Development/Plasticity/Repair

In Vivo Cochlear Hair Cell Generation and Survival by Coactivation of β-Catenin and Atoh1

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The mammalian cochlea exhibit minimal spontaneous regeneration, and loss of sensory hair cells (HCs) results in permanent hearing loss. In nonmammalian vertebrates, spontaneous HC regeneration occurs through both proliferation and differentiation of surrounding supporting cells (SCs). HC regeneration in postnatal mammalian cochleae *in vivo* remains limited by the small HC number and subsequent death of regenerated HCs. Here, we describe *in vivo* generation of 10-fold more new HCs in the mouse cochlea than previously reported, most of which survive to adulthood. We achieved this by combining the expression of a constitutively active form of β-catenin (a canonical Wnt activator) with ectopic expression of Atoh1 (a HC fate determination factor) in neonatal Lgr5$^+$ cells (the presumed SC and HC progenitors of the postnatal mouse cochlea), and discovered synergistic increases in proliferation and differentiation. The new HCs were predominantly located near the endogenous inner HCs, expressed early HC differentiation markers, and were innervated despite incomplete alignment of presynaptic and postsynaptic markers. Surprisingly, genetic tracing revealed that only a subset of Lgr5$^+$ cells that lie medial to the inner HCs respond to this combination, highlighting a previously unknown heterogeneity that exists among Lgr5$^+$ cells. Together, our data indicate that β-catenin and Atoh1 mediate synergistic effects on both proliferation and differentiation of a subset of neonatal cochlear Lgr5$^+$ cells, thus overcoming major limitations of HC regeneration in postnatal mouse cochleae *in vivo*. These results provide a basis for combinatorial therapeutics for hearing restoration.

Key words: canonical Wnt; combinatory therapy; direct conversion; Lgr5 stem cell; mitotic generation; Notch

Significance Statement

Hearing loss in humans from aging, noise exposure, or ototoxic drugs (i.e., cisplatin or some antibiotics) is permanent and affects every segments of the population, worldwide. However, birds, frog, and fish have the ability to recover hearing, and recent studies have focused on understanding and applying what we have learned from them for restoring hearing in humans. However, studies have been hampered by low efficiency, limited cell numbers, and subsequent death of these newly generated auditory cells. Here, we describe a combinatorial approach, which results in the generation of auditory cells in greater numbers than previously reported, with most of them surviving to adult ages *in vivo*. These results provide a basis for combinatorial therapeutics for hearing restoration efforts.

Introduction

The mammalian hearing organ, the organ of Corti, is comprised of both mechanosensory hair cells (HCs) and nonsensory supporting cells (SCs), but the loss of HCs results in permanent hearing loss (Bohne et al., 1976; Hawkins et al., 1976; Kelley, 2007; Oesterle et al., 2008). In contrast, nonmammalian vertebrates are capable of HC regeneration by either direct SC to HC transdifferentiation or SC mitosis followed by SC to HC transdifferentiation (termed mitotic regeneration), and subsequently recover hearing function (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Stone and Cotanche, 2007; Rubel et al., 2013). Despite recent advances, mitotic regeneration in postnatal mammalian

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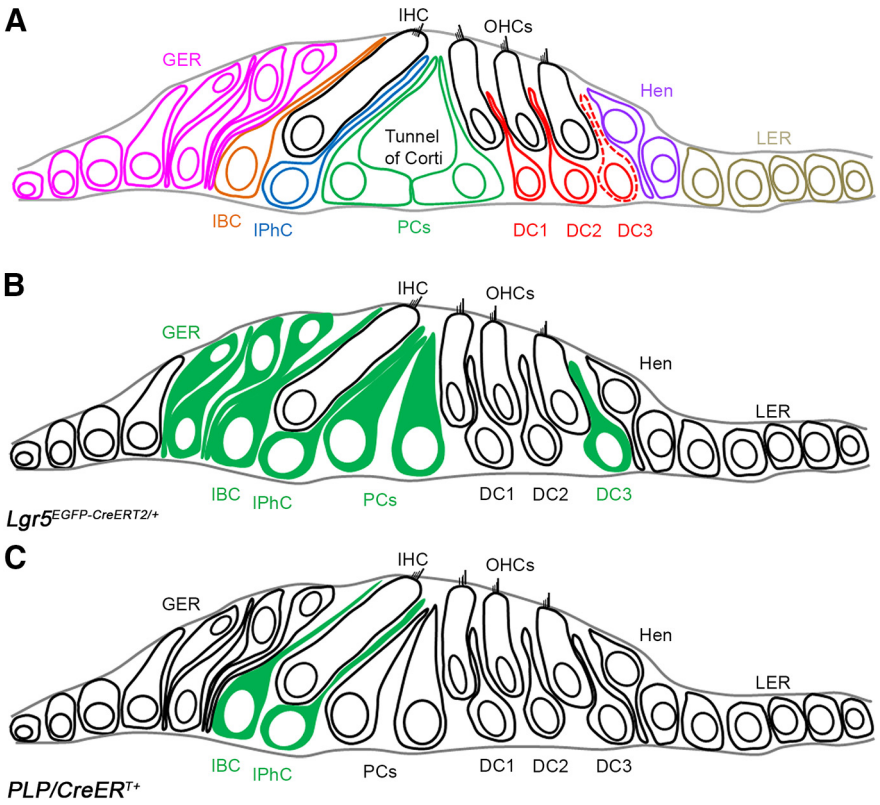

Figure 1. Diagram of the organ of the Corti and its SC subtypes. ***A***, Schematic cross-section of organ of Corti of an adult mouse cochlea depicting the positions of SCs and the nonsensory epithelial cell subtypes relative to the IHC and OHCs. Red dotted line indicates the third row of Deiters' cells, where Lgr5 expression remains throughout adulthood. ***B***, Schematic representation of Lgr5 $^{EGFP\text{-}CreERT2/+}$-targeted cells in green in neonatal cochlea. ***C***, Schematic representation of PLP/CreER $^{T+}$-targeted SCs in green in neonatal cochlea. OHCs, Outer hair cells; IHC, inner hair cell; GER, greater epithelial ridge; IBC, inner border cell; IPhC, inner phalangeal cell; PCs, pillar cells; DC1, first row of Deiters' cell; DC2, second row of Deiters' cell; DC3, third row of Deiters' cell; Hen, Hensen's cells; LER, lesser epithelial ridge.

cochleae *in vivo* remains limited by both insufficient cell number and survival.

The canonical Wnt signaling pathway, mediated by β-catenin, is critical for proper development and maturation of the cochlea (Dabdoub et al., 2003; Ohyama et al., 2007; Jayasena et al., 2008; Munnamalai and Fekete, 2013; Shi et al., 2014). In the chicken basilar papilla and the zebrafish lateral line, Wnt activation plays a key role in promoting SC proliferation in response to ototoxic insult and the subsequent generation of new HCs (Head et al., 2013; Jacques et al., 2014). In the mouse, *in vivo* studies have shown that the expression of an active form of β-catenin only results in a transient proliferation response in Lgr5 $^{+}$ cells using the *Lgr5CreER* mouse line, whereas new HCs are generated from Lgr5 $^{+}$ cells using the *Sox2CreER* mouse line (Chai et al., 2012; Shi et al., 2013). However, it is unclear whether *Sox2* haploinsufficiency contributes to the differential responses observed between the two aforementioned mice lines.

Lgr5 is characterized as a stem cell marker in the intestine and the hair follicle (Oshima et al., 2001; Barker et al., 2007). In the neonatal mammalian cochlea, Lgr5 is expressed in some nonsensory epithelial cells in the greater epithelial ridge (GER), the inner border cells (IBCs), the inner phalangeal cells (IPhCs), pillar cells, and the third row of Deiters' cells (DCs) (Fig. 1) (Chai et al., 2011; Shi et al., 2012). Isolated Lgr5 $^{+}$ cells from the neonatal organ of Corti are able to both proliferate and transdifferentiate into HCs *in vitro*, and recent reports have linked Lgr5 $^{+}$ cells to neonatal HC regeneration (Chai et al., 2012; Shi et al., 2012; Cox et al., 2014). These data suggest that Lgr5 $^{+}$ cells could represent progenitor-like cells in postnatal cochleae for regeneration.

The basic helix-loop-helix transcription factor, Atoh1, plays a key role in HC fate determination (Bermingham et al., 1999; Chen et al., 2002; Mulvaney and Dabdoub, 2012; Jarman and Groves, 2013; Jahan et al., 2015), and its ectopic expression in neonatal SCs induces SC to HC transdifferentiation. However, it is a slow and inefficient process, with most cells failing to transdifferentiate (<150 new HCs per cochlea) (Zheng and Gao, 2000; Kawamoto et al., 2003; Kelly et al., 2012; Liu et al., 2014).

To achieve both proliferation and transdifferentiation in postnatal cochleae, we ectopically coexpressed a constitutively active form of β-catenin and Atoh1 in neonatal cochlear Lgr5 $^{+}$ cells. We discovered that this combination acts synergistically on a subset of Lgr5 $^{+}$ cells by inducing both proliferation and differentiation, with the formation of 1338.48 ± 271.98 new HCs per cochlea, most of which survive to adulthood, thus illustrating an inherent heterogeneity that exists among the Lgr5 $^{+}$ cells. Together, our studies provide a foundation for future research using combinatorial therapeutics for mitotic regeneration in mammals for hearing restoration efforts.

Materials and Methods

Mice. *Lgr5* $^{EGFP\text{-}CreERT2/+}$ and *PLP/CreER* $^{T+}$ mice were purchased from the The Jackson Laboratory (stock #008875 and #005975). Conditional Lgr5 deletion (*Lgr5* $^{fl/+}$) mouse was obtained from Dr. Clevers (Hubrecht Institute, Utrecht, The Netherlands) and described previously (de Lau et al., 2011). *Hes5nLacZ* mouse was obtained from Dr. Kageyama and described previously (Imayoshi et al., 2010). *Atoh1-HA* mouse was described previously (Liu et al., 2012). Refer to the aforementioned references for details regarding the PCR genotyping. Tamoxifen (T5648-5G, Sigma; 3 mg/40 g body weight) was given by intraperitoneal injections at postnatal (P) day 0 (P0) and 1 (P1). Sample sizes were $N = 3$–7 mice of either sex for each group (control and experimental) at each time point for all experiments (except for P42-P44 where $N = 2$). All animal work conducted during the course of this study was approved by the Institutional Animal Care and Use Committee at St. Jude Children's Research Hospital and was performed according to National Institutes of Health guidelines.

Tissue preparation, immunofluorescence, and analysis. Isolated cochleae were fixed in 2% (v/v) PFA (Electron Microscopy Services) in PBS (P3813, Sigma) for 3–4 h at room temperature or at 4°C overnight, and subsequently washed in PBS (3 × 5 min). Whole-mount preparations were performed as described previously (Liu et al., 2010). The following primary antibodies were used: anti-myosin-VII (rabbit, 1:200, 25-6790, Proteus Biosciences), anti-HA (rat,1:100, 11867423001, Roche), anti-Ctbp2 (mouse,1:500, 612044, BD Transduction Laboratories), anti-GFP (chicken, 1:1000, ab13970, Abcam), anti-GluR2 (mouse, 1:200, MAB397, Millipore), anti-prestin (goat, 1:200, sc-22692, Santa Cruz Biotechnology), anti-Sox2 (goat, 1:1000, sc-17320, Santa Cruz Biotechnology), vGlut3 (rabbit, 1:500, 135203, Synaptic Systems), and anti-Tuj1

(mouse, 1:1000, MMS-435P, Covance). All secondary antibodies were purchased from Invitrogen and used at 1:1000, except for AlexaFluor-405 goat anti-rabbit IgG and AlexaFluor-647 goat anti-rat IgG (1:500). BrdU detection was achieved using AlexaFluor-647-conjugated antibody (mouse, 1:20, B35133, Invitrogen). Confocal imaging was performed on a Zeiss LSM 700 or 710, and image processing was performed with Photoshop CS6 (Adobe Systems).

Quantification of ectopic HCs. Cochleae were cut into two parts with the cut site close to the end of the first apical turn. The apical, middle, and base regions were first imaged <20× to identify regions of interest, and at least two *Z*-stacks from nonoverlapping regions were then obtained for each turn using the 40× objective (1 μm step). For each genotype and age, at least 3 cochleae from different mice were analyzed using Zeiss LSM image browser. We determined the number of ectopic HCs by scoring HA^+ and HA^+/Myosin7a$^+$ cells along the *z*-axis and determined the average number of cells per 300 μm section based on an average measured cochlear length of 5.4 mm (from P8 and P21 whole mounts).

RNA isolation and real-time PCR. A minimum of 3 littermates from at least 2 litters were assayed. Pups were killed by decapitation, and the ears were dissected in ice-cold HBSS. RNA was isolated using the RNAqueous-Micro RNA Isolation Kit (Ambion). cDNA was generated using Superscript First-Strand cDNA Synthesis system for RT-PCR (Invitrogen) with random primers. Relative expression levels were assayed using TaqMan Gene Expression Master Mix and TaqMan probes (Applied Biosystems) for *Notch1*, *Sox2*, *Jagged1*, *Delta-like 1*, and *18S*. Reactions were run in triplicate in an Eppendorf Realplex^2 Mastercycler System, which has a C_T cutoff value of 40. In our analysis, we set the upper most limit of C_T at 38 to be included in the analysis. Each sample was run in triplicate on each plate, and each sample was also run on multiple plates to ensure the validity of the results. The level of *18S* was used as an internal control and was run as a multiplex reaction with each assayed gene. The difference in C_T between the assayed gene and *18S* for any given sample was defined as $\Delta C_{T(X)}$. The difference in $\Delta C_{T(x)}$ between two samples was defined as $\Delta\Delta C_{T(X)}$, which represents a relative difference in expression of the assayed gene. The fold change of the assayed gene relative to *18S* was defined as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). DataAssist software (Applied Biosystems) was used for statistical analysis and to confirm $\Delta C_{T(X)}$ calculation.

Statistical analysis. Statistical analysis was performed using the GraphPad Prism 5.0 (Graphpad Software). Nonparametric Student's *t* test with Bonferroni correction or two-way ANOVA with Bonferroni correction (for auditory brainstem response [ABR] measurements) was used to determine statistical significance.

Results

β-Catenin and Atoh1 synergistically increase the proliferation of Lgr5$^+$ cells

Our group and others have previously shown that *in vivo* expression of a constitutively active form of β-catenin in neonatal cochlear Lgr5$^+$ cells results in a transient proliferative response with the formation of replication foci closely abutting the inner HCs (IHCs) (Chai et al., 2012; Shi et al., 2013). However, unlike cultured Lgr5$^+$ cells (Chai et al., 2012), this induction paradigm did not generate new HCs *in vivo*. Atoh1 is critical for normal HC differentiation (Bermingham et al., 1999; Chen et al., 2002; Dabdoub et al., 2003; Mulvaney and Dabdoub, 2012; Jarman and Groves, 2013; Jahan et al., 2015); and because its ectopic expression induces the transdifferentiation of SCs *in vivo*, we generated *Lgr5$^{EGFP\text{-}CreERT2/+}$;Catnb$^{flox(exon3)/+}$;CAG-loxP-Stop-loxP-Atoh1-HA* mice (thereafter refer to as *Lgr5CreER;β-catenin;HA+*) to induce the transdifferentiation of β-catenin-induced proliferating Lgr5$^+$ cells. The HA tag in the *Atoh1-HA* transgenic mice allowed for lineage tracing and provides a way to distinguish between ectopic and endogenous Atoh1 expression (Liu et al., 2012, 2014). We first assessed the effect of this combination on proliferation by performing a BrdU pulse-chase experiment. Tamoxifen induction was first performed at postnatal day 0 and 1 (P0-P1), followed by intraperitoneal injections of BrdU (4 injections 2 h apart) at P4. The resulting total of BrdU$^+$ cells from P4 samples showed a significant increase (statistical significance was assessed by Student's *t* test with Bonferroni correction) in *Lgr5CreER;β-catenin;HA*$^+$ mice compared with *Lgr5CreER;β-catenin* and *Lgr5CreER;HA*$^+$ mice (Tables 1, 2). Similarly, the number of HA^+/BrdU$^+$ cells was significantly higher in *Lgr5CreER;β-catenin;HA*$^+$ mice compared with *Lgr5CreER;HA*$^+$ mice (Tables 1, 2). Of note, the number of HA^-/BrdU$^+$ cells is unexpectedly higher compared with the number of HA^+/BrdU$^+$ cells, which suggests the potential of a non–cell-autonomous affect via the recruitment of Cre-negative cells to the foci without subsequent proliferation. However, given that all cells in the foci were GFP$^+$ (Cre$^+$) in our previous study (Chai et al., 2012), this scenario is unlikely and we attribute this lower than expected number of HA^+/BrdU$^+$ cells to the low sensitivity of the HA antibody instead. Nonetheless, we conclude that β-catenin and Atoh1 act synergistically on the proliferation of Lgr5$^+$ cells.

We next performed whole-mount analyses as described previously (Chai et al., 2012), with tamoxifen induction at P0-P1, and samples collected at P8, P15, and P21. The results show that replication foci (we use this term based on the results from the BrdU pulse-chase experiments) formed in *Lgr5CreER;β-catenin;HA*$^+$ and *Lgr5CreER;β-catenin* mice, but not in *Lgr5CreER;HA*$^+$ mice. The proliferation response was transient in both β-catenin ectopic expression models (the foci are no longer present at P21; Fig. 2*F*,*L*), but the replication foci were larger with poorly defined boundaries at P8 (Fig. 2*D*) and P15 (Fig. 2*H*) in *Lgr5CreER;β-catenin;HA*$^+$ mice compared with *Lgr5CreER;β-catenin* mice (Chai et al., 2012) (Fig. 2*B*,*F*). Nonetheless, these results further support that the combination of β-catenin and Atoh1 synergistically increases the proliferation response in *Lgr5CreER;β-catenin;HA*$^+$ mice, compared with *Lgr5CreER;β-catenin* mice.

Table 1. Synergistic effect of β-catenin and Atoh1 on Lgr5$^+$ cells' proliferation in the IHC region[a]

Genotype	No. of BrdU$^+$ cells/300 μm (P4)	Sample size (*n*)	
Lgr5CreER	ND	3	
Lgr5CreER; β-catenin$^+$	9.60 ± 2.09	7	*($p = 0.0148$; df = 9)
Lgr5CreER; HA$^+$	1.00 ± 0.54	4	
Lgr5CreER; β-catenin$^+$;HA$^+$	21.13 ± 4.04	6	**($p = 0.0041$; df = 8)

[a]Coexpression of β-catenin and Atoh1-HA in Lgr5$^+$ cells results in a synergistic proliferation response. Number of BrdU$^+$ cells per 300 μm section at P4 after 4 consecutive injections (2 h apart) of BrdU are shown with mean ± SEM. ND, Not detected. $N = 3$–7 for each time point and each genotype.

*$p < 0.017$ (unpaired Student's *t* test with Bonferroni correction).

**$p < 0.003$ (unpaired Student's *t* test with Bonferroni correction).

Table 2. Synergistic effect of β-catenin and Atoh1 on Lgr5$^+$ cells' proliferation in the IHC region[a]

Genotype	No. of HA^+/BrdU$^+$ cells/300 μm (P4)	Sample size (*n*)
Lgr5CreER	ND	3
Lgr5CreER; β-catenin$^+$	ND	3
Lgr5CreER; HA$^+$	0	3
Lgr5CreER; β-catenin+; HA$^+$	3.40 ± 0.59	5

[a]Coexpression of β-catenin and Atoh1-HA in Lgr5$^+$ cells results in a synergistic proliferation response. Number of HA^+/BrdU$^+$ cells per 300 μm section at P4 after 4 consecutive injections (2 h apart) of BrdU are shown with mean ± SEM. ND, Not detected. $N = 3$–7 for each time point and each genotype.

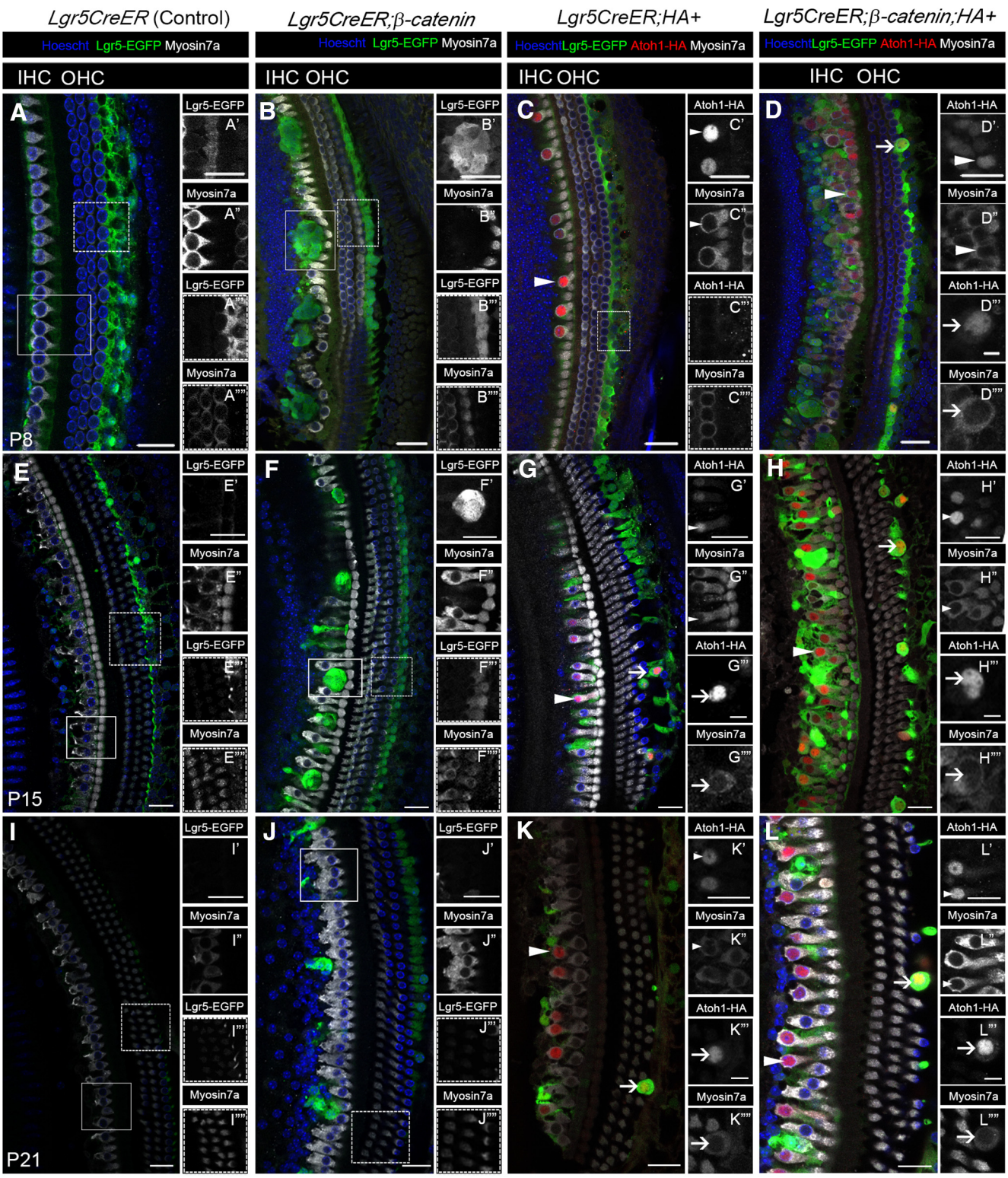

Figure 2. Lgr5 $^{+}$ cells differentiate into HCs in response to β-catenin and Atoh1. ***A–L***, Confocal images of cochleae from *Lgr5CreER* (Control; similar phenotype observed in *Lgr5CreER*-negative; *β-catenin;HA*$^{+}$) (***A***, ***E***, ***I***), *Lgr5CreER;β-catenin* (***B***, ***F***, ***J***), *Lgr5CreER;HA*$^{+}$ (***C***, ***G***, ***K***), and *Lgr5CreER;β-catenin;HA*$^{+}$ (***D***, ***H***, ***L***) mice that were induced with tamoxifen at P0-P1 and analyzed at P8 (***A–D***), P15 (***E–H***), and P21 (***I–L***). ***G***, ***L***, The optical sections do not show the entire third row of outer hair cells, although present. ***B***, Lgr5 $^{+}$ cells only transiently proliferate and form foci in response to ectopic β-catenin at P8. ***C***, Conversely, Lgr5 $^{+}$ cells differentiate into HCs (arrowheads) in response to Atoh1, but without apparent proliferation at P8. ***D***, Replication foci from concurrent β-catenin and Atoh1 expression in Lgr5 $^{+}$ cells appear less defined (compared with β-catenin expression alone, ***B***), suggesting an increased proliferation. In addition, Lgr5 $^{+}$ cells medial to the IHC and the third row of Deiters' cells lateral to the OHCs differentiate into HCs at P8 (***D***, arrowhead and arrow, respectively). The differentiation of the third row of Deiters' cells occurs at P8 only in the presence of β-catenin and Atoh1 (***D***, arrow). New HCs that formed medial to the IHC following Atoh1 alone or β-catenin and Atoh1 ectopic expression persist at P15 (***G***, ***H***, arrowhead, respectively) and P21 (***K***, ***L***, arrowhead, respectively). New HCs derived from the third row of Deiters' cells similarly persist at P15 (***G***, ***H***, arrow, respectively) and P21 (***K***, ***L***, arrows, respectively) following the same paradigm. ***L***, At P21, the ectopic HCs adjacent to the endogenous IHCs have now formed an additional row of HCs. Scale bars, 25 μm. Scale bars: ***D″′***, ***D″″***, ***G″′***, ***G″″***, ***H″′***, ***H″″***, ***K″′***, ***K″″***, ***L″′***, ***L″″***, 10 μm.

Table 3. Synergistic effect of β-catenin and Atoh1 on Lgr5$^+$ cells' differentiation in the IHC region[a]

Genotype	No. of HA + cells/300 μm (P8)	Sample size (*n*)	Genotype	No. of HA+/Myo7a + cells/300 μm (P8)	Sample size (*n*)
Lgr5CreER	ND	3	*Lgr5CreER*	ND	3
Lgr5CreER; β-catenin$^+$	ND	3	*Lgr5CreER;β-catenin$^+$*	ND	3
Lgr5CreER; HA$^+$	17.15 ± 3.65	4	*Lgr5CreER; HA$^+$*	3.70 ± 1.51	4
Lgr5CreER; β-catenin$^+$; HA$^+$	107.50 ± 17.68	7 *($p = 0.0045$; df = 9)	*Lgr5CreER; β-catenin$^+$; HA$^+$*	64.41 ± 11.49	7 **($p = 0.0036$; df = 9)

[a]β-Catenin and Atoh1-HA synergistically promote the differentiation of Lgr5$^+$ cells located near the IHC region. Cell counts per 300 μm section for the number of HA$^+$ cells and HA$^+$/Myosin7a$^+$ double-positive cells are shown with mean ± SEM. ND, Not detected. $N = 3$–7 for each time point and each genotype.

*$p < 0.017$ (unpaired Student's t test with Bonferroni correction).

**$p < 0.003$ (unpaired Student's t test with Bonferroni correction).

Table 4. Synergistic effect of β-catenin and Atoh1 on Lgr5$^+$ cells' differentiation in the IHC region[a]

Genotype	No. of HA$^+$ cells/300 μm	Sample size (*n*)	Genotype	No. of HA$^+$/Myo7a$^+$ cells/300 μm	Sample size (*n*)
P15					
Lgr5CreER	ND	3	*Lgr5CreER*	ND	3
Lgr5CreER; β-catenin$^+$	ND	3	*Lgr5CreER; β-catenin$^+$*	ND	3
Lgr5CreER; HA$^+$	27.15 ± 7.19	4	*Lgr5CreER; HA$^+$*	13.90 ± 3.72	4
Lgr5CreER; β-catenin$^+$; HA$^+$	103.30 ± 20.17	5 *($p = 0.0149$; df = 7)	*Lgr5CreER; β-catenin$^+$; HA$^+$*	74.36 ± 15.11	5 *($p = 0.0105$; df = 7)
P21					
Lgr5CreER	ND	3	*Lgr5CreER*	ND	3
Lgr5CreER; β-catenin$^+$	ND	3	*Lgr5CreER; β-catenin$^+$*	ND	3
Lgr5CreER; HA$^+$	14.25 ± 3.79	4	*Lgr5CreER; HA$^+$*	5.43 ± 1.58	4
Lgr5CreER; β-catenin$^+$; HA$^+$	48.44 ± 10.30	5 *($p = 0.0259$; df = 7)	*Lgr5CreER; β-catenin$^+$; HA$^+$*	31.30 ± 4.61	4 **($p = 0.002$; df = 7)

[a]β-Catenin and Atoh1-HA synergistically promote the differentiation of Lgr5$^+$ cells located near the IHC region. Cell counts per 300 μm section for the number of HA$^+$ cells and HA$^+$/Myosin7a$^+$ double-positive cells are shown with mean ± SEM. ND, Not detected. $N = 3$–7 for each time point and each genotype.

*$p < 0.017$ (unpaired Student's t test with Bonferroni correction).

**$p < 0.003$ (unpaired Student's t test with Bonferroni correction).

Lgr5$^+$ cells expressing β-catenin and Atoh1 give rise to new HCs

Because the proliferation response in *Lgr5CreER;β-catenin;HA$^+$* mice is increased, we next determined whether the coexpression of β-catenin and Atoh1 resulted in the transdifferentiation of proliferating Lgr5$^+$ cells into HCs, using the Atoh1-HA-tag for lineage-tracing. At P8, new HCs expressing both the HA tag and Myosin7a (thereafter referred to as HA$^+$/Myo7a$^+$ HCs) were present in the IHC region along the cochleae of *Lgr5CreER;HA$^+$* mice (Fig. 2*C*, arrowhead), whereas new HCs in both the IHC and outer HC (OHC) regions along the cochleae (Fig. 2*D*, arrowheads and arrow, respectively) were present in *Lgr5CreER;β-catenin;HA$^+$* mice. The HA$^+$/Myo7a$^+$ HCs were still present in the IHC and OHC regions at P15 (Fig. 2*G*,*H*) and P21 (Fig. 2*K*,*L*). There was no significant difference in new HC numbers between cochlear turns (Student's *t* test with Bonferroni correction).

Because no new HCs formed in *Lgr5CreER;β-catenin* mice (Chai et al., 2012; Shi et al., 2013), we counted the numbers of HA$^+$ and HA$^+$/Myo7a$^+$ cells in *Lgr5CreER;HA$^+$* and *Lgr5CreER;β-catenin;HA$^+$* mice (Tables 3, 4). In the IHC region, the numbers of HA$^+$ and HA$^+$/Myo7a$^+$ cells per 300 μm section were significantly higher (statistical significance was assessed by Student's *t* test with Bonferroni correction) in *Lgr5CreER;β-catenin;HA$^+$* mice compared with *Lgr5CreER;HA$^+$* mice (Tables 3, 4). The total number of new HCs (HA$^+$/Myo7a$^+$) in the medial IHC region was as high as 1338.48 ± 271.98 cells per cochlea in *Lgr5CreER;β-catenin;HA$^+$* mice at P15 (e.g., 74.36 ± 15.11 cells/300 μm section; average measured cochlear length of 5.4 mm), and a significant number of the new HCs were also present at P21 (i.e., 563.4 ± 82.98 cells per cochlea). Given that no new HCs formed in the presence of ectopic β-catenin alone, our data suggest a potential synergistic effect between β-catenin and Atoh1-HA on the transdifferentiation of Lgr5$^+$ cells in the IHC region. It is of interest to note that the total numbers of Atoh1-HA$^+$ cells in Lgr5$^+$ cells are more than those we reported previously in *PLP/CreER^{T+}; Atoh1-HA$^+$* mice also induced at P0-P1 (Liu et al., 2014). This difference is most likely because *Lgr5* is expressed in more nonsensory epithelial cells (GER, IBCs, and IPhCs) than in the *PLP/CreER^{T+}* model (IPhCs and IBCs only) (Fig. 1).

Because *Lgr5CreER;β-catenin;HA$^+$* mice have severely reduced body size and a high mortality rate (~80%), which is likely due to detrimental effects on the intestinal lining, this greatly limited our long-term studies. Nonetheless, we managed to analyze two mice that survived to P42-P44 and observed an average of 28.60 ± 1.00 HA$^+$/Myo7a$^+$ new HCs per 300 μm cochlear section or 514.80 ± 18.00 new HCs per cochlea at P42-P44 (Fig. 3*J*,*K*), indicating that the new HCs that were observed at P21 survived up to those ages. Furthermore, HA$^+$/Myo7a$^+$/BrdU$^+$ cells from animals that received BrdU at P4 (immunoprecipitation injections, 4 injections 2 h apart) were also present at P21 (18 ± 10 cells per 300 μm section) (Fig. 3*L*, arrowhead), suggesting that they were derived from proliferating and transdifferentiating Lgr5$^+$ cells that coexpress β-catenin and Atoh1-HA.

We analyzed the effect of β-catenin and Atoh1-HA on the transdifferentiation of the third row of Deiters' cells (Fig. 1*A*, dotted lined, *B*, SC-labeled DC3). We observed the formation of new HCs by P8 (Fig. 2*D*, arrow), which is in contrast to the *Prox1CreER^{T2+};Atoh1-HA* mouse line reported previously, where the transdifferentiation of similar SCs did not occur until P22 (Liu et al., 2012). HA$^+$ and HA$^+$/Myo7a cell counts (Table 5) showed no significant differences (statistical significance was assessed by Student's *t* test with Bonferroni correction) between *Lgr5CreER;β-catenin;HA$^+$* and *Lgr5CreER;HA$^+$* mice. We conclude that, although the ectopic expression of

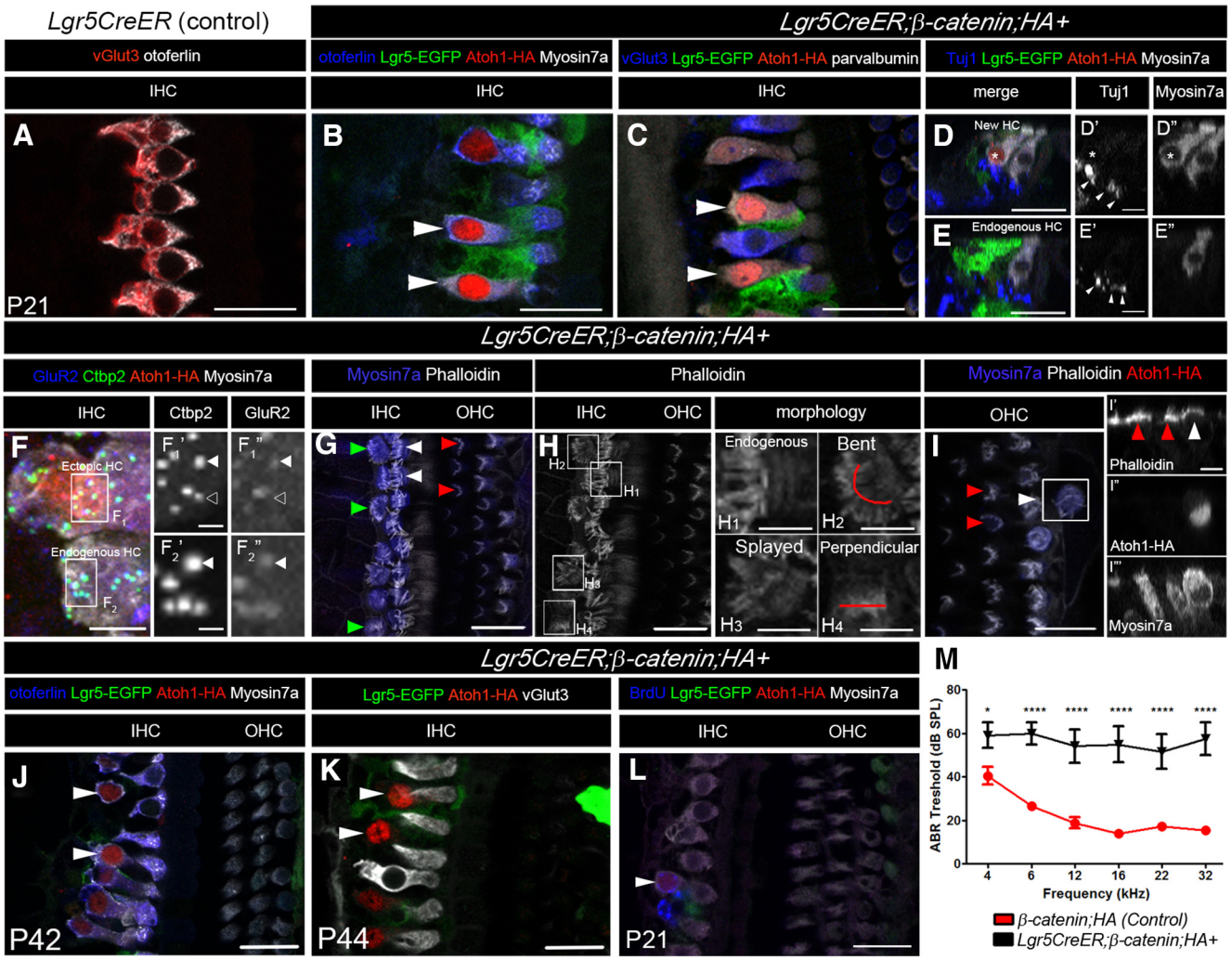

Figure 3. Lgr5$^+$ cells derived new HCs remain immature. ***A–L***, Confocal images of immunostaining with multiple markers in cochleae from *Lgr5CreER* (Control) (***A***) and *Lgr5CreER;β-catenin;HA*$^+$ (***B–I***) mice that were induced with tamoxifen at P0-P1 and analyzed at P21. ***A***, IHCs express otoferlin and vGlut3 in control mice. New HCs (HA$^+$) adjacent to the IHCs express Myosin7a (arrowheads in ***B*** and asterisk in ***D″***), otoferlin (***B***, arrowheads), parvalbumin (***C***, arrowheads), but not the terminal IHC marker vGlut3 (***C***, arrowheads). In the new HCs, Tuj1 projections were present on the basolateral side of the ectopic HCs (***D*** and arrowheads in ***D′***). ***D–D″***, Asterisk indicates the ectopic HC. In the endogenous IHCs, the synaptic markers Ctbp2 and GluR2 are both expressed in a matching pattern (arrowheads in ***F2′*** and ***F2″***, respectively). In the new HCs, some GluR2 puncta overlap with Ctbp2 puncta (arrowheads in ***F1′*** and ***F1″***, respectively), whereas others do not (open arrowhead in ***F1′*** and ***F1″***, respectively). ***G***, The hair bundles form in the new HCs abutting the IHCs (green arrowheads in ***G***), but their morphology differs from that of the endogenous IHCs (white arrowheads in ***G***) and OHCs (red arrowheads in ***G***). ***H***, Phalloidin staining of the IHC region from ***G***. Relative to the endogenous HCs (***H$_1$***), the medially located new HCs display bent (***H$_2$***), splayed (***H$_3$***), or perpendicular morphology (***H$_4$***). ***I***, The ectopic HCs located in the OHC region (white arrowhead in ***I*** and ***I′***) also display a peculiar hair cell bundle morphology, which does not resemble that of the traditional "V" shape found in normal endogenous OHCs (red arrowheads in ***I***). ***I′–I‴***, Cross-sectional view in which there is accumulation of actin at the cuticular plate in the endogenous (red arrowheads in ***I′***) and ectopic HCs (white arrowhead in ***I′***). ***J***, ***K***, New HCs are still present at P42-P44 (514.8 ± 18 per cochlea). Immunostaining of otoferlin (blue), Lgr5-EGFP (green), Atoh1-HA (red), Myosin7a (white in ***J***), and vGlut3 (white in ***K***) in the cochlea of a *Lgr5CreER;β-catenin;HA*$^+$ mouse at P42-P44. ***L***, New HCs generated from β-catenin and Atoh1 with BrdU pulse at P4 is still present at P21. ***M***, ABR thresholds (± SEM) from P21 animals. Scale bars, 25 μm. Scale bars: ***D′***, ***D″***, ***E′***, ***E″***, ***H$_1$–H$_4$***, ***I′–I‴***, 10 μm; ***F***, 5 μm; ***F$_1$′***, ***F$_2$″***, 2 μm. *$p < 0.05$ (two-way ANOVA with Bonferroni correction). ***$p < 0.0001$ (two-way ANOVA with Bonferroni correction). Refer to text for actual *p* values. $N = 9$ (control: *β-catenin;HA*$^+$) and $N = 6$ (*Lgr5CreER; β-catenin;HA*$^+$).

β-catenin and Atoh1-HA accelerated the transdifferentiation of the third Deiters's cells, it had little to no effect on proliferation or conversion efficiency of these cells.

Lgr5-derived HCs from β-catenin and Atoh1-HA ectopic expression remain immature

To address the level of maturation of the new HCs, we analyzed the expression of various HC markers and synaptic components at P21 in *Lgr5CreER;β-catenin;HA*$^+$ mice. The new HCs express the following HC markers: otoferlin (Fig. 3*B*, arrowheads), Myosin7a (Fig. 3*B*, arrowheads, *D″*, asterisk) and parvalbumin (Fig. 3*C*, arrowheads), but do not express the mature/terminal IHC marker, vGlut3 (Fig. 3*C*, arrowheads) or the mature/terminal OHC marker, prestin, in either the IHC or OHC regions, while retaining a 2.09 ± 0.38-fold increase in *Sox2* expression compared with control littermates ($p = 3.04$ E-09; df = 46;$n = 4$ using a Student's *t* test with Bonferroni correction) (see Fig. 5*H*). The persistence of Sox2 is consistent with a previous study using the Atoh1-HA mouse line (Liu et al., 2012, 2014). The new HCs remained immature even at P44 (Fig. 3*K*).

The new HCs closely abutting the endogenous IHCs were innervated as shown by the presence of Tuj1$^+$ projection on their basolateral side (Fig. 3*D*,*D′*, arrowheads, where the asterisk denotes the new HC), and also expressed the presynaptic ribbon component, C-terminal binding protein 2 (Ctbp2) (Fig. 3*F$_1$′*, arrowheads), as well as the postsynaptic marker glutamate receptor type 2 (GluR2) (Fig. 3*F$_1$″*, arrowheads). However, there was some incomplete overlap/match of Ctbp2 and GluR2 pu-

Table 5. Limited synergistic effect from β-catenin and Atoh1 on the third row of Deiters' Lgr5 $^+$ cells[a]

Genotype	No. of HA $^+$ cells/300 μm	Sample size (*n*)	Genotype	No. of HA $^+$/Myo7a $^+$ cells/300 μm	Sample size (*n*)
P8					
Lgr5CreER	ND	3	*Lgr5CreER*	ND	3
Lgr5CreER; β-catenin $^+$	ND	3	*Lgr5CreER; β-catenin* $^+$	ND	3
Lgr5CreER; HA $^+$	13.15 ± 2.38	4	*Lgr5CreER; HA* $^+$	0	4
Lgr5CreER; β-catenin $^+$*; HA* $^+$	21.33 ± 0.39	7 (NS, $p = 0.100$; df = 9)	*Lgr5CreER; β-catenin* $^+$*; HA* $^+$	0.74 ± 0.30	7 (NA, p = NA; df = NA)
P15					
Lgr5CreER	ND	3	*Lgr5CreER*	ND	3
Lgr5CreER; β-catenin $^+$	ND	3	*Lgr5CreER; β-catenin* $^+$	ND	3
Lgr5CreER; HA $^+$	14.15 ± 1.27	4	*Lgr5CreER; HA* $^+$	2.40 ± 0.52	4
Lgr5CreER; β-catenin $^+$*; HA* $^+$	29.69 ± 4.18	5 *($p = 0.0156$; df = 7)	*Lgr5CreER; β-catenin* $^+$*; HA* $^+$	10.90 ± 3.17	5 (NS, $p = 0.0513$; df = 7)
P21					
Lgr5CreER	ND	3	*Lgr5CreER*	ND	3
Lgr5CreER; β-catenin $^+$	ND	3	*Lgr5CreER; β-catenin* $^+$	ND	3
Lgr5CreER; HA $^+$	11.95 ± 2.18	4	*Lgr5CreER; HA* $^+$	2.95 ± 0.17	4
Lgr5CreER; β-catenin $^+$*; HA* $^+$	12.34 ± 3.71	5 (NS, $p = 0.9351$; df = 7)	*Lgr5CreER; β-catenin* $^+$*; HA* $^+$	3.84 ± 1.08	4 (NS, $p = 0.4946$; df = 7)

[a]Limited synergistic effect was observed on the number of HA $^+$ cells and the number of HA $^+$/myo7a $^+$ cells, except for the number of HA $^+$/myo7a $^+$ at P15. ND, Not detected; NS, not significant. $N = 3$–7 for each time point and each genotype. Data are mean ± SEM.

*$p < 0.017$ (unpaired *t* test with Bonferroni correction).

ncta (Fig. 3F_1',F_1'', open arrowheads, for Ctbp2 and GluR2, respectively).

Stereocilia were present at the apical surface of the new HCs located in the IHC region (Fig. 3*G*, green arrowheads), but their morphology differed from that of endogenous IHCs and OHCs (Fig. 3*G*, white and red arrowheads, respectively). We observed 3 types of stereocilia morphology relative to the endogenous HCs (Fig. 3H_1): bent (Fig. 3H_2), splayed (Fig. 3H_3), and perpendicular (Fig. 3H_4). Finally, the new HCs derived from the third row of Deiters' cells formed stereocillia as reflected by the accumulation of actin at the cuticular plate (Fig. 3*I* and white arrowhead for the new HCs vs red arrowheads for the endogenous HCs in Fig. 3*I*,*I'*). Auditory brainstem responses (ABR) measurements at P21 from *Lgr5CreER;β-catenin;HA*$^+$ mice show significant higher hearing thresholds than littermate control mice (two-way ANOVA: 4 kHz: $p = 0.293$,df = 78; 6, 12, 16, 22, 32 kHz: $p < 0.0001$,df = 78) (Fig. 3*M*). This is consistent with our previous studies where the addition of immature new HCs resulted in elevated hearing thresholds (Liu et al., 2014). Together, these data indicate that the new HCs express some HC markers and synaptic components, are innervated, and contain stereocilia bundles. However, these cells remain immature and their presence results in elevated ABR thresholds.

Only a subset of Lgr5 $^+$ cells proliferate and differentiate in response to β-catenin and Atoh1 expression

To assess the potential heterogeneity among Lgr5 $^+$ cell populations, we used another independent inducible Cre line, *PLP/CreER*$^{T+}$ at P0-P1 (Gómez-Casati et al., 2010; Liu et al., 2014). *PLP/CreER*$^{T+}$ targets the two SC subtypes directly abutting the IHCs, the IPhCs and IBCs (Fig. 1*C*), whereas *Lgr5CreER* targets a broader range of nonsensory epithelial cell subtypes (Fig. 1*B*) but includes the IPhCs and IBCs (Fig. 1*B*). Unlike the *Lgr5CreER;β-catenin* mice, we did not observe the formation of replication foci at P8 or P21 in any of the 3 genotypes generated: *PLP/CreER*$^{T/+}$*;β-catenin* (Fig. 4*B*,*F*), *PLP/CreER*$^{T+}$*;CAG-loxP-Stop-loxP-Atoh1-HA* (thereafter referred to as *PLP/CreER*$^{T/+}$*;HA*$^+$; Fig. 4*C*,*G*), and *PLP/CreER*$^{T+}$*;Catnb*$^{flox(exon3)/+}$*;CAG-loxP-Stop-loxP-Atoh1-HA* (thereafter referred to as *PLP/CreER*$^{T/+}$*;β-catenin;HA*$^+$; Fig. 4*D*,*H*). As expected, and based on previously published results (Liu et al., 2014), new HCs (HA $^+$/Myo7a $^+$) formed in the *PLP/CreER*$^{T/+}$*;HA*$^+$ (Fig. 4*C*,*G*, arrowhead, at P8 and P21, respectively) and in the *PLP/CreER*$^{T/+}$*;β-catenin;HA*$^+$ mice at P8 and P21 (Fig. 4*D*,*H*, arrowhead, at P8 and P21, respectively). HA $^+$ and HA $^+$/Myo7a $^+$ cell counts showed no significant differences (statistical significance was assessed by Student's *t* test with Bonferroni correction) between *PLP/CreER*$^{T/+}$*;β-catenin;HA* $^+$ and *PLP/CreER*$^{T/+}$*;HA*$^+$ mice (Table 6). Because this line activates Atoh1-HA equally well in *PLP/CreER*$^{T/+}$*;β-catenin;HA* $^+$ and *PLP/CreER*$^{T/+}$*;HA*$^+$ mice, as reflected by the formation of new HCs, it is unlikely that low Cre efficiency underlies the lack of proliferation. Additionally, we were able to confirm the *PLP/CreER*$^{T/+}$-mediated deletion of exon 3 in the β-catenin allele with the successful detection of the WT and the exon3-deleted β-catenin alleles in cochleae-extracted DNA by PCR from *PLP/CreER*$^{T/+}$*;β-catenin* mice (Fig. 4*I*, WT and Δex3) as described by Harada et al. (1999). It may be of interest to note that we did observe differences in the number of ectopic HA $^+$/Myo7a $^+$ cells in *PLP/CreER*$^{T/+}$*;HA*$^+$ compared with a previous study (Liu et al., 2014), and these differences are likely due to variation between the mixed background strains used for the different studies or differences in sample processing for fixation and HA antibody labeling.

We next asked whether Lgr5 haploinsufficiency could contribute to the absence of proliferation in the *PLP/CreER*$^{T/+}$*;β-catenin* and *PLP/CreER*$^{T/+}$*;β-catenin;HA*$^+$ mice. To address this, we bred the *Lgr5*$^{fl/+}$ mouse (de Lau et al., 2011) for the conditional deletion of one *Lgr5* allele in *PLP/CreER*$^{T/+}$*; β-catenin; Lgr5* $^{fl/+}$ mice. In these mice, we were unable to detect a proliferative response as reflected by the lack of IHC displacement at P8 (Fig. 4*K*,*L*), which we typically observed in the *Lgr5CreER;β-catenin* mice (Fig. 2). Therefore, this indicates that Lgr5 haploinsufficiency does not have a significant role in preventing Wnt mediated mitogenic effect on the IBCs/IPhCs, but rather that the IBCs/IPhCs are unresponsive to ectopic Wnt signaling. The lack of a GER-specific CreER mouse line further prevented us from testing the impact of Lgr5 haploinsufficiency on the proliferative response in these cells. Together, these data indicate that the Lgr5 $^+$, but PLP/CreER-negative nonsensory epithelial cells located medially to the IBCs or IPhCs in the GER region, are responsible for the synergistic proliferation and differentiation responses mediated by ectopic β-catenin and Atoh1 expression.

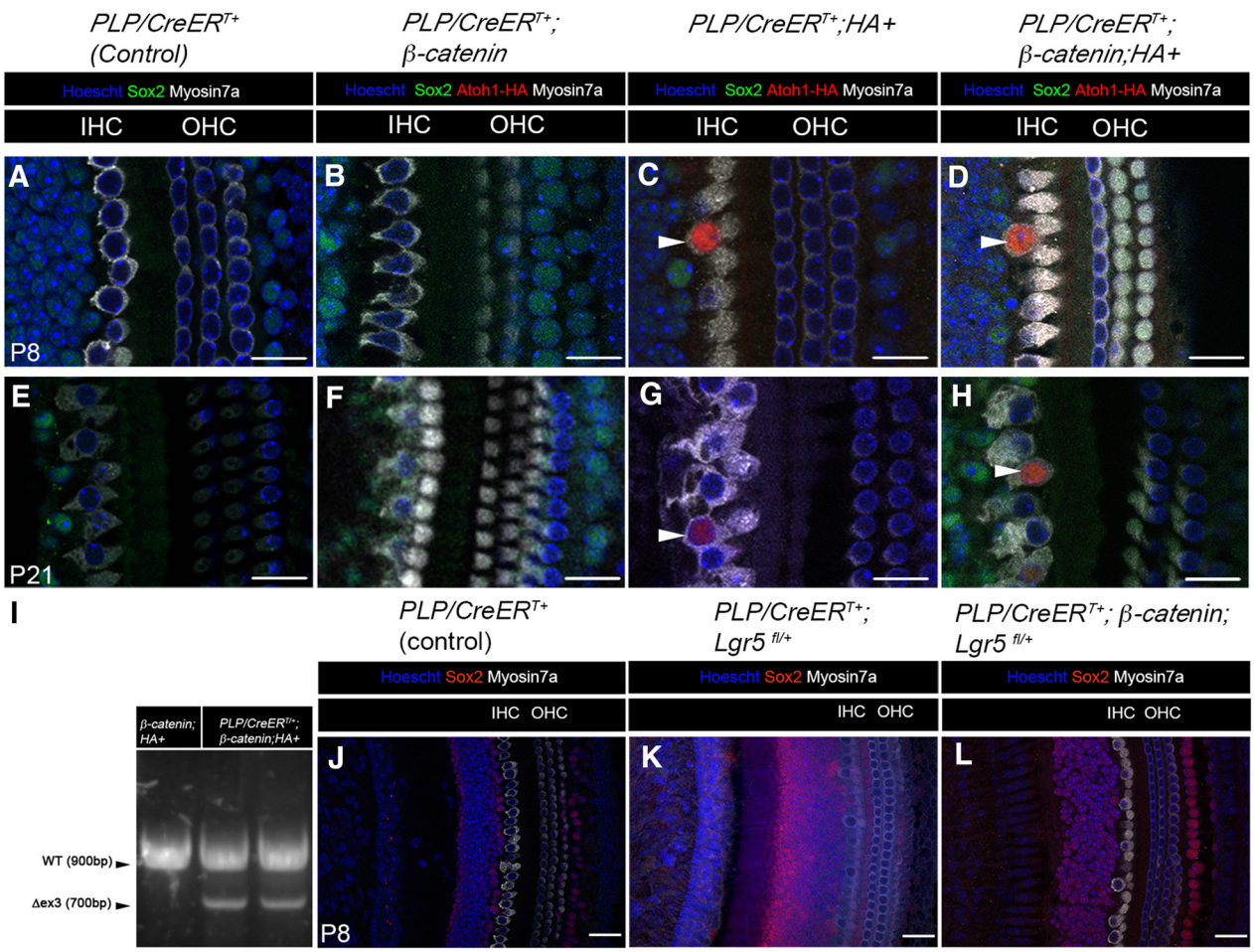

Figure 4. Lack of synergistic effects on proliferation and differentiation on the IPhCs/IBCs. ***A–H***, Confocal images of immunostained cochleae from *PLP/CreER*$^{T+}$ (control) (***A***, ***E***), *PLP/CreER*$^{T+}$;*β-catenin* (***B***, ***F***), *PLP/CreER*$^{T+}$;*HA*$^{+}$ (***C***, ***G***), and *PLP/CreER*$^{T+}$;*β-catenin;HA*$^{+}$ (***D***, ***H***) mice induced with tamoxifen at P0-P1 and analyzed at P8 (***A–D***) and P21 (***E–H***). There is no proliferative response at P8 (***B***) or P21 (***F***) in *PLP/CreER*$^{T+}$;*β-catenin* mice. However, IPhCs/IBCs still transdifferentiate into HCs in the presence of Atoh1-HA at P8 (***C***, arrowhead) and persist at least until P21 (***G***, arrowhead). New HCs (HA^{+}/Myo^{+}) form at P8 (***D***, arrowhead) and persist at least until P21 (***H***, arrowhead) in *PLP/CreER*$^{T+}$;*β-catenin;HA*$^{+}$ mice. ***I***, Both wild-type (WT; 900 bp) and mutant β-catenin alleles (Δex3; 700bp) were detected by PCR from the cochleae of *PLP/CreER*$^{T/+}$;*β-catenin; HA*$^{+}$ mice, whereas only the wild-type (WT) allele is detected in *β-catenin; HA*$^{+}$ mice at P4. To address whether *Lgr5* haploinsufficiency plays a role in the lack of proliferative response of the IBCs and IPhCs, we analyzed *PLP/CreER*$^{T+}$;*β-catenin;Lgr5*$^{fl/+}$ mice bearing one conditionally deleted Lgr5 allele to replicate the *Lgr5*$^{EGFP\text{-}CreERT2/+}$ knock-in mice. Following tamoxifen induction at P0-P1, no replication foci were observed, and the IHC layer shows no endogenous HC displacement at P8 in the *PLP/CreER*$^{T+}$ (***J***; control), *PLP/CreER*$^{T+}$;*Lgr5*$^{fl/+}$ (***K***), or the *PLP/CreER*$^{T+}$;*β-catenin;Lgr5*$^{fl/+}$ (***L***) mice. Scale bars, 25 μm.

Table 6. Lack of synergistic effects on proliferation and differentiation of IPhCs/IBCs[a]

Genotype	No. of HA^{+} cells/300 μm	Sample size (*n*)	Genotype	No. of HA^{+}/Myo7a^{+} cells/300 μm	Sample size (*n*)
P8					
PLP/CreERT/$^{+}$;	ND	3	*PLP/CreERT/*$^{+}$;	ND	3
PLP/CreERT/$^{+}$; *β-catenin*	ND	3	*PLP/CreERT/*$^{+}$; *β-catenin*	ND	3
PLP/CreERT/$^{+}$; *HA*$^{+}$	35.23 ± 10.05	3 (NS, $p = 0.7223$; df = 5)	*PLP/CreERT/*$^{+}$; *HA*$^{+}$	4.43 ± 2.15	3 (NS, $p = 0.7013$; df = 5)
PLP/CreERT/$^{+}$; *β-catenin; HA*$^{+}$	30.63 ± 7.54	4	*PLP/CreERT/*$^{+}$; *β-catenin; HA*$^{+}$	5.98 ± 2.82	4
P21					
PLP/CreERT/$^{+}$	ND	3	*PLP/CreERT/*$^{+}$	ND	3
PLP/CreERT/$^{+}$; *β-catenin*	ND	3	*PLP/CreERT/*$^{+}$; *β-catenin*	ND	3
PLP/CreERT/$^{+}$; *HA*$^{+}$	36.62 ± 9.02	5 (NS, $p = 0.9439$; df = 9)	*PLP/CreERT/*$^{+}$; *HA*$^{+}$	23.40 ± 7.20	5 (NS, $p = 0.4691$; df = 9)
PLP/CreERT/$^{+}$; *β-catenin; HA*$^{+}$	35.70 ± 8.84	6	*PLP/CreERT/*$^{+}$; *β-catenin; HA*$^{+}$	17.17 ± 4.59	6

[a]There is no synergistic effect on the proliferation or differentiation of the IPhCs/IBCs SCs at P8 or P21 in the presence of β-catenin and Atoh1. Statistical analysis of HA^{+} and HA^{+}/Myo7a^{+} cell counts per 300 μm sections did not show any significant differences between *PLP/CreER*$^{T+}$;*Atoh1-HA*$^{+}$ *and PLP/CreER*$^{T+}$;*β-catenin; HA*$^{+}$ mice at P8 and P21. ND, Not detected; NS, not significant using unpaired Student's t test with Bonferroni correction. $N = 3$–6 for each time point and each genotype. Data are mean ± SEM.

Notch upregulation by β-catenin is abrogated by β-catenin and Atoh1 in Lgr5^{+} cells

To address the potential mechanism underlying the synergistic effect of β-catenin and Atoh1-HA on the proliferation and differentiation of Lgr5^{+} cells, we focused on the Notch signaling pathway because it has been shown to regulate both β-catenin and Atoh1 (Lanford et al., 2000; Zheng et al., 2000; Zine and de Ribaupierre, 2002; Takebayashi et al., 2007; Sikandar et al., 2010; Kwon et al., 2011; VanDussen et al., 2012; Petrovic et al., 2014). Using *Hes5nLacZ* reporter mice (Imayoshi et al., 2010), we analyzed the expression of *Hes5*, one of the Notch

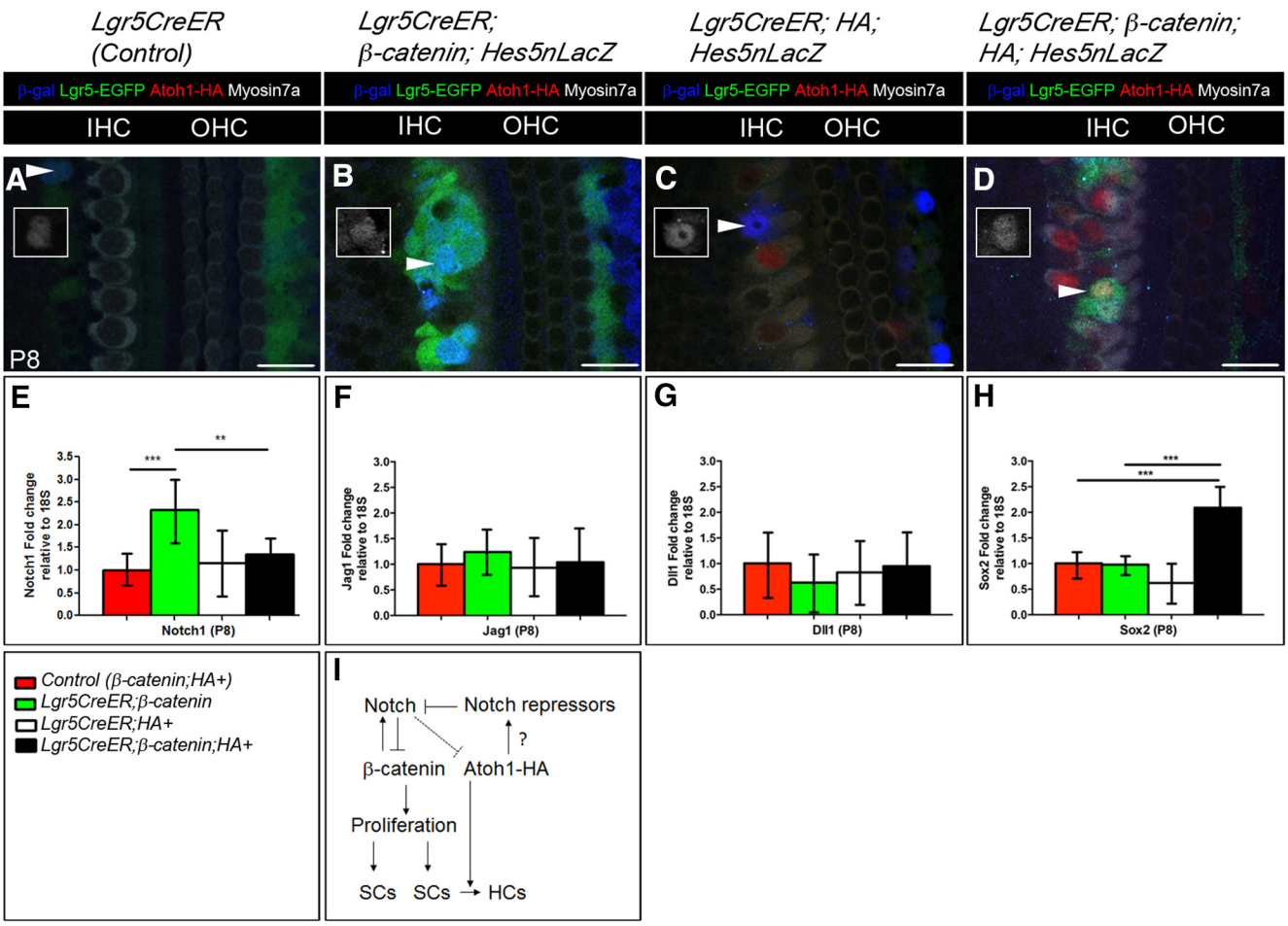

Figure 5. β-Catenin upregulates Notch, but its expression is abrogated by β-catenin and Atoh1-HA in Lgr5$^+$ cells. ***A–D***, Confocal images of immunostained cochleae from *Lgr5CreER* (Control) (***A***), *Lgr5CreER;β-catenin;Hes5nLacZ* (***B***), *Lgr5CreER;HA*$^+$*;Hes5nLacZ* (***C***), and *Lgr5CreER;β-catenin;HA*$^+$*;Hes5nLacZ* (***D***) mice that were induced with tamoxifen at P0-P1 and analyzed at P8. Hes5 upregulation in Lgr5$^+$ foci from *Lgr5CreER;β-catenin;Hes5nLacZ* mouse (arrowhead and inset) suggests active Notch signaling mediated by β-catenin. Very few Hes5nLacZ-positive cells were observed in *Lgr5CreER;HA*$^+$ (***C***, arrowhead and inset) and *Lgr5CreER;β-catenin;HA*$^+$*;Hes5nLacZ* (***D***, arrowhead and inset). ***E***, qPCR analysis of *Notch1* from isolated P8 cochlea shows an increase and decrease in *Lgr5CreER;β-catenin* mice (C_T value = 29.81 ± 0.98; 18S C_T value = 20.59 ± 0.61) and *Lgr5CreER;β-catenin;HA*$^+$ mice (C_T value = 30.68 ± 0.96; 18S C_T value = 20.68 ± 0.55) relative to control littermates (C_T value = 31.13 ± 0.87; 18S C_T value = 20.69 ± 0.47), respectively. For *Lgr5CreER;HA*$^+$ mice: C_T value = 30.56 ± 1.15; 18S C_T value = 20.34 ± 0.49. ***F***, ***G***, qPCR analysis of *Jagged1* (*Jag1*) and *Delta-like 1* (*Dll1*) ligands from isolated P8 cochlea shows no change in expression between all genotypes. In the case of *Jagged1*: for control littermates: C_T value = 31.11 ± 0.50; 18S C_T value = 20.61 ± 0.75, for *Lgr5CreER;HA*$^+$: C_T value = 31.01 ± 0.84; 18S C_T value = 20.42 ± 0.60, for *Lgr5CreER;β-catenin*$^+$: C_T value = 30.26 ± 0.79; 18S C_T value = 20.08 ± 0.53, and for *Lgr5CreER;β-catenin;HA*$^+$: C_T value = 30.94 ± 1.10; 18S C_T value = 20.51 ± 0.70. In the case of *Dll1*: for control littermates: C_T value = 35.58 ± 1.22; 18S C_T value = 20.76 ± 0.58, for *Lgr5CreER;HA*$^+$: C_T value = 35.28 ± 0.90; 18S C_T value = 20.19 ± 0.35, for *Lgr5CreER;β-catenin*$^+$: C_T value = 35.68 ± 1.14; 18S C_T value = 20.19 ± 0.45, and for *Lgr5CreER;β-catenin;HA*$^+$: C_T value = 35.56 ± 1.29; 18S C_T value = 20.67 ± 0.63. ***H***, qPCR analysis of *Sox2* from isolated P8 cochlea shows a 2.09 ± 0.38 increase in *Lgr5CreER;β-catenin;HA*$^+$ mice. The statistical significance of this increase was assessed by Student's *t* test with Bonferroni correction where the $p = 3.04$ E-09; df = 46; $n = 4$ (Control vs *Lgr5CreER;β-catenin;HA*$^+$) and $p = 7.52$ E-04; df = 46; $n = 3$ (*Lgr5CreER:HA*$^+$ vs *Lgr5CreER;β-catenin;HA*$^+$). In this case, control littermates: C_T value = 34.45 ± 0.41; 18S C_T value = 21.50 ± 0.38, for *Lgr5CreER;HA*$^+$: C_T value = 35.04 ± 0.61; 18S C_T value = 21.41 ± 0.37, for *Lgr5CreER;β-catenin*$^+$: C_T value = 34.37 ± 0.28; 18S C_T value = 21.39 ± 0.30, and for *Lgr5CreER;β-catenin;HA*$^+$: C_T value = 33.36 ± 0.69; 18S C_T value = 21.47 ± 0.29. ***I***, Proposed model for the synergism of β-catenin and Atoh1 involving Notch upregulation (by β-catenin) and its downregulation by Atoh1 in *Lgr5CreER;β-catenin;HA*$^+$ mouse. Dashed line of Notch to Atoh1-HA indicates the presumed low repression of Notch on the overexpressed Atoh1-HA. Question mark on the arrow between Atoh1-HA and Notch repressors indicates our current lack of targets. $^{**}p < 0.003$ (unpaired Student's *t* test). $^{***}p < 0.0003$ (unpaired Student's *t* test). Refer to text for actual *p* values. $N = 3$ or 4 for each time point and each genotype. Data are mean ± SEM. Scale bars, 25 μm.

downstream effectors (Jarriault et al., 1995; Kageyama and Ohtsuka, 1999; Ohtsuka et al., 1999) involved in HC differentiation (Zine et al., 2001; Zine and de Ribaupierre, 2002; Tateya et al., 2011). We observed the presence of Hes5$^+$ cells in *Lgr5CreER;β-catenin; Hes5nLacZ* mice (Fig. 5*B*, arrowhead) compared with control mice (Fig. 5*A*). This suggests that Notch signaling is indeed activated by the canonical Wnt signaling effector, β-catenin, in the organ of Corti, when it is normally downregulated during the first week of postnatal development (Hartman et al., 2009; Maass et al., 2015). In contrast, there were very few Hes5$^+$ cells in *Lgr5CreER;HA+; Hes5nLacZ* and *Lgr5CreER;β-catenin; HA*$^+$*;Hes5nLacZ* mice (Fig. 5*C*,*D*).

In support of the observed β-catenin-mediated Notch activation (see above), we analyzed the expression of *Notch1* since it has been implicated in cochlear development (Lanford et al., 1999; Zine et al., 2000). We analyzed *Notch1* expression by qPCR analysis (C_T values included in Fig. 5 legend) of P8 microdissected organ of Corti and found a significant increase in *Notch1* expression in *Lgr5CreER;β-catenin*$^+$ mice compared with littermate controls (2.33 ± 0.67 ($n = 3$); $p = 0.001$; df = 36 using a Student's *t* test with Bonferroni correction) (Fig. 5*E*), further supporting the notion that Wnt activation results in Notch activation. However, *Notch1* expression is significantly decreased in *Lgr5CreER;β-catenin; HA*$^+$ mice compared with *Lgr5CreER;β-*

catenin ($p = 0.0068$; df = 39 using a Student's *t* test with Bonferroni correction) (Fig. 5*E*). We also analyzed the expression of Notch1 ligands, *Jagged 1* (Fig. 5*F*) and *Delta-like 1* (Fig. 5*G*) and found no significant differences between all genotypes (statistical significance was assessed by Student's *t* test with Bonferroni correction). Nonetheless, these data combined with the Hes5nlacZ results (Fig. 5*A–D*) suggest that Notch activation mediated by β-catenin is inhibited by the ectopic expression of Atoh1 in Lgr5$^+$ cells, which may play a role in the synergistic effects that we described earlier. Understanding these opposing roles of Notch and Atoh1 in the inner ear warrants future investigation.

Discussion

In vivo HC regeneration in postnatal mammalian cochleae has been hampered by limited differentiation efficiency and spontaneous mitotic regeneration, and subsequent cell death of regenerated HCs (ranging from 12 to 150 new HCs generated per cochlea) (Kelly et al., 2012; Liu et al., 2012; Mizutari et al., 2013; Shi et al., 2013; Cox et al., 2014; Liu et al., 2014). Here we have shown that β-catenin and Atoh1 have synergistic effects on proliferation and transdifferentiation of neonatal Lgr5$^+$ cells *in vivo*. Compared with the aforementioned studies, our results show new IHC numbers reaching 1338.48 ± 271.98 per cochlea, twice as many new HCs as endogenous IHCs (Ou et al., 2000). Moreover, most of these new HCs survive to adulthood with 514.80 ± 18.00 new HCs per cochlea remaining at P42-P44. Our study is the first to achieve substantial proliferation and transdifferentiation in a mammalian cochleae *in vivo*, which parallels the efficient mitotic HC regeneration found in nonmammalian vertebrates and therefore represents a significant advancement for *in vivo* generation of new auditory HCs in a mammalian system.

β-Catenin has been shown to positively regulate the expression of *Atoh1* (Shi et al., 2010, 2013), so the absence of no new HCs generated in *Lgr5CreER;β-catenin*$^+$ mice was puzzling (Chai et al., 2012; Shi et al., 2013). Because β-catenin promotes Notch activation in *Lgr5CreER;β-catenin*$^+$ mice, we therefore attributed the absence of new HCs to Notch repression on β-catenin and presumed β-catenin-mediated Atoh1 activation. Indeed, Notch has been reported to post-translationally repress β-catenin activity (Kwon et al., 2011; Kim et al., 2012), and this further suggests the existence of a negative feedback loop to limit β-catenin activity to possibly prevent uncontrolled growth as observed in various cancers (Reya and Clevers, 2005). In addition, there is evidence that Hes family members, including Hes5, repress HC differentiation via *Atoh1* repression in both auditory sensory epithelia and other tissues (Lanford et al., 2000; Zheng et al., 2000; Zine and de Ribaupierre, 2002; Takebayashi et al., 2007; Sikandar et al., 2010; VanDussen et al., 2012; Petrovic et al., 2014). Because we observed a decrease in Notch signaling with the synergistic effects of β-catenin and Atoh1-HA on proliferation and transdifferentiation in Lgr5$^+$ cells in *Lgr5CreER;β-catenin;HA*$^+$ compared with *Lgr5CreER;β-catenin*, this suggests a repressive role for Atoh1 on Notch signaling (incidentally activated by β-catenin). Repression of Notch would presumably promote the β-catenin mitogenic effect and provide an indirect role on proliferation. This could possibly be through the modulation of Notch repressors (i.e., Numb, Fbxw7) or activators (i.e., Rbpj or Mastermind) (for review, see Ranganathan et al., 2011) (Fig. 5*I*). Nonetheless, we speculate that the ectopic expression of the *Atoh1-HA* transgene in *Lgr5CreER;β-catenin;HA*$^+$ abrogates the β-catenin-mediated Notch repression of endogenous Atoh1 and β-catenin. Furthermore, the ectopic expression of Atoh1 likely increases the levels of endogenous Atoh1, closer to a threshold from which the nonsensory epithelial cells can undergo HC differentiation. Consequently, this leads to an increase in β-catenin-expressing SCs competent to respond to pro-HC differentiation cues regulated by ectopic Atoh1. Of note, a recent report described the importance of Notch repression in the context of Wnt-dependent proliferation and HC generation in Sox2$^+$ and Lgr5$^+$ cells, which is largely consistent with our results (Li et al., 2015). Although the aforementioned report described an increase in Wnt signaling that promotes mitotic HC generation after Notch deletion alone, we attribute such discrepancy to several factors, including the use of *Sox2CreER* line *in vivo*, explant cultures for *Lgr5CreER;Notch1*$^{f/f}$, and/or the use of use of γ-secretase inhibitors, which could affect additional signaling pathways. Nonetheless, Notch inhibition, through γ-secretase inhibitors, has been described to promote HC formation and maturation in the damaged cochlea (Korrapati et al., 2013; Mizutari et al., 2013; Bramhall et al., 2014), thus highlighting the impact of this pathway on HC regeneration. Together, we conclude that the combination of β-catenin and Atoh1 provides a platform from which to promote proliferation and differentiation of SCs, although this paradigm still requires further manipulation(s) for terminal maturation.

Although we describe an indirect role for Atoh1 on proliferation, via Notch repression, targetome analyses provide an extensive list of Atoh1-associated cellular processes (e.g., transcription, proliferation, migration, cytoskeletal rearrangements) (Klisch et al., 2011; Yoon et al., 2011; Cai et al., 2015). This suggests that Atoh1 is involved in other cellular events, which may also influence the synergistic effect observed in our model, although these cellular processes are also likely affected by β-catenin, especially in the context of proliferation.

Cellular heterogeneity has been shown for the Lgr5$^+$ cells in the intestine, whereby only 5% of isolated Lgr5$^+$ cells give rise to organoid (Sato et al., 2009), and lineage-tracing experiments have shown that only high GFP-expressing Lgr5$^+$ cells exhibit clonal expansion capability (Snippert et al., 2010). Our results are consistent with this notion since. For instance, there is an absence of synergic effect on the proliferation and transdifferentiation rate in the third row of Deiters' cells, and in a subset of Lgr5$^+$ cells, the IPhCs and IBCs. For the former, we attribute the lack of synergy to the presumed high Lgr5 expression (reflected by EGFP expression) in these cells (Chai et al., 2011), which could prevent their proliferation and transdifferentiation. In support, the new HCs generated in this study and those derived from isolated Lgr5$^+$ cells cultured in the presence of Wnt3a no longer express EGFP (Chai et al., 2012). For the latter, we conjecture that IPhCs and IBCs may respond differently to β-catenin and Atoh1 compared with other Lgr5$^+$ nonsensory epithelial cells, possibly because the endogenous β-catenin promoter may already be downregulated at the time of induction. This is important because the activation of the β-catenin allele used in this study is dependent upon the endogenous β-catenin promoter (Harada et al., 1999). In this scenario, Lgr5-EGFP expression could merely reflect residual Wnt activity because *Lgr5* expression is normally downregulated in IPhCs and IBCs during the first postnatal week. This inherent cellular heterogeneity within the Lgr5$^+$ cells is also supported by other studies (Liu et al., 2012; Shi et al., 2013). Regardless, because the Lgr5$^+$ cells are thought to be the stem/progenitor cells of the neonatal cochlea with higher proliferation and differentiation potential than the broader population of p27^{Kip1+} cells (White et al., 2006), these cells remain of high interest for hearing restoration efforts.

Similar to previous reports (Kelly et al., 2012; Liu et al., 2012, 2014), the Atoh1-induced new HCs described here underwent incomplete maturation because they do not express the terminal IHC (vGlut3) and OHC (prestin) markers and have some incomplete alignment of presynaptic and postsynaptic markers, Ctbp2 and GluR2. We propose the following possible explanations for the lack of terminal differentiation in the new HCs from this study. Ectopic Atoh1 and/or Wnt/β-catenin expressions are unnatural to HC terminal differentiation, as both genes are normally downregulated during development (Lanford et al., 2000; Lumpkin et al., 2003; Layman et al., 2013; Munnamalai and Fekete, 2013; Jansson et al., 2015). In our model, Atoh1-HA expression is driven by a constitutive promoter and is therefore unlikely to be downregulated after tamoxifen induction (Liu et al., 2012, 2014). In contrast, the expression of the *Catnb*$^{flox(exon3)/+}$ allele is controlled by the *β-catenin* endogenous promoter, but because Lgr5 (and presumably Wnt) is downregulated after the first postnatal week, it is unlikely that *de novo* production of stable β-catenin is sustained past the first week of postnatal development in Lgr5^{+} cells. Nonetheless, transient ectopic expression of Atoh1 and β-catenin may be more effective in promoting HC maturation. Of note, Cre-mediated activation of β-catenin (using the *Catnb*$^{flox(exon3)/+}$ mouse line), and the subsequent evaluation of the effects of Atoh1 and β-catenin in mature SCs will require different strategies because of the inactive β-catenin endogenous promoter in mature cochleae.

Traditionally, HC regeneration strategies have focused on manipulating individual pathways or genes, such as Wnt (Chai et al., 2012; Jacques et al., 2012; Shi et al., 2013; Jacques et al., 2014), Notch (Kiernan et al., 2005; Yamamoto et al., 2006; Takebayashi et al., 2007; Korrapati et al., 2013), Eya1-Six1 (Ahmed et al., 2012), or Atoh1 (Izumikawa et al., 2005; Kelly et al., 2012; Liu et al., 2012; Liu et al., 2014). However, multiple pathways work in concert to promote HC mitotic regeneration in nonmammalian vertebrates (Jiang et al., 2014; Ku et al., 2014; Steiner et al., 2014), further supporting the use of combinatorial therapies. The success of this type of combinatorial approach for the generation of new HCs has been successfully illustrated by Minoda et al. (2007), with the adenovirus-mediated expression of *Skp2* and *Atoh1*; Masuda et al. (2012), with the combination of Atoh1 with TCF3 or GATA3; and Costa et al. (2015), with the combination of Atoh1, Gfi1, and Pou4f3. Moreover, successful regeneration in other systems (e.g., cardiomyocytes, β islet cells, and CNS neurons) also uses a combinatorial approach (Niu et al., 2013; Xin et al., 2013; Ziv et al., 2013). Therefore, the combination of β-catenin and Atoh1 is likely critical for successful HC regeneration and provides the basis for combinatorial therapeutics for hearing restoration in humans.

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