Retinoic acid signaling is required to maintain supporting cell plasticity for differentiation into cochlear hair cells

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Retinoic acid signaling is required to maintain supporting cell plasticity for differentiation into cochlear hair cells

Directed by Professor Chul Hoon Kim

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ABSTRACT

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In mammals, during the neonatal period, cochlear hair cells can be regenerated from supporting cells. However, after the neonatal period, hearing loss is irreversible because of lack of regenerative abilities of cochlear hair cells in the postnatal stage. The mechanisms of how supporting cells can regenerate hair cells during early period has not been completely revealed. Retinoic acid (RA), a metabolite of vitamin A (VA), is known to be crucial for the development of various organs, including the inner ear. However, the role of retinoic acid in hair cell regeneration in the postnatal period remains unclear. In this study, I investigated the effect of RA signaling on hair cell differentiation in the postnatal stage using a cochlear organoid model. The process of generating the organoid model consists of two sequential steps: the expansion phase in which supporting cells proliferate and the differentiation phase in which supporting cells are converted into mature hair cells. When I removed VA in the organoid culture medium, hair cell maturation that occurs in the differentiation phase was significantly impaired. When RA was added instead of VA, there was no significant difference in hair cell differentiation compared to the condition of VA in the culture medium,

indicating that VA induces hair cell differentiation through RA signaling. To know whether VA is required during either the proliferation of supporting cells or the differentiation of hair cells, I removed VA during the expansion phase or the differentiation phase, respectively. Interestingly, even when VA was provided only during the expansion phase, subsequent hair cell differentiation normally occurred. I found the omission of VA in this critical phase results in a decrease in the expression of essential hair cell maturation transcription factors (TFs) such as *Atoh1*, *Pou4f3*, and *Gfi1* and subsequent hair cell maturation during the differentiation phase. To activate RA signaling, VA should be converted into RA by enzymes such as retinol dehydrogenases (RDHs) and retinal dehydrogenases (RALDHs). To identify whether supporting cells have those enzymes, I analyzed the mRNA levels of RA synthesis enzymes. I found that supporting cells have their own RA synthesis enzymes. These data suggest RA signaling in the cochlea is required for maintaining the plasticity of supporting cells to differentiate into hair cells.

Key words: hair cell, retinoic acid, cochlear organoids

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I. INTRODUCTION

Hair cells and supporting cells are components of the sensory epithelium known as the organ of Corti. There are two kinds of hair cell differentiation. First, in the developmental stage, prosensory cells differentiate into hair cells and supporting cells by cell fate decision which involves the regulation of various signaling pathways such as Notch signaling and Wnt signaling[3,4,5]. Second, in the postnatal stage, when the hair cells get damaged by environmental factors such as noise and drugs, supporting cells can trans-differentiate or mitotically differentiate into hair cells[6,7,8]. However, in mammals, supporting cells lose the capacity to generate hair cells after the neonatal period so that damaged hair cells cannot be regenerated.

Retinoic acid (RA), a metabolite of vitamin A(VA), plays an important role in regulating the transcription of various genes by binding to receptors located inside the cell nucleus[10]. RA is essential in the developmental process and the deficiency in RA results in developmental disorders [11]. RA can affect the development of the brain and eyes[12,13] as well as the formation of the heart, lungs, and limb budding[15,16,17]. Additionally, RA is crucial for the development of the inner ear[14, 18, 21]. RARα and RARγ, the receptors for RA, are required for the inner ear development[19] and RA signaling affects inner ear compartmentalization along the anterior-posterior axis[20]. Also, RA promotes the differentiation of hair cells during the embryonic stage[42]. In contrast, the role of RA in hair cell differentiation during the postnatal stage remains unclear. In a previous report, after exposure to neomycin, damaged hair cells were regenerated with the supplement of RA in the organ of Corti of rats at postnatal day 3[22]. In contrast, the other group reported that under similar conditions, RA could not help regenerate hair cells[23]. Subsequent studies related to the role of RA in hair cell regeneration have been lacking, probably because of the absence of a suitable model for further validation. Therefore, the effect of RA on the hair cell regeneration remains unclear.

In 2017, McLean *et al* established the method to generate cochlear organoids using epithelial stem cell marker Lgr5-positive supporting cells [9]. The method consists of two sequential phases, expansion and differentiation. During the expansion phase, supporting cells proliferate by activation of Wnt signaling and Notch signaling. In the differentiation phase, inhibition of notch signaling converts supporting cells into hair cells. This method has the advantage of being able to determine the impact of regulatory factors on two distinct phases, the proliferation of supporting cells and the differentiation of hair cells, respectively.

In this study, I investigated the role of RA signaling in hair cell differentiation from supporting cells in the postnatal stage using a cochlear organoid model. I discovered VA is required for supporting cells during the expansion phase to subsequently differentiate into hair cells. Furthermore, RA enzymes which participate in synthesizing RA from VA were found in supporting cells in the organoids. These data indicate that the RA signaling is important to maintain the plasticity of supporting cells.

II. MATERIALS AND METHODS

1. Animals

Lgr5-EGFP-IRES-Cre-ER mice (The Jackson Laboratory, strain 8875)[1] were used to analyze cochlear stem cell expansion. Atoh1-nGFP mice (MGI:3703598)[2] were used to identify differentiated hair cells. C57BL/6N mice were used for the rest of the experiments.

2. Cochlear organoid culture

The cochlear organoid culture method was adopted from a recently published protocol[9]. Cochlea of postnatal day 1 to 3 mice were isolated in Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY, USA). Reissner's membrane was removed to obtain the organ of Corti. The isolated organ of Corti was singularized using TrypLE (Gibco). Dissociated cells were triturated with matrigel (Corning, Corning, NY, USA). During expansion, DMEM/F12 (Gibco) with B27 (Gibco) and N2 (Gibco) supplemented with 50 ng/ml of EGF (Peprotech, Rocky Hill, New Jersey, USA), 50 ng/ml of bFGF (R&D Systems, Minneapolis, MN, USA), 50 ng/ml of IGF (Peprotech), 3μM CHIR99021 (Tocris, Minneapolis, MN, USA), 1mM Valproic acid (Sigma-Aldrich, St Louis, MO), 3μg/ml of pVc (Sigma-Aldrich) and 2μM 616352 (Merk millipore, Burlington, MA, USA). For differentiation, DMEM/F12 (Gibco) with B27 (Gibco) and N2 (Gibco) was supplemented with 3μM CHIR99021 and 5μM LY411575 (Sigma-Aldrich). Media changed every other day.

3. Immunohistochemistry

Matrigel was removed using cell recovery solution (Corning) for 45min at 4°C. Isolated organoids were fixed with 4% paraformaldehyde for 45 min at 4°C and permeabilized with 0.1% Tween20 for 20min at 4°C. Organoids were blocked with 0.1% bovine serum albumin (BSA) in 0.2% TX-100 solution for 20min at 4°C incubated for 2 overnights at 4°C with primary antibodies diluted in the blocking solution. Antibodies are listed in Table 1. After incubation, organoids were washed three times with the blocking solution and incubated with secondary antibodies diluted 1: 400 in the blocking solution.

Table 1. List of primary antibodies

Antibodies	Source	Identifier	Dilution
Myo7a	Our lab	-	1:200
Prestin	Santa cruze	sc-22694	1:200
vGlut3	Synaptic System	135 203	1:500
Otoferlin	Abcam	ab53233	1:300
Sox2	Santa cruze	sc-365823	1:200
Pou4f3	Santa cruze	sc-81980	1:200
EPS8	BD bioscience	610144	1:200
Harmonin	Our lab	-	1:200
Myo6	Proteus	25-6791	1:200
GRXCR2	Sigma-Aldrich	HPA059421	1:200
TPRN	Sigma-Aldrich	HPA020899	1:200

PMCA2	Abcam	ab3529	1:200
NPTN	R&D systems	AF7818	1:200

4. RNA extraction and real-time PCR (qRT-PCR)

RNA was extracted with TriZol (Thermo Fisher Scientific, Rutherford, NJ, USA). cDNA was synthesized from RNA samples by PrimeScript™ 1st strand cDNA Synthesis Kit (TAKARA, Shiga, Japan) following the manufacturer's instructions. The gene expression levels of organoids were tested by quantitative real-time PCR (qRT-PCR) using SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) on a Quantstudio3 Real-Time PCR system (Applied Biosystems). The primers used in this study are listed in Table 2.

Table 2. List of primers

Gene	Forward (5'-3')	Reverse (5'-3')
Atoh1	GGGTGAGCTGGTAAGGAGAA	ACTACAACCCCACCCTTCAG
Pou4f3	ATTCTCCAGCCTACACTCCG	ATGATTCTTGCCGTGGGAGA
Gfi-1	TGTTCCAGCCATCCTTCATC	GCAATGCCAGGGTACATAGT
Lgr5	TCTTTGAAGGCAAAGACATGG	TTATGCTCCTGCGCTTTCTT
Sox2	GTCCACCCTCCTGGCTTT	AAACTTGTTGGTGCCCATCT

5. Transcriptome analysis

Read alignment and processing were performed using the TruSeq Standard mRNA Reference Guide (1000000040498). Raw reads were trimmed with

Trimmomatic 0.38(http://www.usadellab.org/cms/?page=trimmomatic), aligned against reference using HISAT2 version 2.1.0 (https://ccb.jhu.edu/software/hisat2/index.shtml), and quantified with StringTie version 2.1.3b (https://ccb.jhu.edu/software/stringtie/). Differential analyses were performed after adjusting batches using the R package Limma-Voom (v3.54.0, PMID: 24485249). Gene set enrichment analysis (GSEA) was conducted with R package clusterProfiler (v4.6.2, PMID: 22455463). Pathway terms were obtained from MsigDB Gene Ontology (PMID 14681407).

6. Statistical analysis

All data analysis was conducted with GraphPad Prism version 10 (GraphPad Software, Inc., La Jolla, CA, USA). *P*-values were calculated via an unpaired Student's *t-test* or one-way analysis of variance (ANOVA) followed by multiple comparisons with the Bonferroni *post hoc* test. All data are presented as means ± standard error of the mean (SEM). The significance level was set at $p < 0.05$.

III. RESULTS

1. Generation of mouse cochlear organoids

I generated cochlear organoids using a recently developed culture system [9]. In mature mice, supporting cells lose their ability to differentiate into hair cells. Therefore, I performed the experiment with neonatal mice (postnatal day 1 to 3). The culture method is composed of two sequential phases, the expansion phase and the differentiation phase. First, during 10 days of the expansion phase, Sox2-positve supporting cells are proliferated. Second, 10 days of the differentiation phase, supporting cells are converted into Atoh1-positive hair cells (Fig 1). Using this method, I generated the cochlear organoid model. By performing immunostaining, I found most cells in organoids are Sox2-positive supporting cells at day 10 (Fig. 2A). At day 20, Sox2-positive supporting cells were converted into Myo7a and Pou4f3-positive hair cells (Fig. 2B). Prestin, known to be located in the membrane of outer hair cells, was expressed on the membrane of Myo7a-positive hair cells in the organoids[46] (Fig. 2C). Additionally, organoids expressed inner hair cell markers, vesicular glutamate transporter3 (vGlut3) and Otoferlin[47,48] (Fig. 2C). To determine the time points of hair cell markers expression, I examine expression of Atoh1, Pou4f3, and Myo7a at day 10, 12, and 14. For this experiment, Atoh1-nGFP mouse line was used for generating organoids. At day 10, expression of Atoh1 was observed slightly even before the differentiation phase. Then the expression of Atoh1 was significantly increased within two days of differentiation. Pou4f3 was weakly expressed at day 12 and expressed in most cells of the organoids at day 14. Lastly, Myo7a, which is known as a mature hair cell marker, was detected from day

14 (Fig. 3). Mammalian hair cells have actin-based hair bundles, stereocilia. After the differentiation phase, most cells comprising the organoids were Myo7a-positive and majority of them contained phalloidin-positive actin bundles (Fig. 4A, B). Moreover, differentiated hair cells of organoids had several stereciliary proteins that are essential for processing sound wave into electrical signals (Fig. 5A, B). Stereociliary proteins in organoids recapitulated their localization in *in vivo*. Myo7a, EPS8, and Harmonin are located on the top of stereocilia[24,25], GRXCR2, Taperin (TPRN) and Myosin6 (Myo6) are located on base of stereocilia[26,27] and, PMCA2 and Neuroplastin (NPTN) located throughout the entire stereocilia[28] (Fig. 5A, B). These data validated that this organoid model can recapitulate many key events in hair cell regeneration, expansion of SOX2-positive supporting cells, the expression of hair cell maturation markers, the formation of hair bundles, and the localization of stereociliary proteins.

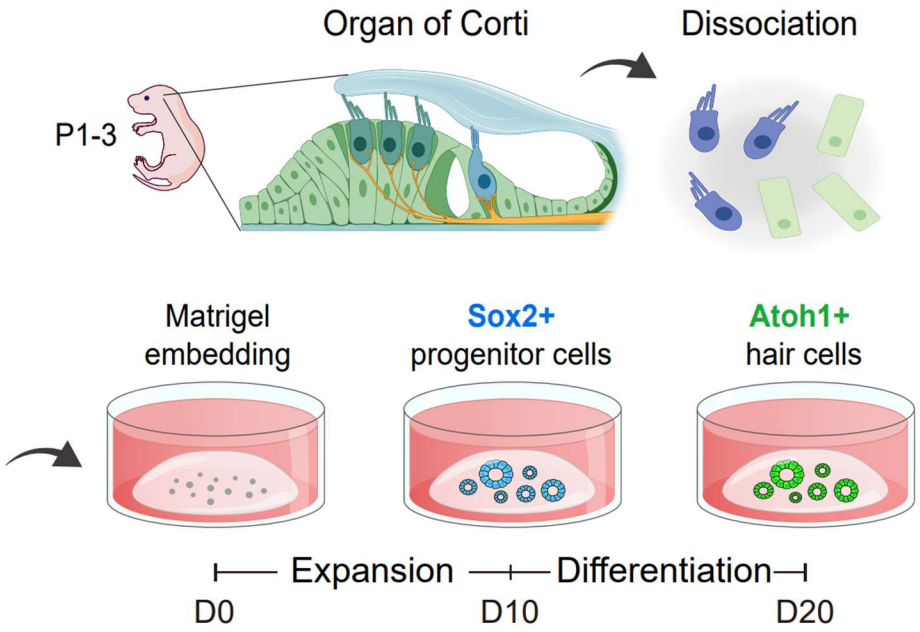

Figure 1. Schematic diagram of mouse cochlear organoid culture methods. Cochlear organoids were generated from the organ of Corti of postnatal day 1 to 3 mice. Sox2-positive supporting cells were proliferated during 10 days of the expansion phase and supporting cells are converted into Atoh1-positive hair cells during following 10 days of the differentiation phase.

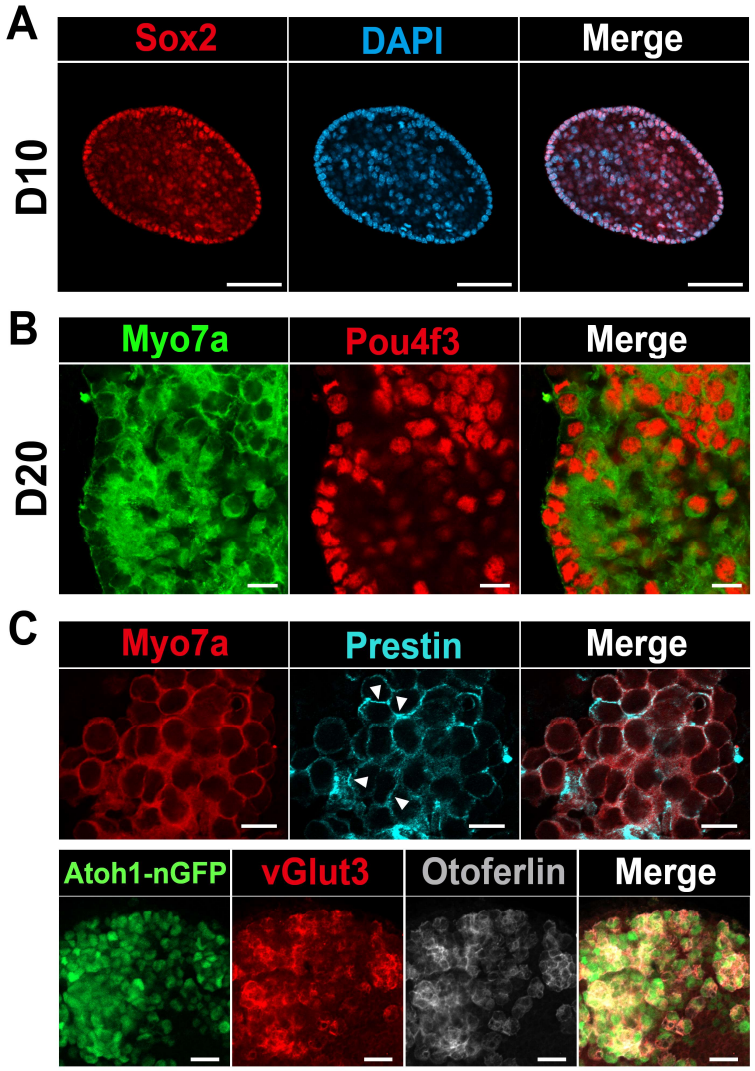

Figure 2. Generation of cochlear organoids. (A) Representative confocal images of immunolabeling for Sox2 (at day 10). Scale bars, 50 μm. (B) Representative confocal images of immunostaining for Myo7a and Pou4f3 (at day 20). Scale bars, 10 μm. (C) Representative confocal images of immunolabeling for Myo7a and Prestin (upper), and Atoh1-nGFP, vGlut3, and Otoferlin (lower) (at day 20). Scale bars, 10 μm.

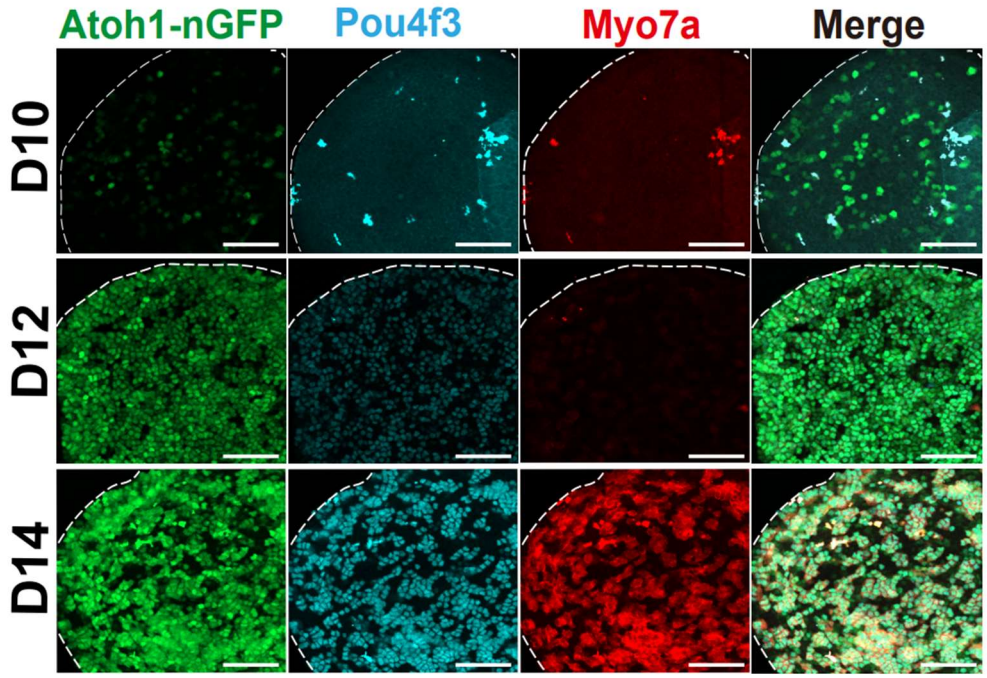

Figure 3. The time points of hair cell differentiation markers expression. Representative confocal images of immunolabeling for Atoh1-nGFP, Pou4f3 and Myo7a at day 10, 12, and 14. Scale bars, 50 μm.

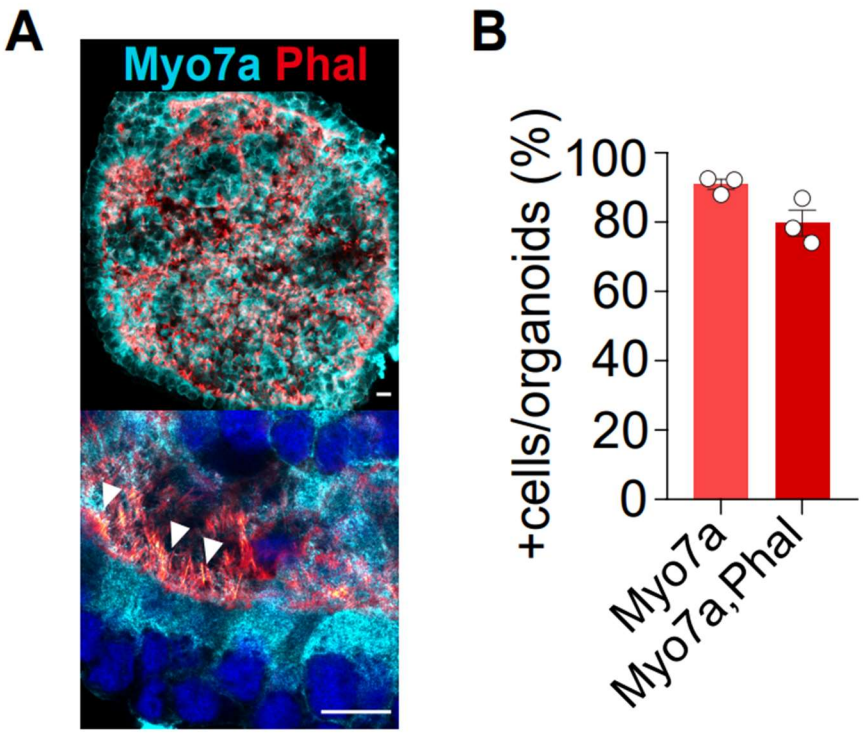

Figure 4. Cochlear organoids contain actin-based stereocilia. (A) Representative confocal images of immunolabeling for Myo7a and phalloidin. Scale bars, 10 μm. (B) Quantification of Myo7a-positive cells and Myo7a-positive cells with Phalloidin-positive stereocilia. Phal, phalloidin.

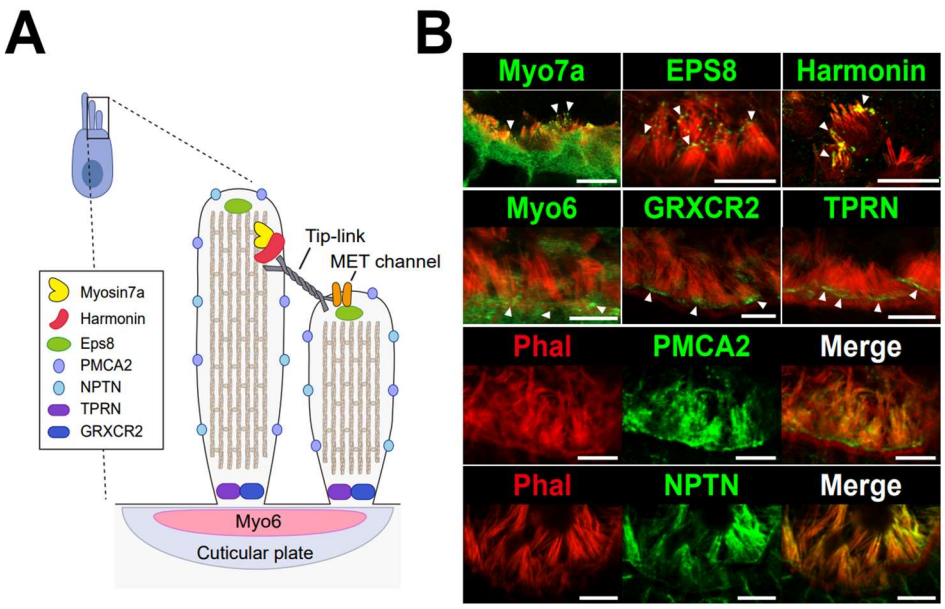

Figure 5. Cochlear organoids contain stereociliary proteins. (A) Schematic illustration of stereociliary proteins localization. (B) Representative confocal images of immunostaining of Myo7a, EPS8, Harmonin, Myo6, GRXCR2, TPRN, PMCA2, and NPTN. Scale bars, 5 μm. Phal, phalloidin; TPRN, Taperin; NPTN, Neuroplastin.

2. RA signaling is required for hair cell differentiation

To determine whether RA signaling affects hair cell differentiation, I conducted VA withdrawal test with the cochlear organoid model. The organoid culture medium was supplemented with the B27 supplement. There are two types of B27 supplement, with VA and without VA. I performed VA withdrawal test using the B27 with vitamin A (+VA) or B27 without vitamin A (-VA). To display differentiated hair cells, Atoh1-nGFP mice were used for generating cochlear organoids. After 20 days of culture, 70% organoids cultured with VA contained Atoh1-nGFP cells. In contrast, less than 10% of Atoh1-nGFP positive organoids was observed in the absence of VA. To determine whether the effect of VA was due to RA signaling, RA was added to the culture medium with B27-VA. The results showed that when cultured with RA instead of VA, the expression of positive Atoh1-nGFP was similar compared to when cultured with B27+VA. However, additional RA supplementation did not affect Atoh1 expression in organoids. (Fig. 6A, B). This result indicates that RA signaling is required for hair cell differentiation.

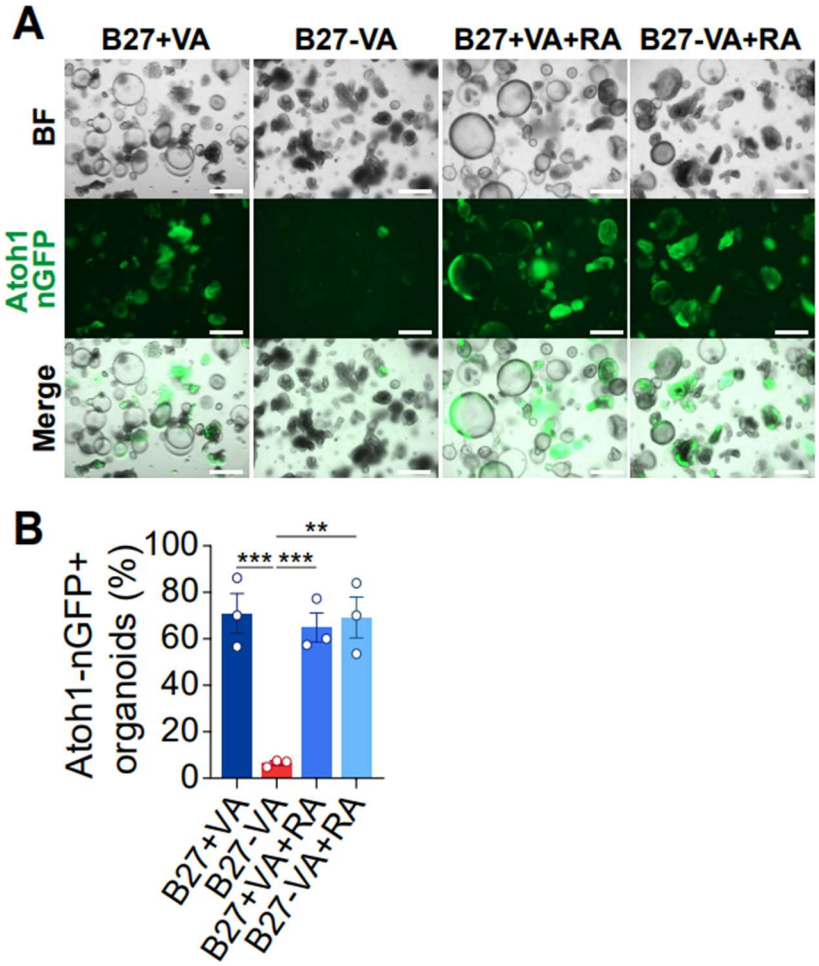

Figure 6. The number of Atoh1-positive organoids decreased in the absence of VA. (A) Representative images of Atoh1-nGFP-positive cochlear organoids cultured in B27+VA, B27-VA, B27+VA+RA, and B27-VA+RA, respectively (at day 20). (B) Quantification of Atoh1-positive organoids in each condition. Scale bars, 500 μm. *P*-value was calculated by one-way ANOVA, followed by Bonferroni *post hoc* tests for multiple comparisons. Results are presented as means ± SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

3. The presence of VA in the expansion phase is required for hair cell differentiation

To identify whether RA signaling is required in the expansion phase or the differentiation phase, VA was selectively removed from each phase (Fig. 7A). When VA was only in the expansion phase, even though VA was not in the differentiation phase, the population of Atoh1-positive organoids was not different from that cultured with VA during the entire culture period. In contrast, despite the presence of VA in the differentiation stage, the absence of VA in the expansion phase suppressed the expression of Atoh1 (Fig. 7B, C). This result indicates that VA is required to supporting cells in organoids during the expansion phase. To differentiate hair cells, sequential expression of key transcription factors Atoh1, Pou4f3, and Gfi1 is required. To observe the expression of transcription factors in the organoids cultured with or without VA, immunolabeling was performed for Atoh-nGFP and Pou4f3 (Fig. 8A). The number of Atoh1-positive cells and Pou4f3-positive cells decreased in -VA conditions compared to +VA conditions (Fig. 8B). The mRNA expression of Atoh1, Pou4f3, and Gfi-1 was downregulated in -VA conditions, consistent with protein expression. (Fig. 8C). Next, I compared the maturity of hair cells within the organoids by immunostaining for Myo7a and phalloidin. Organoids cultured in the absence of VA during the expansion phase, had very few Myo7a-positive hair cells (Fig. 9A, B). However, even in the absence of VA, most Myo7a-positive cells had phalloidin-positive actin bundles (Fig. 9A, B). Overall, these data indicate that VA is required during the expansion phase and the absence of VA impairs subsequent hair cell maturation during the differentiation phase.

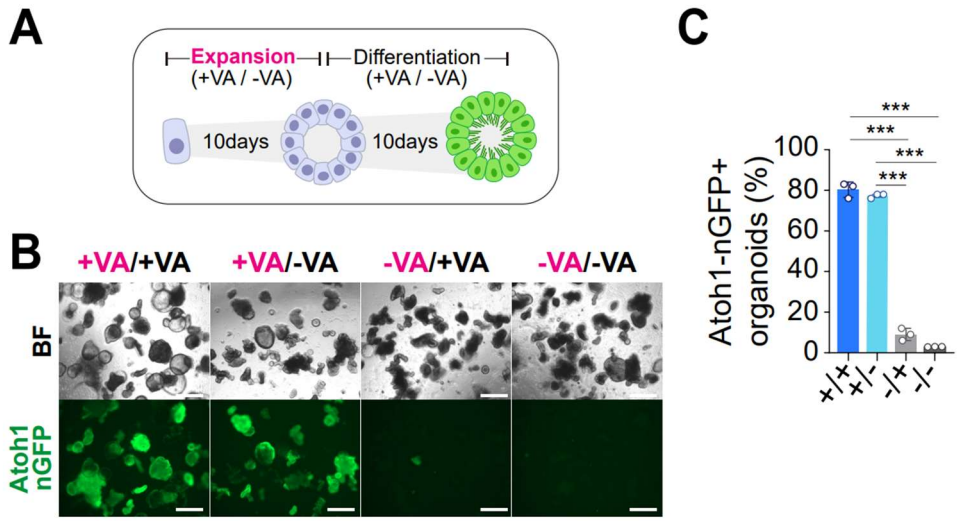

Figure 7. The absence of VA during the expansion phase reduced Atoh1 expression. (A) Schematic illustration of withdrawal test with VA. (B) Representative images of Atoh1-nGFP-positive organoids cultured in +VA or –VA during expansion and differentiation, respectively (at day 20). (B) Quantification of Atoh1-positive organoids in each condition. Scale bars, 500 μm. *P*-value was calculated by one-way ANOVA, followed by Bonferroni *post hoc* tests for multiple comparisons. Results are presented as means ± SEM of three independent experiments. *** $p < 0.001$.

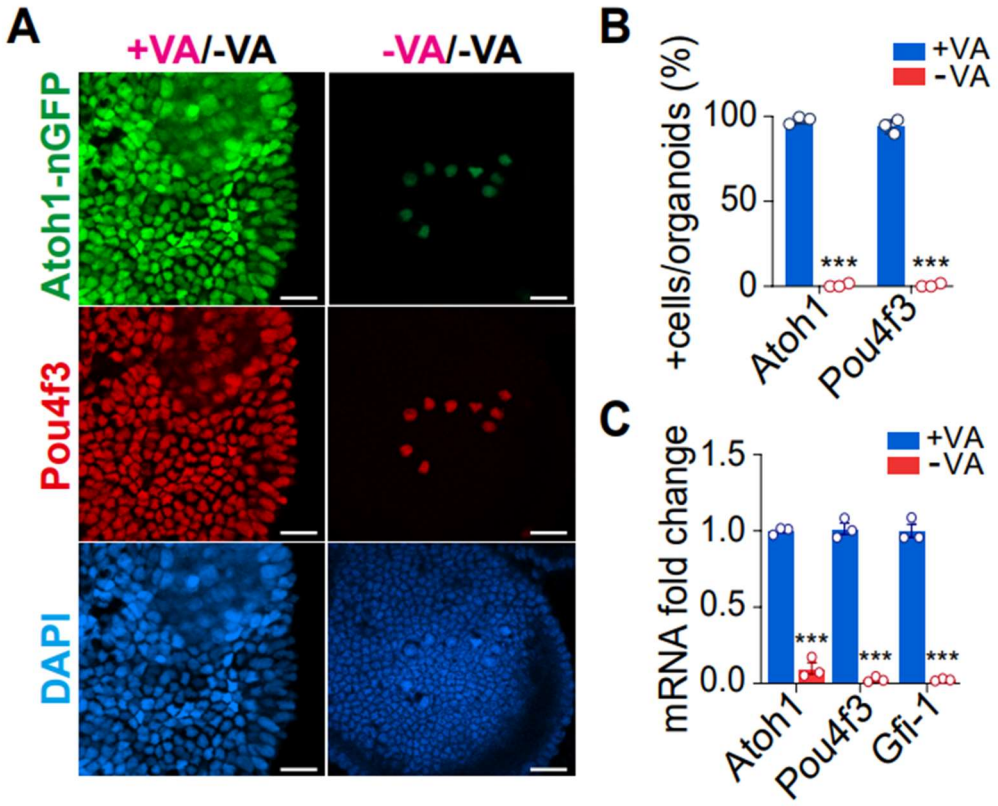

Figure 8. Expressions of key transcription factors regulating hair cell differentiation were decreased in the absence of VA. (A) Representative confocal images of immunostaining in cochlear organoids for Atoh1-nGFP and Pou4f3 (at day 20). (B) Quantification of Atoh1-positive cells and Pou4f3 positive cells per organoids cultured in +VA and –VA during the expansion phase. (C) mRNA fold changes of Atoh1, Pou4f3 and Gfi-1 in organoids cultured in +VA and -VA (at day 20). Scale bars, 20 μm. *P*-value was calculated by unpaired Student's *t-test*. Results are presented as means ± SEM of three independent experiments. *** $p < 0.001$.

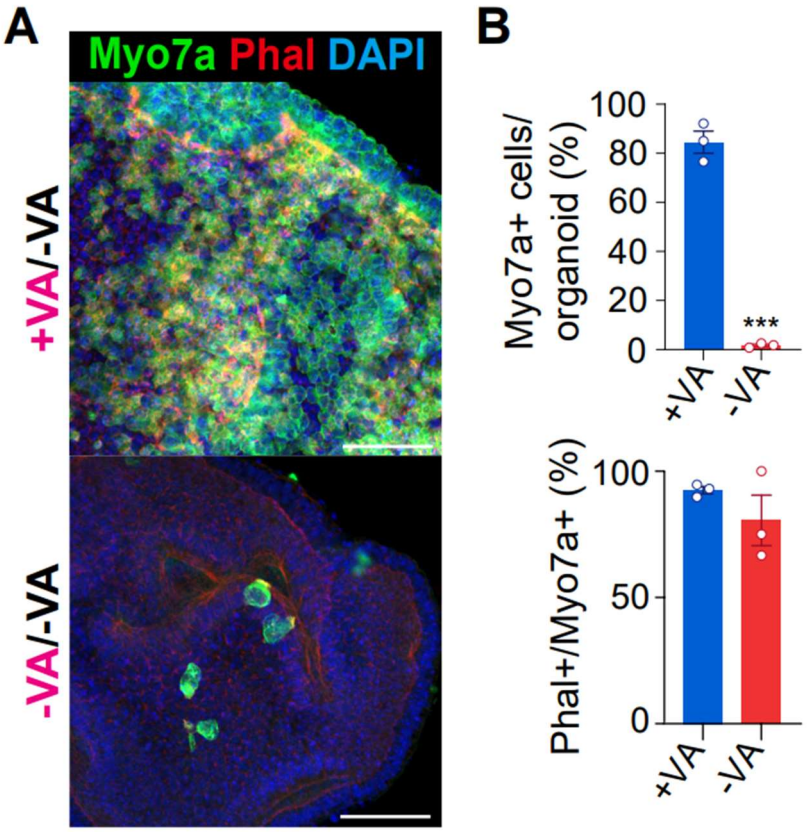

Figure 9. Myo7a-positive hair cells were decreased in the absence of VA. (A) Representative confocal images of immunostaining for Myo7a and phalloidin (at day 20). (B) Quantification of Myo7a-positive cells and Phalloidin-positive cells out of Myo7a-positive cells. Scale bars, 50 μm. Phal, phalloidin. *P*-value was calculated by unpaired Student's *t-test*. Results are presented as means ± SEM of three independent experiments. *** $p < 0.001$.

4. Removal of VA does not affect proliferation of supporting cells

To verify whether VA promotes proliferation of supporting cells during the expansion phase, I analyzed organoid forming efficiency and organoid size. The number of organoids and average organoid diameter were not significantly different between the organoids cultured in +VA and –VA conditions. (Fig. 10A, B). To indicate proliferating cells in organoids, cell proliferation assay with EdU (5-ethynyl-2´-deoxyuridine) was conducted. For this experiment, organoids received a 1.5-hour pulse of EdU at day 10. As a result, there was no significant difference in the population of EdU-positive cells in organoids cultured in –VA, compared to those cultured in +VA (Fig. 11). To determine whether organoids grown in –VA condition lack progenitor-like features, I compared the expression of Lgr5 and Sox2 in organoids cultured in –VA and +VA. For this experiment, I used Lgr5-GFP mice for generating the organoids. As a result, most populations in the organoids were Lgr5-positive and Sox2-positive in both conditions, and at day 10, the mRNA expression levels of Lgr5 and Sox2 were similar in –VA and +VA (Fig. 12A, B). These data suggest that omission of VA does not affect proliferation of supporting cells during the expansion phase. Additionally, the expressions of progenitor cell markers of supporting cells are also unaffected by VA.

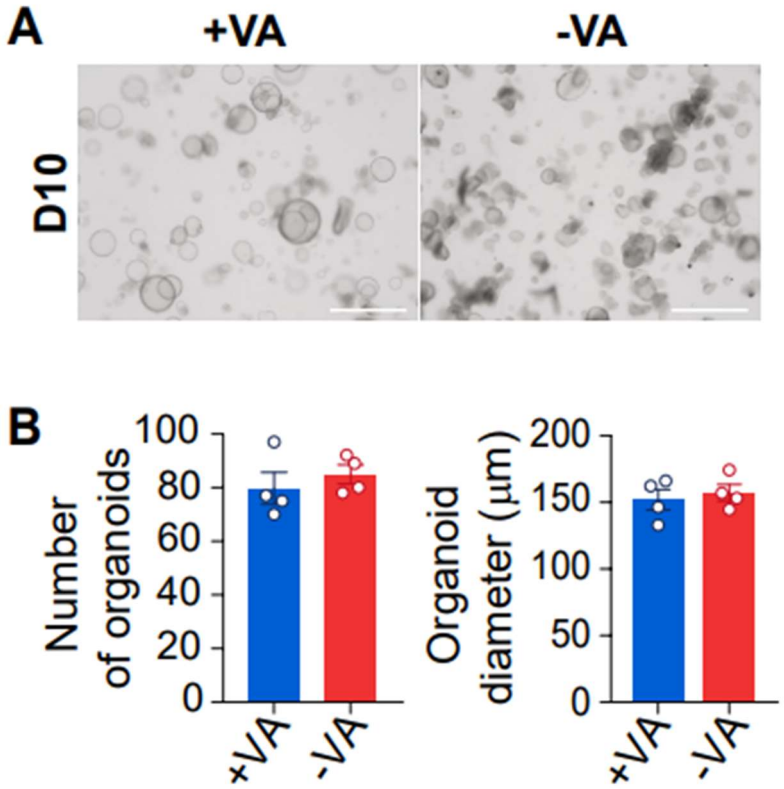

Figure 10. Quantification of organoid forming efficiency and size. (A) Bright-field images of the organoids cultured in +VA and -VA (at day 10). (B) Quantification of the number of organoids and average of organoid diameters of the organoids cultured in +VA and -VA. Scale bars, 500 μm. *P*-value was calculated by unpaired Student's *t-test*. Results are presented as means ± SEM of three independent experiments.

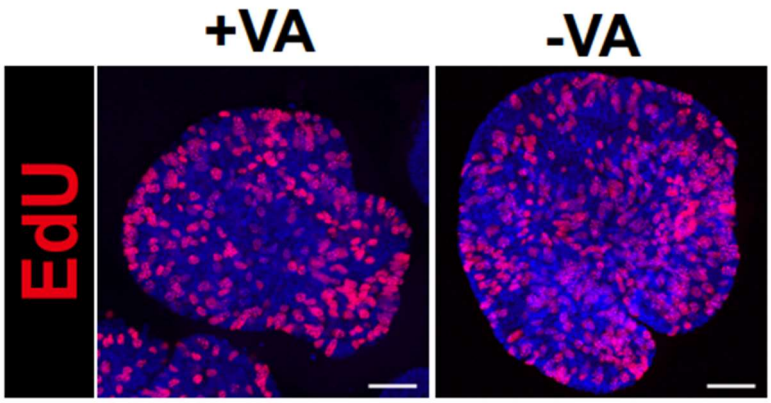

Figure 11. EdU cell proliferation assay with cochlear organoids. Representative confocal images of EdU staining for organoids cultured in +VA and –VA (at day 10). Scale bars, 50 μm.

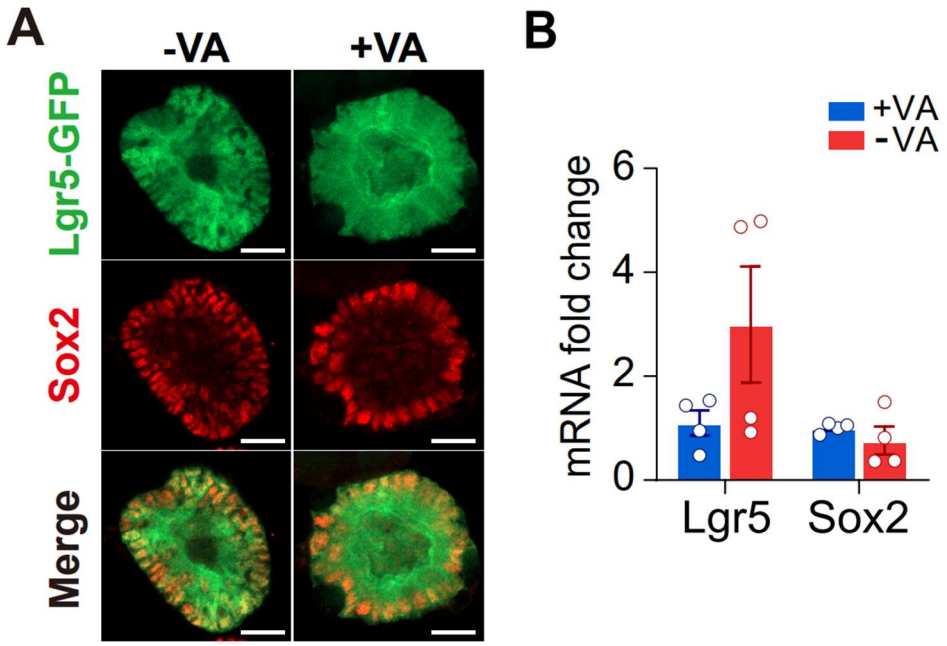

Figure 12. Supporting cells cultured in the absence of VA maintain expression of progenitor cell markers. (A) Representative confocal images of Lgr5-GFP and Sox2 labeling in organoids cultured in –VA and +VA (at day 10). (B) mRNA fold change of *Lgr5* and *Sox2* of organoids cultured in –VA and +VA. Scale bars, 20 μm. *P*-value was calculated by unpaired Student's *t-test*. Results are presented as means ± SEM of four independent experiments.

5. Transcriptome analysis of cochlear organoids

To determine differentially expressed genes (DEG) between cochlear organoids cultured in +VA and –VA, I performed bulk mRNA sequencing at day 10. DEG analysis showed 833 upregulated genes and 769 downregulated genes in organoids cultured in +VA compared to those cultured in –VA (Fig. 13A). Retinoic acid receptor, *Rarb* was significantly increased in +VA condition. *Dhrs3*, known to be essential for preventing excessive formation of RA, was also increased in +VA. The expression of RNA binding protein, *Rbm24*, also known as a hair cell specific marker, was upregulated by VA. Interestingly, *Otop1*, a vestibular supporting cell markers was upregulated in the presence of VA (Fig. 13A). To identify pathways and biological processes that altered by VA, I performed a gene ontology (GO) analysis and gene set enrichment analysis (GSEA). Through GO analysis and GSEA, it was confirmed that gene sets of ear development, ear morphogenesis, embryonic organ morphogenesis, hair cell differentiation, and inner ear morphogenesis were upregulated during the expansion phase when VA was present (Fig. 13B, C).

During organoid culture, supporting cells are differentiated into hair cells by Notch signaling inhibition. To identify whether supporting cells cultured in -VA have defects with Notch signaling pathway, Notch signaling genes in cochlear supporting cells and cochlear hair cells were analyzed at day 10 and day 12. For these experiments, during 10 days of expansion, organoids were cultured in –VA or +VA condition, then for 2 days of differentiation, organoids cultured under both conditions were supplemented with VA. As a result, at day 10, the Notch ligand in supporting cells *Jag1* and target of Notch cascade, *Hes5*, *Hes1* and *Heyl* were not significantly increased in +VA except for the Notch receptor regulator *Lfng* (Fig. 14). After two days of

differentiation, *Jag1*, *Hes5*, *Hes1*, and *Heyl* were downregulated in both condition of -VA and +VA during the expansion phase. In contrast, Notch ligand genes in hair cells *Jag2*, *Dll3*, and *Dlk2* were significantly increased only in +VA during the expansion phase (Fig. 14). Overall, these data indicate that VA regulate gene sets of hair cell development and hair cell differentiation during expansion of supporting cells. The absence of VA does not impair the expression of Notch ligands and target genes in cochlear supporting cells and cochlear hair cells.

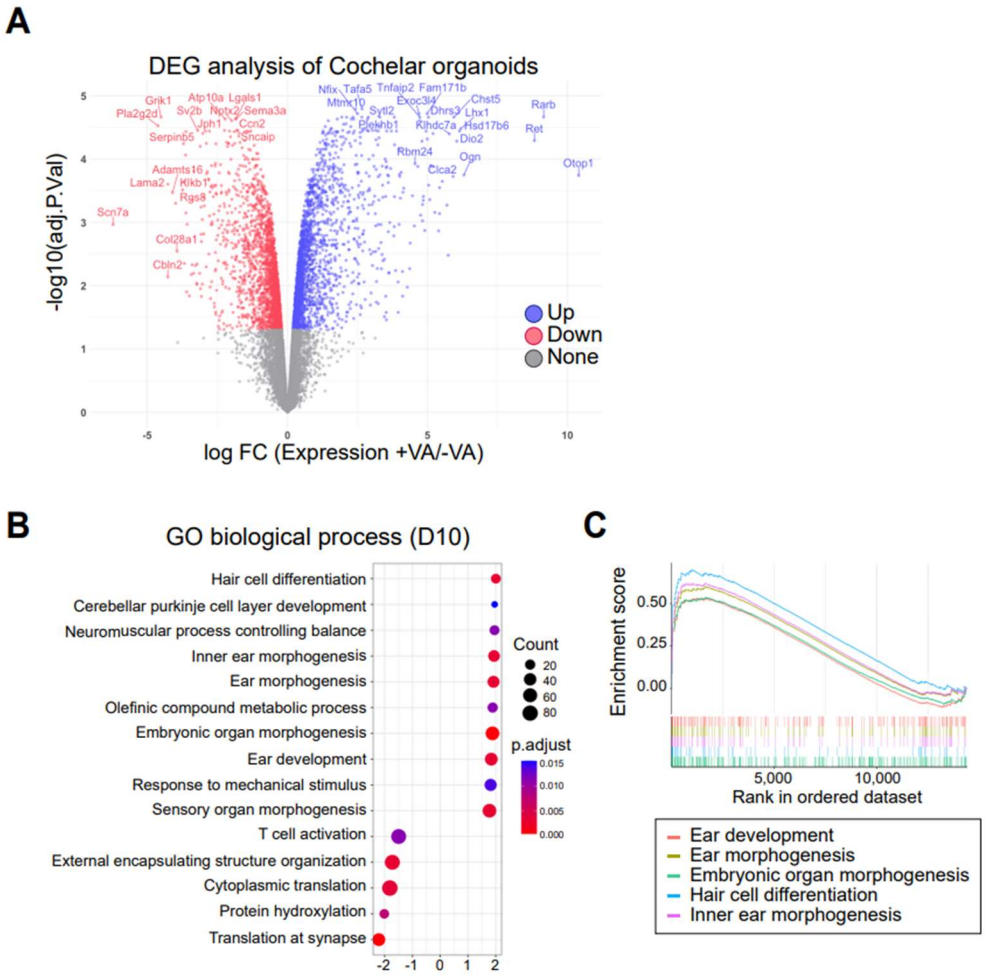

Figure 13. Transcriptome analysis of the cochlear organoids cultured with or without VA during expansion. (A) Volcano plot of DEG analysis of cochlear organoids (at day 10). (B) GO analysis in the biological process of cochlear organoids (at day 10). (C) Gene set enrichment plot of ear development, ear morphogenesis, embryonic organ morphogenesis, hair cell differentiation, and inner ear morphogenesis (at day 10).

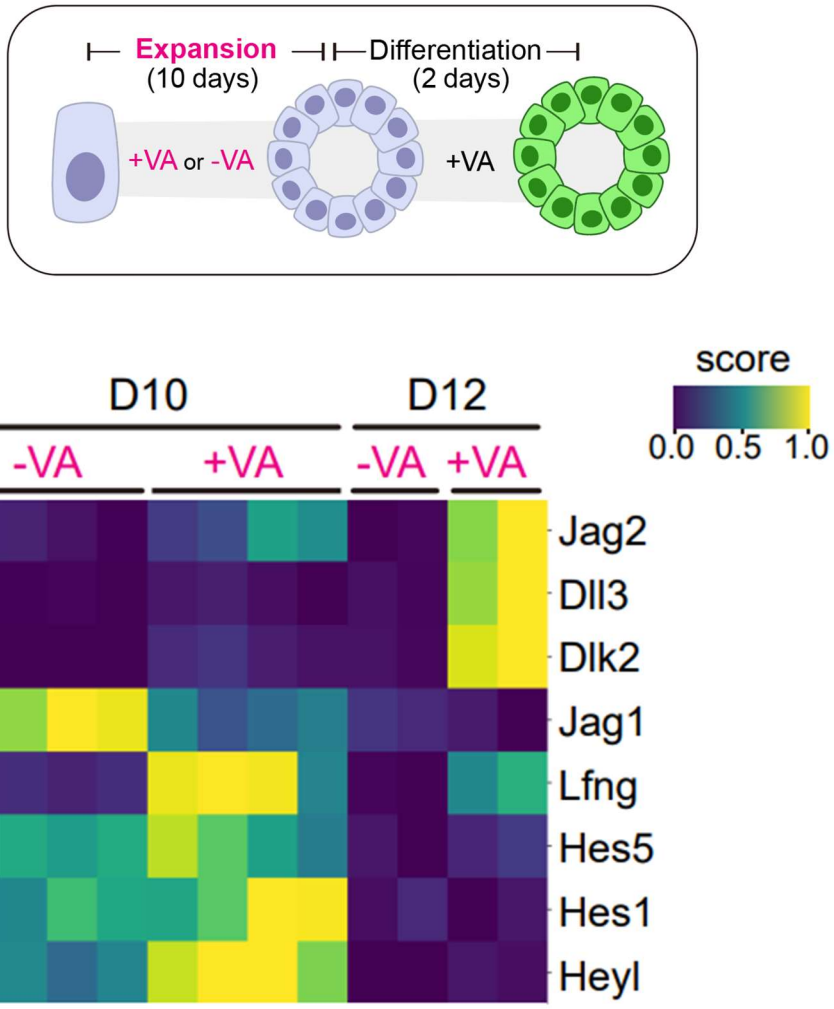

Figure 14. Expression of Notch pathway genes of the organoids cultured with or without VA during expansion. Heatmap of relative expression of mRNA computed for DEGs between –VA and +VA during the expansion phase at day 10 and 12.

6. RA signaling regulatory enzymes in the organoids

In order to activate RA signaling, VA should be converted into RA by RA synthesis enzymes, retinol dehydrogenases (RDHs) which convert VA(retinol) into retinal in a reversible manner, and retinal dehydrogenases (RALDHs) which irreversibly convert retinal into RA[49,50]. To determine whether supporting cells have ability to convert VA to RA, I examine the expression of RA synthesis enzymes in the organoids during the expansion phase in which VA was required. As a result, various enzymes which regulate RA signaling were detected in the organoids at day 10 cultured with VA. Among RDHs, *Rdh1*, *Rdh10*, *Dhrs3*, and *Rdh11* were expressed in cochlear organoids at day10. In addition, in the family of RALDHs, *Aldh1a1* and *Aldh1a3*, which encode Raldh1 and Raldh3, were highly expressed (Fig. 15). Additionally, there is a RA degradation enzyme cytochrome P450 family 26 (CYP26) [51]. To determine whether supporting cells in organoids express this enzymes, I examined the expression of CYP26s at day 10 during the expansion phase. As a result, among the members of CYP26 family, Cyp26b1 was expressed in the organoids (Fig. 15). These data indicate that cochlear supporting cells express all types of enzymes that enable precise regulation of RA signaling. These enzymes may contribute to the fine-tuning of RA signaling in supporting cells.

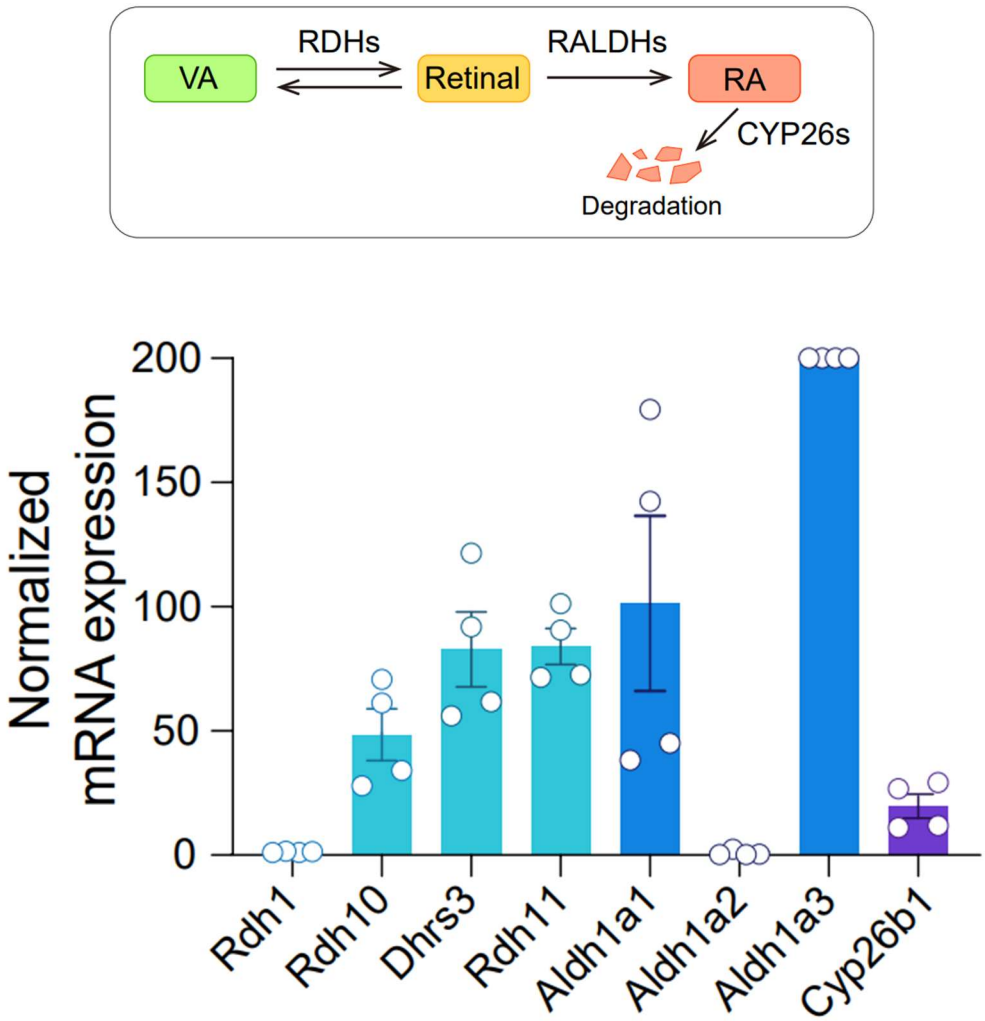

Figure 15. mRNA levels of the enzymes that regulate RA signaling. Relative mRNA expressions of RA signaling regulatory enzyme at day 10. Data exceeding 200 was indicated as 200.

IV. DISCUSSION

Mammalian hair cells cannot be regenerated in the postnatal stage. Immature supporting cells can trans-differentiate or mitotically differentiate into hair cells by hair cell fate-inducing cues such as inhibition of Notch signaling and activation of Wnt signaling[40,41]. However, supporting cells lose their capacity of hair cell regeneration after neonatal period. In this study, using a cochlear organoid model, I investigated the effect of RA signaling on hair cell differentiation. By specifically removing VA from the expansion or the differentiation phase during organoid culture, I found VA affects the plasticity of supporting cells to form hair cells.

RA, an active derivative from VA, is synthesized by two key enzymes. First, retinol dehydrogenases (RDHs) convert VA into retinal and then aldehyde dehydrogenases (ALDHs) convert retinal into RA[38, 39]. Therefore, for using VA to activate RA signaling, these enzymes are essential. Because it has not been discovered whether there are RA synthesis enzymes in the cochlear supporting cells, I confirmed the expression of RA enzymes during the expansion phase when supporting cells expanded. This finding supports the idea that cochlear supporting cells express RA synthesis enzymes so that they can synthesize RA themselves. Once supporting cells undergo maturation, they lose regenerative capacity for forming new hair cells when hair cell loss occurs. According to our knowledge, RA is required to maintain the ability of supporting cells to differentiate into hair cells in the neonatal period. Perhaps, the loss of regenerative capacity of mature supporting cells after the neonatal period may be due to a reduced activation of RA signaling caused by the absence of RA synthesis enzymes. To test this interesting hypothesis, it would be necessary to verify the expression of the enzymes in the supporting cells during and after the neonatal period in both mouse in vivo and cochlear organoids in the future study.

Xiao-Jun-Li *et al* suggested that one of the reasons why mature supporting cells cannot produce hair cells is that they cannot de-differentiate into prosensory cells[33]. When the organoids cultured with the sensory epithelium of postnatal day 5 (P5) mice, the time when cochlear supporting cells lose their regenerative capacity, both organoid forming efficiency and organoids size were decreased during the expansion phase compared to the organoids derived from neonatal mice (postnatal day 2). Also, subsequent hair cell differentiation was compromised during the differentiation phase in the same organoids. Xiao-Jun-Li *et al*. suggested that the expression of Lin28b, which promotes hair cell production at neonatal stage, is necessary for supporting cells to de-differentiate into prosensory cells. However, we found no significant differences in Lin28b expression between organoids cultured with VA or without VA in DEGs analysis of mRNA-sequencing data. Therefore, the defect in organoids cultured in the absence of VA may have a different mechanism other than the failure to return to prosensory cells.

Rbm24 is a RNA-binding protein that can regulate alternative splicing, RNA stability, and translation efficiency[35-37]. Rbm24 is expressed in mouse cochlear hair cells and vestibular hair cells[43]. Also, Rbm24 is expressed in some of Lgr5-positive supporting cells, inner phalangeal cells, border cells, and greater epithelial of ridge cells in neonatal mouse cochlear[30] indicating that expression of Rbm24 may be required not only for hair cells but also for supporting cells during the neonatal period. Rbm24 has been recognized as a target of Atoh1, which is the master regulator of hair cell differentiation[44]. Recently, it was demonstrated that Rbm24 affects the alternative splicing of various hair cell-specific markers such as *Cdh23*, *Myo7a*, *Ush1c* and *Ptprq*[34]. Furthermore, *Rbm24* knockout mice shows degeneration of hair cells by postnatal day 19[45]. Therefore, Rbm24 is one of the important factors regulating hair cell differentiation and protecting hair cells from degeneration. In our data, *Rbm24*

expression was downregulated in supporting cells cultured in the absence of VA. This downregulation of *Rbm24* may cause defects in supporting cells in expressing proteins essential for hair cell differentiation in the subsequent differentiation phase. Although the role of Rbm24 in regulating the expression of Atoh1 has not yet been discovered, whether Rbm24 has the potential to regulate the mRNA stability of Atoh1 still needs to be investigated. Also, increased Rbm24 expression by Atoh1 may act as a positive feedback mechanism to further increase Atoh1 and other essential proteins for hair cell differentiation. Further studies will examine whether overexpression of Rbm24 can rescue the capacity of supporting cells to differentiate into hair cells in the absence of VA.

Albert A.B Edge's research group which developed the organoid culture method reported that some of vestibular supporting cell genes were observed in the organoids by single-cell mRNA sequencing[32]. Due to limitations in detailed annotation, cell types composing the organoid are unknown. In our data, a vestibular supporting cell specific marker *Otop1*[31] is upregulated in organoids cultured with VA, even though the organoids were generated from cochlear epithelium. Hence, to determine whether vestibular supporting cells in organoids were derived from cochlear epithelium, single cell mRNA analysis with detailed annotation of the organoid model is necessary.

These studies will provide insight into the mechanisms of how RA signaling regulates the plasticity of supporting cells. Moreover, it will help subsequent research on the effects of RA signaling on the loss of regenerative capacity of mature supporting cells.

We conducted bulk mRNA sequencing to identify differentially expressed genes in supporting cells cultured with or without VA at day 10, just before the dramatic increase in Atoh1 expression induced by Notch inhibition. However, the sequencing data showed an increase of gene-sets associated with hair cell development and

differentiation in the presence of VA. This result suggests that the cell fate has been determined already, at day 10. To complement this, it would be useful to analyze transcriptome through single cell mRNA sequencing at several time points during the organoid culture process, from the early expansion phase to the differentiation phase. This allows us to explore the diverse networks within the transcription factors and molecular signaling pathways involved in maintaining the plasticity of supporting cells regulated by RA signaling.

V. CONCLUSION

Overall, these data suggest that RA signaling is required for cochlear supporting cells to differentiate into hair cells. Here, it was revealed that supplement of VA only during the expansion phase is sufficient for supporting cells to maintain their capacity to differentiate into hair cells. This result indicated that VA does not directly affect cochlear hair cell differentiation stage but is necessary for priming cochlear supporting cells for the ensuing differentiation process. RA synthesis enzymes, which convert VA to RA, are observed in the supporting cells of the organoid model. These data demonstrate that RA signaling plays an important role in maintaining the plasticity of supporting cells which is required for their differentiation into hair cells.

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ABSTRACT (IN KOREAN)

달팽이관 지지세포의 가소성 유지에 있어 레티노산 신호전달의 필요성

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최 지 혜

포유류의 경우, 신생기 동안 달팽이관 유모세포는 지지세포에 의해 재생 가능하다. 하지만, 신생기 이후에는 유모세포의 재생능력이 감소하여 환경에 의한 청력 상실은 되돌릴 수 없다. 지지세포가 생후 초기에 유모세포를 형성할 수 있는 메커니즘은 완전히 밝혀지지 않았다. 레티노산은 내이를 비롯한 다양한 장기의 발달에 필수적인 것으로 알려져 있다. 그러나 출생 후 유모세포 분화에 대한 레티노산의 효과는 명확하게 밝혀지지 않았다. 본 연구에서는 달팽이관 오가노이드 모델을 이용하여 레티노산이 출생 후 유모세포 분화에 어떤 영향을 미치는지 검증해보고자 하였다. 오가노이드 모델은 확장과 분화라는 두 가지

순차적 단계에 의해 형성된다. 먼저, 레티노산 신호전달이 유모세포의 분화에 영향을 주는지 확인하기 위해 비타민A (VA)를 이용해 withdrawal test를 진행하였다. 오가노이드 배양 중에 VA가 없으면, 유모세포 수가 현저히 감소하는 것을 발견했다. 그 다음으로, 확장과 분화 구간 중 어떤 구간에서 VA가 영향을 주는지 확인하기 위해 구간을 나누어 VA를 제거하였다. 흥미롭게도 확장 기간 중에만 VA를 제거했을 때, 분화 기간에 VA를 주더라도, 유모세포 분화에 필수적인 전사 인자의 발현과 그에 따른 유모세포 분화 정도에 영향을 주는 것을 확인 하였다. 그러나 분화 중에만 VA를 제거했을 때는 전사 인자 발현과 유모세포 분화도에 영향을 주지 않았다. 레티노산 신호전달에서, VA는 레티노산 합성 효소인 retinol dehydrogenases (RDHs) 와 retinal dehydrogenases (RALDHs) 에 의해 레티노산으로 변환된다. 지지세포가 VA를 레티노산으로 전환할 수 있는지 확인하기 위해 확장기간 중 레티노산 합성 효소의 발현을 비교했다. 그 결과, 오가노이드의 VA가 필요한 시기인 확장기에 오가노이드에서 다양한 레티노산 합성 효소가 발현 하는 것을 알 수 있었다. 이러한 결과는 레티노산 신호 전달이 달팽이관 유모 세포를 형성할 수 있는 지지세포의 가소성을 유지하는 데 중요하다는 것을 시사한다.

핵심되는 말 : 유모세포, 레티노산, 달팽이관 오가노이드