the Shanghai Medical Experimental Animal Administrative Committee (Permit Number: 2009-0082). All efforts were made to minimize suffering and reduce the number of animals used.

References

1. Whitfield TT. Development of the inner ear. Curr Opin Genet Dev 2015; 32: 112–118
2. Chen P, Johnson JE, Zoghbi HY, Segil N. The role of Math1 in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. Development 2002; 129(10): 2495–2505
3. Lee YS, Liu F, Segil N. A morphogenetic wave of p27Kip1 transcription directs cell cycle exit during organ of Corti development. Development 2006; 133(15): 2817–2826
4. Zak M, Klis SF, Grolman W. The Wnt and Notch signalling pathways in the developing cochlea: formation of hair cells and induction of regenerative potential. Int J Dev Neurosci 2015; 47(Pt B): 247–258
5. Chai R, Kuo B, Wang T, Liaw EJ, Xia A, Jan TA, Liu Z, Taketo MM, Oghalai JS, Nusse R, Zuo J, Cheng AG. Wnt signaling induces proliferation of sensory precursors in the postnatal mouse cochlea. Proc Natl Acad Sci USA 2012; 109(21): 8167–8172
6. Shi F, Hu L, Edge AS. Generation of hair cells in neonatal mice by β-catenin overexpression in Lgr5-positive cochlear progenitors. Proc Natl Acad Sci USA 2013; 110(34): 13851–13856
7. Jacques BE, Puligilla C, Weichert RM, Ferrer-Vaquer A, Hadjantonakis AK, Kelley MW, Dabdoub A. A dual function for canonical Wnt/β-catenin signaling in the developing mammalian cochlea. Development 2012; 139(23): 4395–4404
8. Chai R, Xia A, Wang T, Jan TA, Hayashi T, Bermingham-McDonogh O, Cheng AG. Dynamic expression of Lgr5, a Wnt target gene, in the developing and mature mouse cochlea. J Assoc Res Otolaryngol 2011; 12(4): 455–469
9. Zhang Y, Chen Y, Ni W, Guo L, Lu X, Liu L, Li W, Sun S, Wang L, Li H. Dynamic expression of Lgr6 in the developing and mature mouse cochlea. Front Cell Neurosci 2015; 9: 165
10. Cox BC, Chai R, Lenoir A, Liu Z, Zhang L, Nguyen DH, Chalasani K, Steigelman KA, Fang J, Rubel EW, Cheng AG, Zuo J. Spontaneous hair cell regeneration in the neonatal mouse cochlea *in vivo*. Development 2014; 141(4): 816–829
11. Chai R, Kuo B, Wang T, Liaw EJ, Xia A, Jan TA, Liu Z, Taketo MM, Oghalai JS, Nusse R, Zuo J, Cheng AG. Wnt signaling induces proliferation of sensory precursors in the postnatal mouse cochlea. Proc Natl Acad Sci USA 2012; 109(21): 8167–8172
12. Shi F, Kempfle JS, Edge AS. Wnt-responsive Lgr5-expressing stem cells are hair cell progenitors in the cochlea. J Neurosci 2012; 32 (28): 9639–9648
13. Wang T, Chai R, Kim GS, Pham N, Jansson L, Nguyen DH, Kuo B, May LA, Zuo J, Cunningham LL, Cheng AG. Lgr5^{+} cells regenerate hair cells via proliferation and direct transdifferentiation in damaged neonatal mouse utricle. Nat Commun 2015; 6(1): 6613
14. Jayasena CS, Ohyama T, Segil N, Groves AK. Notch signaling augments the canonical Wnt pathway to specify the size of the otic placode. Development 2008; 135(13): 2251–2261
15. Zine A, Aubert A, Qiu J, Therianos S, Guillemot F, Kageyama R, de Ribaupierre F. Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. J Neurosci 2001; 21(13): 4712–4720
16. Lewis AK, Frantz GD, Carpenter DA, de Sauvage FJ, Gao WQ. Distinct expression patterns of notch family receptors and ligands during development of the mammalian inner ear. Mech Dev 1998; 78(1-2): 159–163
17. Kiernan AE, Xu J, Gridley T. The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. PLoS Genet 2006; 2(1): e4
18. Yamamoto N, Tanigaki K, Tsuji M, Yabe D, Ito J, Honjo T. Inhibition of Notch/RBP-J signaling induces hair cell formation in neonate mouse cochleas. J Mol Med (Berl) 2006; 84(1): 37–45
19. Doetzlhofer A, Basch ML, Ohyama T, Gessler M, Groves AK, Segil N. Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. Dev Cell 2009; 16(1): 58–69
20. Lin V, Golub JS, Nguyen TB, Hume CR, Oesterle EC, Stone JS. Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. J Neurosci 2011; 31(43): 15329–15339
21. Mizutari K, Fujioka M, Hosoya M, Bramhall N, Okano HJ, Okano H, Edge ASB. Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. Neuron 2013; 77(1): 58–69
22. Zheng JL, Shou J, Guillemot F, Kageyama R, Gao WQ. Hes1 is a negative regulator of inner ear hair cell differentiation. Development 2000; 127(21): 4551–4560
23. Daudet N, Gibson R, Shang J, Bernard A, Lewis J, Stone J. Notch regulation of progenitor cell behavior in quiescent and regenerating auditory epithelium of mature birds. Dev Biol 2009; 326(1): 86–100
24. Li W, Wu J, Yang J, Sun S, Chai R, Chen ZY, Li H. Notch inhibition induces mitotically generated hair cells in mammalian cochleae via activating the Wnt pathway. Proc Natl Acad Sci USA 2015; 112(1): 166–171
25. Ni W, Lin C, Guo L, Wu J, Chen Y, Chai R, Li W, Li H. Extensive supporting cell proliferation and mitotic hair cell generation by *in vivo* genetic reprogramming in the neonatal mouse cochlea. J Neurosci 2016; 36(33): 8734–8745
26. Hartman BH, Reh TA, Bermingham-McDonogh O. Notch signaling specifies prosensory domains via lateral induction in the developing mammalian inner ear. Proc Natl Acad Sci USA 2010; 107(36): 15792–15797
27. Pan W, Jin Y, Stanger B, Kiernan AE. Notch signaling is required for the generation of hair cells and supporting cells in the mammalian inner ear. Proc Natl Acad Sci USA 2010; 107(36):

15798–15803
28. Pan W, Jin Y, Chen J, Rottier RJ, Steel KP, Kiernan AE. Ectopic expression of activated notch or SOX2 reveals similar and unique roles in the development of the sensory cell progenitors in the mammalian inner ear. J Neurosci 2013; 33(41): 16146–16157
29. Liu Z, Owen T, Fang J, Zuo J. Overactivation of Notch1 signaling induces ectopic hair cells in the mouse inner ear in an age-dependent manner. PLoS One 2012; 7(3): e34123
30. Cheng C, Guo L, Lu L, Xu XC, Zhang SS, Gao JY, Waqas M, Zhu CW, Chen Y, Zhang XL, Xuan CY, Gao X, Tang ML, Chen FY, Shi HB, Li HW, Chai RJ. Characterization of the transcriptomes of Lgr5^{+} hair cell progenitors and Lgr5^{-} supporting cells in the mouse cochlea. Front Mol Neurosci 2017; 10: 122
31. Petrovic J, Formosa-Jordan P, Luna-Escalante JC, Abelló G, Ibañes M, Neves J, Giraldez F. Ligand-dependent Notch signaling strength orchestrates lateral induction and lateral inhibition in the developing inner ear. Development 2014; 141(11): 2313–2324
32. Liu Z, Liu Z, Walters BJ, Owen T, Kopan R, Zuo J. *In vivo* visualization of Notch1 proteolysis reveals the heterogeneity of Notch1 signaling activity in the mouse cochlea. PLoS One 2013; 8 (5): e64903
33. Atkinson PJ, Dong Y, Gu S, Liu W, Najarro EH, Udagawa T, Cheng AG. Sox2 haploinsufficiency primes regeneration and Wnt responsiveness in the mouse cochlea. J Clin Invest 2018; 128(4): 1641–1656
34. Waldhaus J, Durruthy-Durruthy R, Heller S. Quantitative high-resolution cellular map of the organ of Corti. Cell Reports 2015; 11 (9): 1385–1399