Roles of sonic hedgehog and follistatin signaling cascade in the establishment of tonotopy in the mammalian cochlea

Hei Yeun Koo

Department of Medical Science

The Graduate School, Yonsei University

Roles of sonic hedgehog and follistatin signaling cascade in the establishment of tonotopy in the mammalian cochlea

Hei Yeun Koo

Department of Medical Science

The Graduate School, Yonsei University

Roles of sonic hedgehog and follistatin signaling cascade in the establishment of tonotopy in the mammalian cochlea

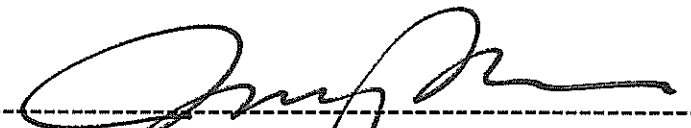
Directed by Professor Jinwoong Bok

The Doctoral Dissertation
submitted to the Department of Medical Science
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

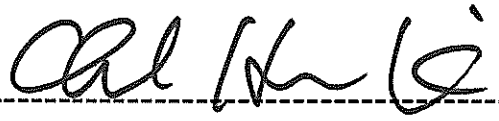
Hei Yeun Koo

December 2021

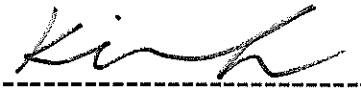
This certifies that the Doctoral Dissertation of Hei Yeun Koo is approved.



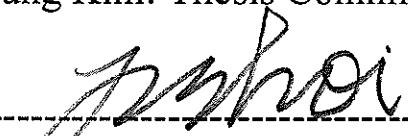
Thesis Supervisor: Jinwoong Bok



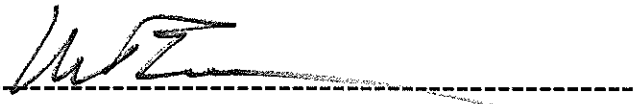
[Chul Hoon Kim: Thesis Committee Member#1]



[Unkyung Kim: Thesis Committee Member#2]



[Jae Young Choi: Thesis Committee Member#3]



[Won Taek Lee: Thesis Committee Member#4]

The Graduate School
Yonsei University

December 2021

ACKNOWLEDGEMENTS

I am so honored and happy to finally receive my Ph.D and convey my gratitude to all the people who gave support and courage for me. There are so many people I would like to express my gratitude to, but first, I would like to thank my supervisor Prof. Jinwoong Bok for guiding me with love and patience for a long time. Thanks to his guidance, I was able to learn and grow a lot during my degree course. Also, I would like to express my gratitude to the other thesis committee members, Prof. Unkyung Kim, Chul Hoon Kim, Jae Young Choi, Won Taek Lee who improved the quality of the thesis with objective and critical comments. I would also like to thank my lab colleagues for their help and support in many ways. Last but not least, I would definitely like to express my gratitude to my family who supported and believed in me from the nearest place during the long years of my degree. It might be not possible to finish my degree without their support and dedication. I apologize for the names have not been personally mentioned one by one and thank you again.

Heiyeun Koo

December 2021

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Roles of sonic hedgehog and follistatin signaling cascade in the establishment of tonotopy in the mammalian cochlea

Hei Yeun Koo

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jinwoong Bok)

The vertebrate cochlea is tonotopically organized to discriminate various frequencies of sounds ranging from low to high frequency. The word “Tonotopy” came from the Greek word. Tono means frequency, topos mean place. Thus, tonotopy in the cochlea means the spatial arrangement of where sounds of different frequency are processed along the cochlear duct. Even though there are many known factors that have morphological, functional gradient along the tonotopic axis, how this tonotopic organization is “established” during cochlear development has begun to be understood recently. Previous reports propose that the tonotopic organization is established by a sequential signaling cascade that is coordinated by an increasing base-to-apex gradient of Sonic hedgehog (SHH) signaling both in chicken and mouse. In chicken basilar papilla, it was shown that Bmp7 and Retinoic acid (RA) are key downstream targets of SHH in

mediating the tonotopic organization. However, in mouse cochlea, the downstream mediator of SHH has not been examined. Here, we suggest that Follistatin (Fst) is the downstream effector of Shh signaling to build tonotopy in mouse cochlea by promoting and maintaining the apical cochlear identity. We revealed that Fst is required for the tonotopically patterned stereociliary morphology along with tonotopically graded gene expression and proper low-frequency sound discrimination by preserving the apical cochlear identity from developing to mature cochlea.

Key words: Tonotopy, basilar papilla, cochlea, Shh signaling, Fst

Roles of sonic hedgehog and follistatin signaling cascade in the establishment of tonotopy in the mammalian cochlea

Hei Yeun Koo

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jinwoong Bok)

I. INTRODUCTION

Auditory stimuli from nature are the mixture of various frequencies of sounds. Thus, the ability to discriminate precise frequency is critical to get meaningful information from mixed sounds and is fundamental to animal communication and survival. The peripheral auditory organ, the cochlea, is expertized to discriminate diverse frequencies of sound and transmit those sound signals to the brain accurately, thanks to a special topographic organization known as the tonotopy. The cochlea is a spiral-shaped tubular structure that contains auditory sensory organ known as the basilar papilla in chicken and the organ of Corti in mouse. Depending on where they are along the cochlear duct, auditory hair cells in the sensory organs are tuned to specific frequencies. High-frequency sounds are more sensitive to hair cells in the basal (proximal) part of the cochlea, while lower-frequency sounds are more sensitive to hair cells in the apical (distal) region. The gradual change in

various morphological and physiological features along the cochlear duct allows the ability to identify sound frequencies. The gradual morphological change of the stereocilia responsible for detecting sound vibrations is the most notable tonotopic trait in hair cells. In many animals, including reptiles, birds, and mammals, the stereocilia in basal hair cells are shorter than those in apical hair cells. Basal hair cells, on the other hand, have more stereocilia per hair cell than apical hair cells [1-4]. Stereocilia in mammalian outer hair cells have a "V-shaped" angle that is larger at the base and gradually narrows at the apex [5,6]. Furthermore, the spiral ligament, tunnel of Corti, basilar membrane, and tectorial membrane structural features have been reported to vary gradually along the tonotopic axis [7-10].

Several genes associated to cochlear physiology are expressed differently along the tonotopic axis, in addition to morphological features. Non-syndromic hearing loss genes such as Slc26a5, Tectb, and Otof, for example, are highly expressed near the apex than at the base of the mouse cochlea [11]. Furthermore, ion channel distribution changes along the cochlea. The voltage-gated potassium channel Kcnq4 is more abundant near the base of the mouse cochlea than at the apex [12], another voltage-gated potassium channel, Kcna10, is also highly expressed at the apex [11]. Moreover, Cacna1d, which encodes the calcium channel CaV1.3a1, and Calb1, which encodes the calcium-binding protein Calbindin, are expressed more highly at the apex of the mammalian cochlea [11,13-16].

Even though many anatomical and physiological features that facilitate frequency discrimination have been identified, the underlying molecular mechanism(s) for establishing this tonotopic organization has only recently begun to be understood. Bone morphogenetic protein 7 (BMP7) and retinoic acid (RA) regulate cellular characteristics of hair cells related with tonotopic functions in the chicken cochlea [17,18]. Both BMP7 and RA signals are graded along the developing cochlea, becoming stronger at the apex and gradually weaker towards the base, favoring apical hair cell morphology in chickens. Subsequently, the gradient of sonic hedgehog (SHH) signaling emanating from the notochord and floor plate, which is stronger at the apex and weaker toward

the base, was revealed to function as an extrinsic signal that determines the BMP7 gradient in the chicken cochlea [3]. Thus, in the chicken cochlea, the SHH-BMP7-RA signaling cascade appears to function in concert to establish tonotopic organization.

The SHH gradient is also important in providing a regional identity to the cochlear primordium in mammalian cochleas [3,19]. However, because Bmp7 is neither graded nor positively regulated by SHH in mice, the downstream mediators of SHH do not appear to be conserved across chicken and mouse, suggesting a divergence in the mechanisms in establishing the tonotopic organization in birds and mammals [3]. Interestingly, SHH has been demonstrated to positively regulate the expression of follistatin (Fst), an antagonist of TGFβ signaling, to establish an FST gradient, stronger at the apex and weaker toward the base in developing mouse cochlea [4,20]. This SHH-induced FST gradient appears as soon as the cochlear primordium develops from the ventral otocyst and lasts until postnatal (P) 8 [6], suggesting a role of FST in the tonotopic organization in mammals.

We propose in this study that FST is critical for the establishment of tonotopic organization in the mammalian cochlea. Our findings reveal that in the absence of FST throughout cochlear development, the apical identity specified by SHH is lost, resulting in a loss of apical characteristics such as stereociliary length, number, and angle in mature cochlea. Also, in the absence of FST, the gene expression profile of the apical cochlea shifts to that of the basal cochlear region. Finally, the lack of FST function causes molecular and morphological alterations in the apical cochlea, resulting in low-frequency specific hearing loss.

II. MATERIALS AND METHODS

1. Mice

Fst heterozygous (*Fst*$^{+/-}$) mice were purchased from Jackson laboratory (JAX.002788) [21] to get Fst Knock-out mice. To make inner ear specific cKO (*Pax2-Cre; Fst*$^{lox/lox}$), Tg(*Pax2-Cre*)1Akg/Mmnc (*Pax2-Cre*; MMRRC, NC) [22] mice were bred with *Fst*$^{lox/lox}$ mice [23] kindly given by Dr. Martin Matzuk from Baylor college of Medicine. For *Pax2-Cre; Smo*$^{M2/+}$, Gt (ROSA)26Sortm1(Smo/EYFP)Amc/J mice (JAX.005130) [24] were bred with *Pax2-Cre* mice. *Emx2-Cre* mice (RIKEN) were bred with Smotm2Amc/J (*Smo*$^{lox/lox}$) mice (JAX.004526). B6.129S2(Cg)-Msx1tm2.1(cre/ERT2)Bero/J mice (JAX.027850) were bred to B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J (JAX.007914) mice to make double hetero. To get Fst KO with the Msx1CreERT2 allele positive, the triple heterozygous, *Msx1*$^{CreERT2/+}$*; Tdtomatolox*$^{/+}$*;Fst*$^{+/-}$ mice were bred to *Tdtomato*$^{lox/lox}$; *Fst*$^{+/-}$ mice. FST transgenic mice samples [25,26] were obtained from Angelika Doetzlhofer, Johns Hopkins University School of Medicine. A cassette encoding the human FST-288 isoform is under control of a tetracycline-responsive promoter element (tetO). The R26-M2rtTA mice were from Jackson Laboratories (JAX.006965) [27]. To induce FST transgene expression, doxycycline (dox) was delivered to time-mated females via ad libitum access to feed containing 2 grams of dox per kilogram feed (Bioserv, NJ, USA). The pups received doxycycline through the milk from the nursing females. Mice were mated and the morning of vaginal plug formation counted as E0.5. All animal protocols were approved by the Institutional Animal Care and Use Committee at Yonsei University College of Medicine.

2. Paint-fill injection

E15.5 embryos were harvested, and the head was fixed overnight in Bodian's fixative. Specimens were dehydrated in ethanol and then cleared in

methyl salicylate. The inner ears were visualized by injection of commercial correction liquid in methyl salicylate (1:800) into the lumen of the inner ear. The micropipette was inserted on the lateral surface of inner ear ampulla.

3. *In situ* hybridization and measurement of *in situ* intensity

In situ hybridization was performed as previously described [28]. The whole or hemi-sectioned heads of embryos from E11.75 to E15.5 were fixed in 4% paraformaldehyde overnight, dehydrated in 30% sucrose for overnignt. Samples were embedded in Tissue-Tek optimum cutting temperature (OCT) compound and sectioned at 12 μm thickness using a cryostat (HM 525, Thermo Scientific, Rockford, IL, USA). An antisense RNA probe for *Ptch1*, *Gli1*, *Sox2*, *Atoh1*, *Fst*, *Msx1*, *Efnb2*, *Slitrk3*, *A2m*, *Inhba* were prepared as previously described [4,6,29]. The micrographs of gene expression patterns were acquired using Leica DM2500 optical microscopes. All in situ hybridization figures are representative of at least three different samples in two or more independent experiments. To quantify the expression of each gene, in situ hybridization signal intensities were measured using Multi Gauge software (FUJIFILM). Images of all cochlear sections were collected and numbered from the base to the apex. The number of cochlear sections were around 40 to 50 in E15.5 control slides, and less for Fst KO mutants. To measure signal intensity of a cochlear section, a rectangle (12X4.5 μm for *Msx1*, *Fst*, *A2m*, *Inhba*, 20X4.5 μm for *Slitrk3*, *Efnb2*, *Ptch1*, and *Gli1*) was positioned on the cochlear epithelial region that shows hybridization signals for the gene of interest (e.g., the lesser epithelial ridge area for *Msx1*), and the signal intensity within the rectangle was measured as a quantum level (Q). Then, the same size rectangle was positioned in a nearby epithelial or mesenchymal region that is not expressing the gene of interest to measure background level (B). The intensity of in situ hybridization signal in each cochlear section was calculated by subtracting the background level (B) from the quantum level (Q) and designated as QB. Cochlear sections that were obliquely sectioned due to the coil structure such as the curvature of the spiral were excluded from the

analysis. For each gene, the lowest QB value among all sections with positive signals from a cochlea was set as 'the threshold QB', and the relative signal intensity of each section was calculated as follows:

% intensity of a section=((QB value of a section)-(the threshold QB value))/((the highest QB value among all sections)-(the threshold QB value))

The *in situ* hybridization signal with the highest QB value among all cochlear sections from a wild type was designated as 100% intensity and the signal with the threshold QB value as 0% intensity. The percent intensities of each section from wild type and mutant cochleae (Y-axis) were plotted according to the section number starting from the base to the apex (X-axis).

4. Immunohistochemistry

E15.5, E18.5 embryos and P5 postnatal mice were harvested. Head of the mice was hemi-sectioned, and the inner ear structure so called peanut was isolated. Isolated peanut was fixed in 4% PFA for overnight, and the cochlea was dissected out from the inner ear. Lateral wall, Reissner's membrane, spiral ganglia were removed. Dissected cochlear was stained with following primary antibodies: Anti-Myo7a antibody (Proteus Biosciences, CA, USA), and Tuj1 (BioLegend, CA, USA). We also used the following secondary antibodies: Alexa 568-Phalloidin (Invitrogen, CA, USA), goat anti-mouse Alexa 488, donkey anti-rabbit Alexa 568. Stained cochlea was mounted by Prolonged Gold anti-fade (Thermo Fisher Scientific, Massachusetts, USA) and was covered with coverslip on the coated slide glass. Imaged were captured using confocal microscopes (Zeiss LSM700 and LSM 780).

5. Scanning electron microscopy (SEM) and measurement

Cochlear samples from 4weeks Fst cKO mice, Fst overexpressed TG mice were prepared for SEM analysis as previously described (Son et al.,

2015). Platinum coated specimens were mounted on a stub holder and imaged using a cold field emission scanning electron microscope (JSM-7001F; JEOL). For measurements of stereociliary lengths along the cochlear duct, images of hair cells were from the lateral side so that the tallest stereocilia of each hair cell could be easily visible for analysis. For measurements of stereociliary angle and numbers, images were taken to V-shaped outer hair cells are easily seen. Images were obtained from entire cochlear regions and were combined using ImageJ (NIH), and the cochlear regions were divided into five regions 10–18%, 28–36%, 46–54%, 64–72%, and 82–90% from the basal end to represent base, mid-base, mid, mid-apex, and apex of the cochlea, respectively. We excluded 0-10% (basal end) and 90-100% (apical end) regions from measurement because of the irregular stereociliary morphology disturbs the reliable measurement results. The lengths of the 3 tallest stereocilia were measured from at least 30 hair cells in each region from 3 control and 3 mutant mice using ImageJ software (NIH). The angles of stereocilia were measured by ImageJ program (NIH) and the numbers of stereocilia were manually counted from at least 30 hair cells in each cochlear region. Averages of the three tallest stereocilia, each measured value of angles and numbers in each hair cell were plotted as a box plot using Prism 7.0 software (GraphPad, USA). In the box plot, individual dots represent individual data values, the points outside the whiskers represent outliers.

6. RNA-Seq sample preparation and Sequencing

For sequencing library construction, RNAs from three different regions, base, middle and apex of mouse cochlea are extracted from both 4weeks Fst floxed and Fst cKO samples. Three replicates from each sample group were used. RNA was prepared for sequencing using the TruSeq RNA Sample Prep Kit v2 with Ribo-Zero (Illumina). Libraries were quantified, pooled, and a 101-cycle paired-end sequence run was performed using TruSeq SBS Kit v3 on a HiSeq 4000 sequencer (Illumina). Sequencing data were generated using HiSeq Control Software v3.3 for system control and base calling through Real

Time Analysis v2.5.2 (Illumina). The BCL (base calls) binary is converted into FASTQ using illumine package bcl2fastq (v2.16.0.10).

7. RNA-Seq data analysis

Sequencing reads in Fastq files with three replicates of each sample group were aligned to Mus musculus genome (assembly GRCm38) with STAR aligner (version 2.5.2b). Read counts and fragments per kilobase of transcript per million mapped reads (FPKM) were calculated based on the mouse CRCm38 Ensembl Release 82 gene annotation. Cuffdiff (version 2.2.1) was used for searching differentially expressed genes (DEGs) setting pairwise comparison for all sample groups at once. Cuffdiff output data were then used for downstream analysis in R (version 3.5.0). Principal component analysis was performed based on FPKM values using “prcomp” and “ggplot2” packages in R. Heatmaps were generated using hierarchical clustering using “heatmap.2” package in R.

8. Differentially expressed genes (DEGs)

To determine significant DEGs for this study, only genes with the value of averaged FPKM >= 1.0 in at least one of all six sample groups (B,M,A for floxed and cKO) were considered. 14,994 (32.2%) genes out of total 46,597 Ensembl annotated genes were in the category. Then, those were categorized depending on the changes of the value of FPKMs as UP (1) and DOWN (2). First, DEGs were searched depending on the regions of mouse cochlea in Fst floxed samples. 1,256 genes that showed at least 1.5-fold change of FPKM with directionality from base to middle to apex were selected, allowing 5% variable against the directionality. Among those, 585 genes showed increase in the value of FPKM either at middle or at apex compared to base and 671 genes showed decrease. Then, these genes further categorized into UP-1, UP-2, UP-3, DOWN-1, DOWN-2, and DOWN-3 as following.

UP-1 - among the 585 genes, genes with the percent increase of FPKM value in cKO is at least 25% smaller than the percent increase in Fst floxed (control) ((FPKM of control Apex -FPKM of control Base)/(FPKM of control Base) ×0.75 ≥(FPKM of cKO Apex - FPKM of cKO Base)/(FPKM of cKO Base)), or genes with the FPKM value in cKO apex is smaller than the one in control apex (FPKM of control Apex×0.75≥FPKM of cKO Apex), or genes with the percent increase of FPKM from Mid to Apex in cKO is smaller than the one in control ((FPKM of control Apex - FPKM of control Mid)/(FPKM of control Mid) ×0.5 ≥ (FPKM of cKO Apex - FPKM of cKO Mid)/(FPKM of cKO Mid))

UP-2 – after excluding UP-1 genes from the 585 genes, genes with both changes in control and cKO are less than 1.5-fold in all regions

UP-3 – genes left by excluding genes in UP-1 and UP-2 category from the 585 genes

DOWN-1 – among the 671 genes, genes with the percent decrease of FPKM value in cKO is at least 25% smaller than the percent decrease in control ((FPKM of control Base -FPKM of control Apex)/(FPKM of control Base) ×0.75 ≥(FPKM of cKO Base - FPKM of cKO Apex)/(FPKM of cKO Base)), or genes with the FPKM value in cKO apex is larger than the one in control apex (FPKM of cKO Apex≥FPKM of control Apex×1.25), or genes with the percent decrease of FPKM from Mid to Apex in cKO is smaller than the one in control ((FPKM of control Mid - FPKM of control Apex)/(FPKM of control Apex) ×0.5 ≥ (FPKM of cKO Mid - FPKM of cKO Apex)/(FPKM of cKO Apex))

DOWN-2 – after excluding DOWN-1 gene from the 671 genes, genes with both changes in control and cKO are less than 1.5 fold in all regions

DOWN-3 – genes left by excluding genes in DOWN-1 and DOWN-2 category from the 671 genes.

9. Auditory Brainstem Response (ABR)

ABR thresholds were measured in a sound-proof chamber using Tucker-Davis Technologies (TDT) RZ6 digital signal processing hardware and the BioSigRZ software package (Alachua, FL, USA). Sub-dermal needles (electrodes) were positioned at the vertex and ventrolateral to the right and left ear of the anesthetized mice. Calibrated click stimulus (10 μs duration) or tone burst stimuli (5 ms duration) at 4, 6, 8, 10, 12, 18, 24, 30 and 42kHz were produced using the SigGenRZ software package and an RZ6 digital signal processor and delivered to the ear canal through a multi-field 1 (MF1) magnetic speaker (TDT). The stimulus intensity was increased from 10 to 95 dB SPL in 5 dB SPL increments. The ABR signals were fed into a low-impedance Medusa Biological Amplifier System (RA4LI, TDT), which then was used to deliver the signal to the RZ6 digital signal processing hardware. The recorded signals were filtered using a 0.5–1 kHz band-pass filter and the ABR waveforms in response to 512 tone bursts were averaged. The ABR thresholds for each frequency were determined using the BioSigRZ software package.

10. Distortion Product Otoacoustic Emission (DPOAE)

DPOAEs were measured using a combined TDT microphone-speaker system. Primary stimulus tones were produced using an RZ6 digital signal processor and the SigGenRZ software package and delivered through a custom probe containing an ER 10B+ microphone (Etymotic, Elk Grove Village, IL, USA) with MF1 speakers positioned in the ear canal. The primary tones were set at a frequency ratio (f2/f1) of 1.2 with target frequencies at 6, 8, 10, 12, 16, 18, 24, and 30 kHz. The f1 and f2 intensities were set at equal levels (L1 = L2) and increased from 20 to 80 dB SPL in 5 dB SPL increments. The resultant sounds in response to the primary tones were received by the ER 10B+ microphone in the custom probe and recorded using the RZ6 digital signal processor. The input/output (I/O) functions for the DPOAEs were determined

at two specific frequencies (6 and 18 kHz). The intensity levels of the primary tones were increased from 20 to 80 dB SPL in 5 dB SPL increments. At each primary tone for each intensity for the I/O functions, the fast Fourier transform (FFT) was performed using the BioSigRZ software package to determine the average spectra of the two primaries, the 2f1-f2 distortion products, and the noise floors.

11. FM1-43 uptake

Mice were harvested at P5. The head of the mice was hemi-sectioned, and the peanut was isolated. The cochlea was dissected out from the peanut. Dissected cochlear was placed on a superfrost slide (Thermo scientific) and treated with FM1-43 (Invitrogen, Massachusetts, USA) (5uM) dye by applying 50ul per cochlea for 20s. Wash with HBSS once, and the cochlea was mounted with Prolonged Gold anti-fade (Thermo Fisher Scientific, Massachusetts, USA). The images were taken within 48hrs using confocal microscopes (Zeiss LSM700 and LSM 780).

12. Alcian blue staining

P0 pups were harvested and proceeded for skeletal staining with 0.015% Alcian blue (A-3157, Sigma, St. Louis, USA). Briefly, the specimens were treated with 95% ethanol for four days at room temperature, stained with Alcian blue for three days. Specimens were cleared with 1% KOH/20% glycerol for two days and were stored in a 1:1 mixture of glycerol and 95% ethanol. Micrographs of dissected middle ear ossicles were acquired using Leica S8APO microscope.

13. Statistical analysis

Statistical comparisons were made using two-way analysis of variance (ANOVA) with Bonferroni corrections for multiple comparisons for the Edu

graph, stereociliary morphologies (length, angle, number), ABRs, DPOAEs, using Prism 7.0 (GraphPad, San Diego, CA, USA). For cochlear length graphs, t-test was applied using Prism 7.0. All graphs with statistical analysis are expressed as the mean ± standard error. Statistical significance is indicated in the figures as n.s., non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

III. RESULTS

1. Apical identity is lost in the absence of FST in the developing mouse cochlea

To test the hypothesis that SHH-induced FST gradient plays a role in tonotopic organization, we first asked whether regional identity is compromised in the developing cochlea of *Fst*$^{-/-}$ mice. First, the inner ear morphology was observed by the paint-fill injection method. The overall morphology was comparable with control, except for the apical end (A-a, B-b, red arrow). The total cochlear length was similar between two genotypes (C). The regional identity was determined by examining expression patterns of genes exhibiting either an increasing or decreasing basal-to-apical gradient along the developing cochlea in mice [3,19]. In E15.5 wild-type cochlea, *Fst*, *Msx1*, *Slitrk3*, and *Efnb2* are strongly expressed at the apical end and gradually weakened toward the base, serving as apical cochlear markers (Fig. 1D-G) [3]. In contrast, *A2m* and *Inhba* expressions are restricted at the base, serving as basal cochlear markers (Fig. 1L, M, arrows) [3]. Each apical marker appears to define distinct cochlear domains: *Msx1* expression is mostly restricted at the apical end (Fig. 1E, arrow), while *Fst* and *Slitrk3* show strongest expression at the apical end and gradually weaker expression up to the middle cochlear region (Fig. 1D, F). *Efnb2* show even broader expression gradient often showing weak expression up to the basal regions (Fig. 1G). We also visualized graded expression patterns of these regional markers along the cochlear duct by measuring signal intensities of *in situ* hybridization from all cochlear sections and plotting them according to section numbers starting from base to apex (Fig. 1Ta-Tf)[19].

In *Fst*$^{-/-}$ cochlea, in which *Fst* expression is absent (Fig. 1H, asterisks), *Msx1* expression, which is normally restricted to the apical end, is completely abolished (Figs. 1E, I). In addition, expression levels of *Slitrk3* and *Efnb2*, although apical-to-basal gradient is maintained, are greatly decreased in *Fst*$^{-/-}$ cochlea compared to wild type cochlea (Fig. 1F-G, J-K). In contrast, *A2m* and

Inhba expressions are indistinguishable between wild type and *Fst*$^{-/-}$ cochleae (Fig. 1L-M, P-Q). These results suggest that apical cochlear identity, especially the apical end, is lost in *Fst*$^{-/-}$ cochlea, whereas the basal identity is not affected.

It was previously shown that apical cochlear identity is specified by strong SHH signaling at the distal region in the developing cochlea in which apical identity is promoted while basal identity is suppressed [19,30]. We thus asked whether the loss of apical identity in *Fst*$^{-/-}$ cochlea is due to defective SHH signaling. Our *in-situ* hybridization results show that *Ptch1* and *Gli1*, readouts of SHH signaling, show graded expression patterns, stronger at the apex and gradually weaker toward the base, in both wild-type and *Fst*$^{-/-}$ cochleae (Fig. 1N-O, R-S, Tg-Th), indicating that the loss of apical cochlear identity in *Fst*$^{-/-}$ cochlea is not due to defective SHH signaling. Together, these results suggest that FST is required for apical cochlear specification even in the presence of normal SHH signaling.

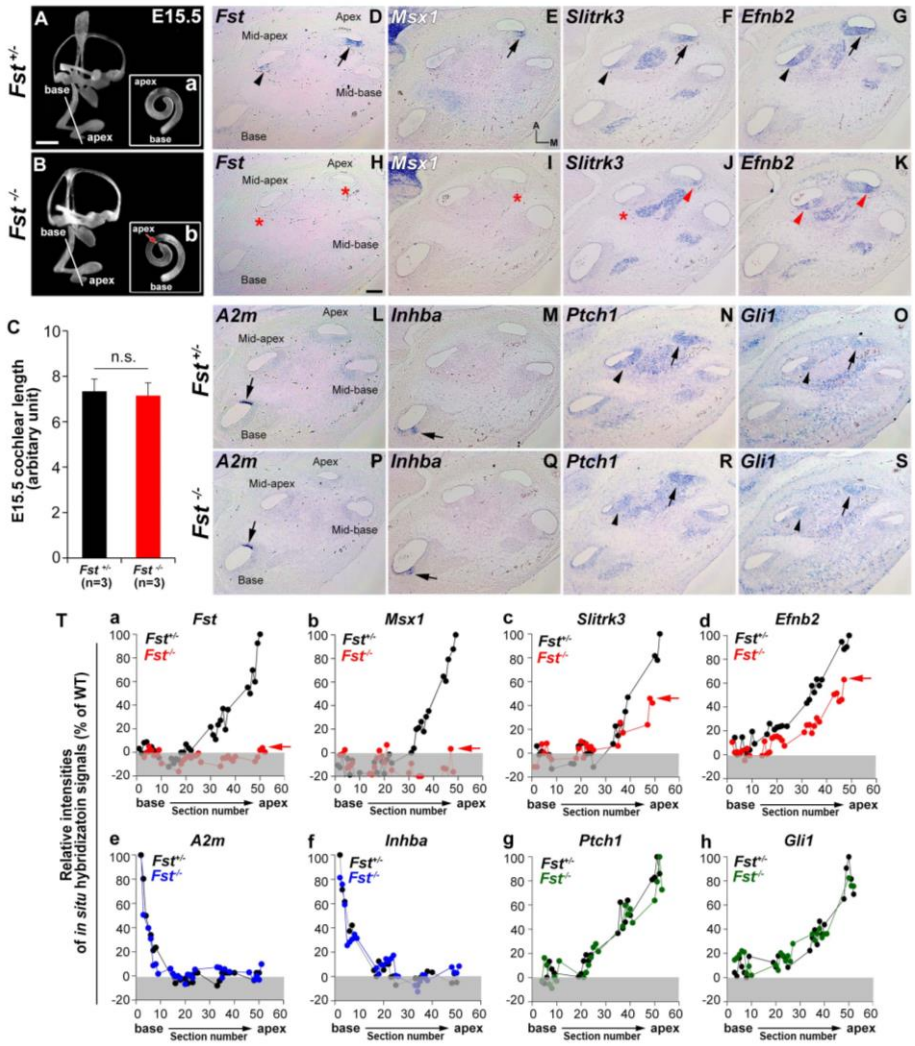


Figure 1. Apical identity is lost in the absence of FST in the developing mouse cochlea. (A-C) The paint-fill analysis shows the normal structure or inner ear except for the apical end (A-a, B-b, red arrow) exhibiting comparable cochlear length between control and *Fst* $^{-/-}$ at E15.5 (C). In *Fst* $^{-/-}$, the absence of *Fst* expression was confirmed (D, H, red asterisks). *Msx1*, the apical marker that are restrictedly expressed at apical end is not expressed in *Fst* $^{-/-}$ (E, I, red asterisk). *Slitrk3* and *Efnb2* are similarly down-regulated in the apical cochlea (F-G, J-K, red asterisk, red arrowheads). On the other hand, the

expression patterns of basal markers, *A2m* and *Inhba*, are comparable with controls (L-M, P-Q, arrow). Shh mediators, *Ptch1* and *Gli1* are indistinguishable with controls (N-O, R-S, arrow, arrowhead). The representative in situ hybridization results shown in D-S are supported by the quantitative analysis of its intensities (apical markers: Ta-d, arrow, basal markers: e-f, Shh mediators: g-h).

2. FST is not sufficient to promote apical cochlear identity

Since *Fst* expression is positively regulated by SHH signaling and both SHH and FST are required for apical specification in the developing cochlea (Fig. 1) [3,19], it is possible that SHH exerts its function in conferring regional identity by establishing signaling gradient to confer regional cochlear identity by promoting apical identity. Thus, we asked whether ectopic FST could promote apical cochlear identity as does ectopic SHH signaling [3]. We overexpressed human *FST* gene in the developing cochlea using *FST*-transgenic (TG) mice that express human *FST* gene in the entire body upon doxycycline administration [25]. Doxycycline was treated staring from E10.5 and overexpression of *FST* in the entire cochlea was confirmed at E15.5 (Fig. 2A, B, H). In *FST-TG* cochlea, apical markers such as *Msx1*, *Slitrk3*, and *Efnb2* are expressed normally, stronger at the apex and gradually weakened toward the base, like controls (Fig. 2D-F, I-K). Consistently, one of the basal cochlear markers *Inhba* is expressed normally in basal cochlear duct (Fig. 2G, L). These results show that FST alone cannot promote apical identity, suggesting that FST is not sufficient for the promotion of apical cochlear identity unlike SHH signaling [3].

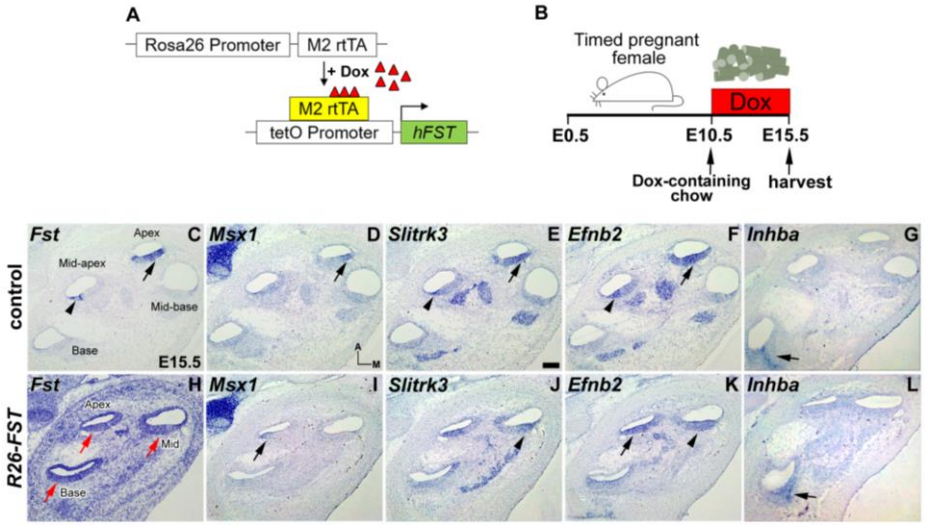


Figure 2. FST is not sufficient to induce apical or inhibit basal cochlear identity. (A-B) Inducible FST transgenic mouse model scheme. FST was overexpressed in whole embryo by crossing the M2rtTA mouse line to an inducible Follistatin (FST) transgenic mouse line, where human FST (hFST) is overexpressed under the control of a Tet-On promoter. M2rtTA tg/+; FST tg/+ mice overexpress hFST ubiquitously upon doxycycline administration. Doxycycline (dox) was treated to pregnant female by dox-containing chow from E10.5 to E15.5. The embryos were harvested at E15.5. The overexpression of Fst is clearly shown (C, H). However, the expression of apical markers is not expanded towards the basal cochlea (D-F, I-K), basal marker Inhba was normally expressed in basal cochlea (G, L).

3. FST is essential for the maintenance of apical cochlear identity

Our loss- and gain-of-function experiments indicate that FST is required for apical cochlear specification in the presence of normal SHH signaling, but FST alone is not sufficient to promote apical identity (Fig. 1 and Fig. 2). To further explore functional relationship between SHH and FST in apical specification, we examined the cochlea of *Fst*$^{-/-}$ mutants at E11.75 when the apical identity is first conferred by strongly SHH signaling [19,30]. Surprisingly, unlike E15.5 cochlea, *Msx1* is strongly expressed at the tip of cochlear primordium of E11.75 embryos (Fig. 3Ah, Ak).

We compared the expression patterns of *Fst*, *Msx1* and *Ptch1* during cochlear development. At E11.5, the earliest age at which apical identity becomes evident by *Msx1* expression that is dependent on strong SHH signaling in mice [3], all three genes are expressed strongly at the apical aspect of cochlear primordium (Fig. 3Aa- Af). At E12.5, when the cochlear duct begins to coil, *Fst* and *Msx1* is exclusively expressed at the apical cochlea but is absent from the base (Fig. 3Ba, Bb) whereas *Ptch1* expression is observed at both apical and mid-basal regions in E12.5 wild-type cochlea (Fig. 3Bc). At E13.5, apical expressions of *Fst* and *Msx1* maintain in wild-type cochlea (Fig. 3Ca, Cb), *Ptch1* is strongly expressed in mid-apical cochlear region compared to basal regions (Fig. 3 Cc). Interestingly, *Msx1* expression, which is completely absent in Fst$^{-/-}$ cochlea at E15.5 (Fig. 1I), is observed in the apical aspect of cochlear primordium at E11.75 in *Fst*$^{-/-}$ cochlea (Fig. 3Ak), suggesting that apical identity is specified in the developing cochlea in the absence of FST function. *Msx1* expression at the apical cochlea is slightly downregulated at E12.5 and greatly weakened by E13.5 (Fig. 3Be, Ce). These results suggest that FST is neither necessary nor sufficient to promote apical cochlear identity but is required for the maintenance of apical identity that is specified by SHH signaling.

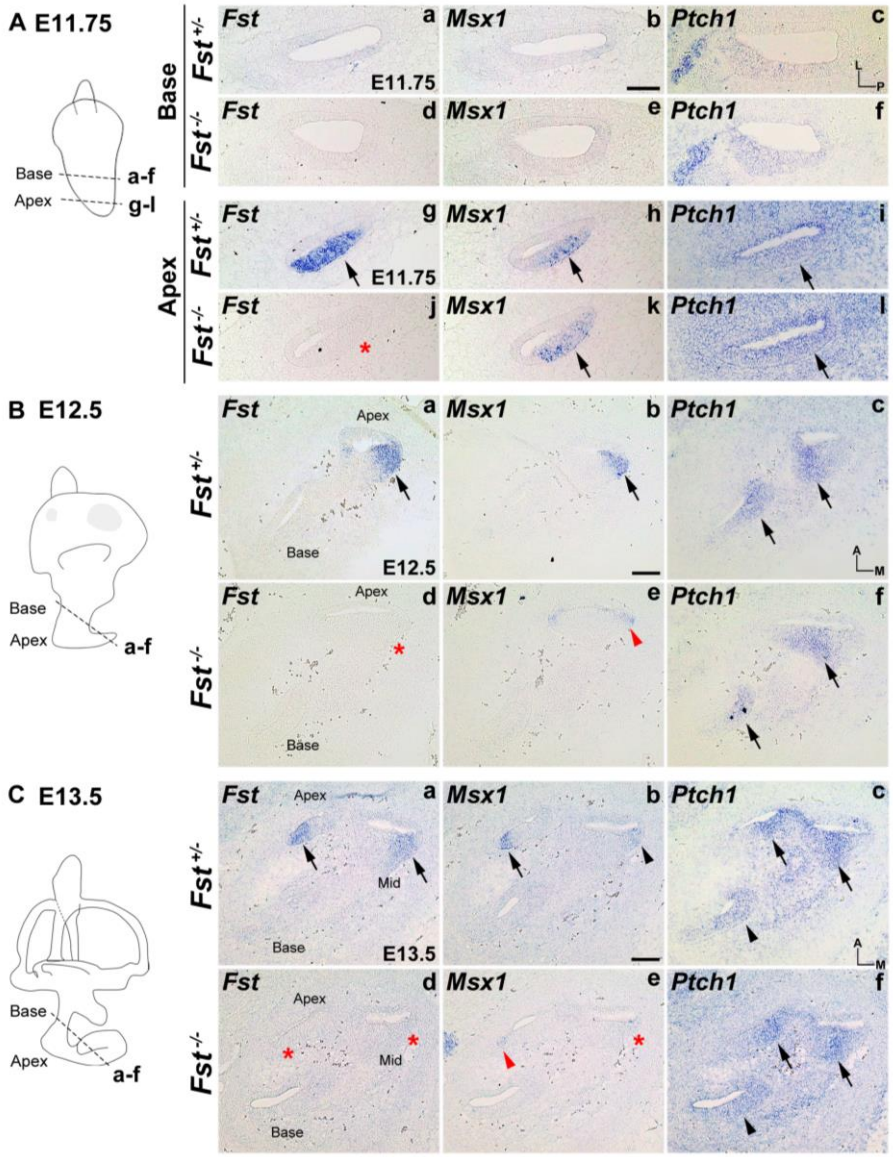


Figure 3. FST is essential for the maintenance of apical cochlear identity. (A) At E11.75, *Fst* and *Msx1* is not expressed in basal cochlea whereas weak *Ptch1* expression is observed in basal epithelium (Aa-Ac) both in control and Fst KO. In apical cochlea, *Fst* and *Msx1* expression are clearly observed, and the *Ptch1* expression was stronger than that of in basal cochlea (Ag-Ai, arrow).

In Fst KO, Fst is not observed in apical cochlea, but *Msx1* and *Ptch1* expression was similar with control (Aj-Al). (B) At E12.5, *Fst* and *Msx1* are strongly expressed in apical turn of cochlea, *Ptch1* shows similar gradient along the base to apex longitudinal axis (Ba-Bc, arrows). In Fst KO, *Msx1* expression are greatly down-regulated (red arrowhead) in apical cochlea despite the normal expression of *Ptch1* (Bd-Bf). (C) At E13.5, the basal to apical increasing expression gradient of *Fst* and *Msx1* is maintained, similar increasing gradient is also shown in *Ptch1* expression (Ca-Cc). In Fst KO, the downregulation of *Msx1* expression is more evident with intact basal to apical increasing gradient of *Ptch1* expression (Cd-Cf).

4. FST is required for the promotion of apical identity induced by SHH signaling

Our results suggest that FST may only be temporarily required for the maintenance of apical identity, or it may actively participate in apical specification by reinforcing the SHH function. To better understand the role of FST in relation to SHH, we analyzed the cochlea of Pax2-Cre; SmoM2 mice in the presence or absence of FST. In the Pax2-Cre; SmoM2 cochlea expressing a constitutively active form of SHH transducer (SmoM2), SHH signaling is ectopically activated, expressing *Ptch1* and *Gli1* along the entire cochlea (Fig. 4S, T)[3]. As expected, apical markers including *Fst*, *Msx1*, *Slitrk3*, and *Efnb2* are expanded along the entire cochlea (Fig. 4E-H), whereas basal markers including *A2m* and *Inhba* are completely downregulated (Fig. 4Q-R)[3]. These results confirmed the previous observation that ectopic SHH activity is sufficient to promote apical identity in the entire cochlea at the expense of basal identity.

We next examined the cochlea of Pax2-Cre; SmoM2; Fstfl/fl mice to see if apical identity promoted by ectopic SHH activity is affected in the absence of FST. In Pax2-Cre; SmoM2; Fstfl/fl cochlea, ectopic expression of *Ptch1* and *Gli1* is maintained in the entire cochlea while *Fst* expression is abolished (Fig. 4W-X, I), suggesting that ectopic SHH signaling is not affected in the absence of FST, similar to endogenous SHH activity (Fig. 4S, T). Interestingly, the expression levels and domains of each apical or basal marker are altered to different degrees. While the expanded expression of *Efnb2* in the entire cochlea is generally unaffected (Fig. 4L), the expression levels of *Msx1* and *Slitrk3* are greatly downregulated at the base, restoring the expression gradient similar to wild-types, stronger at the apex and weaker toward the base (Fig. 4J, K). In addition, *Inhba* and *A2m* is not observed in Pax2-Cre; SmoM2; Fstfl/fl mutant, confirming the fact that the basal cochlear identity requires low concentration of Shh signaling (Fig. 4U, V) [3]. These results indicate that some aspects of apical identity that are regulated by SHH signaling are compromised in the absence of FST, and further suggest that

FST play an active role in tonotopic organization, probably by reinforcing SHH's role in promoting apical cochlear identity.

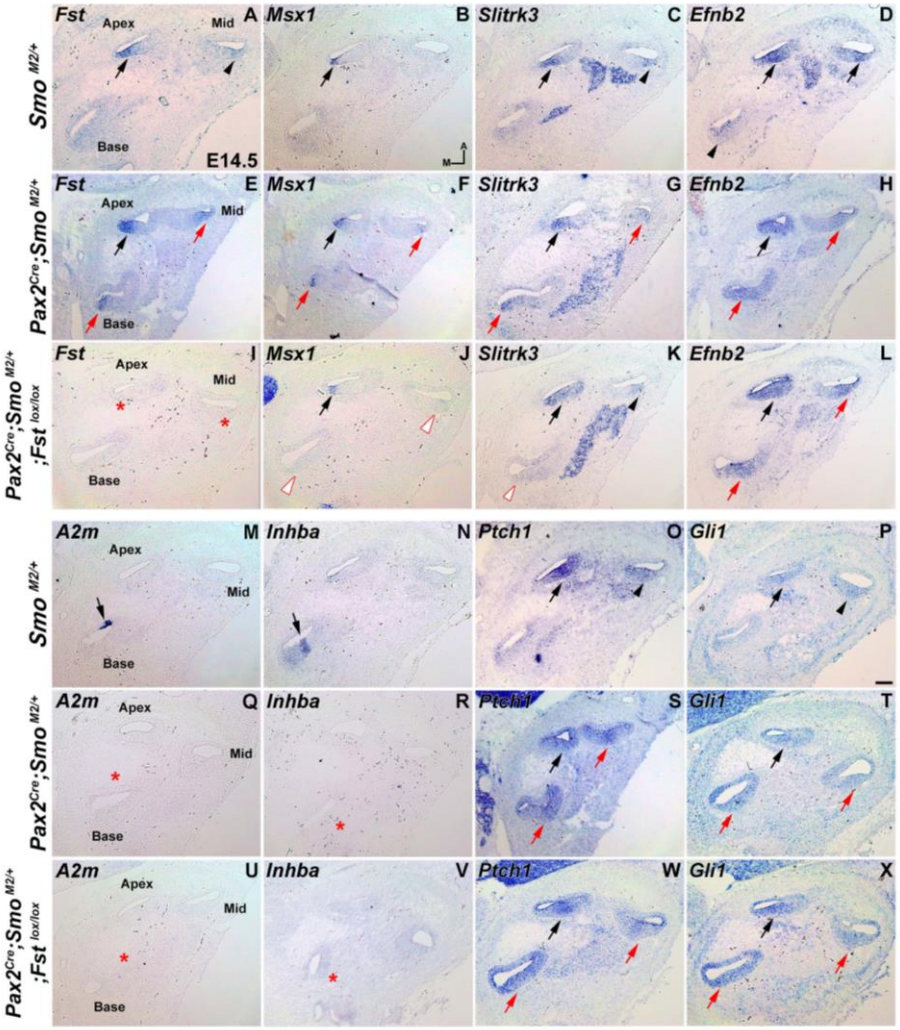


Figure 4. FST cooperates with strong SHH activity to induce the most apical cochlear identity. (A-D) At E14.5, apical cochlear markers show basal to apical increasing gradient; *Fst* and *Msx1* are more restricted to the apical cochlea whereas *Slitrk3* and *Efnb2* are more broadly expressed along the cochlear duct. In Pax2-Cre; SmoM2 mutant cochlea, the expression of all apical markers is expanded to the basal cochlear duct (E-H, red arrows). In Pax2-Cre; SmoM2; Fstfl/fl mutant cochlea however, the expression pattens of *Msx1* and *Slitrk3* are comparable with controls rather than with Pax2-Cre;

SmoM2 mutant cochlea (I-K). The expression of *Efnb2* is expanded to the basal cochlea regardless of Fst (L, red arrows). In control embryos, the basal markers are restricted to the basal cochlear duct (M-N), basal to apical increasing gradient of *Ptch1* and *Gli1* expression (O-P). In Pax2-Cre; SmoM2 mutant cochlea, the basal cochlear identity disappears (Q, R), Strong *Ptch1* and *Gli1* expression is observed in all cochlear turns (S-T). In Pax2-Cre; SmoM2; Fstfl/fl mutant, the basal cochlear markers are not expressed (U, V, red asterisks), expansion of *Ptch1* and *Gli1* expression is clearly observed (W, X, red arrows).

5. The regulation of Fst expression by Shh signaling is dependent on the expression timing

We observed that the Shh signaling promotes the expression of Fst and Fst mediates the function of promoting the apical identity. To further analyze the relationship between Shh signaling and Fst, we next asked whether the Fst expression is diminished in the absence of Shh signaling. Since it is impossible to investigate the regional identities of Smo cKO using Pax2-cre mouse due to its severity [31], we used Smo cKO breeding with Emx2-cre mouse. Interestingly, while the *Msx1* expression is lost in Smo cKO (Fig. 5B, F), *Fst* expression is indistinguishable with control at E15.5 (Fig. 5A, E). According to previous study, the Emx2 cre activation in cochlear epithelium starts around E11.5 [32]. *Fst* starts expression from E10.5 [6], we reasoned that the cre activation was too late to downregulate the Fst expression because the basal to apical increasing gradient is already established at E10.5. Similarly, the expression of other apical markers *Slitrk3* and *Efnb2* is downregulated in Smo cKO (Fig 5I, M, J, N). As expected, the basal identity, marking as *A2m* and *Inhba* is well remained in Smo cKO (Fig. 5K-L, O-P). These results confirm that SHH signaling is required for the initial apical cochlear specification.

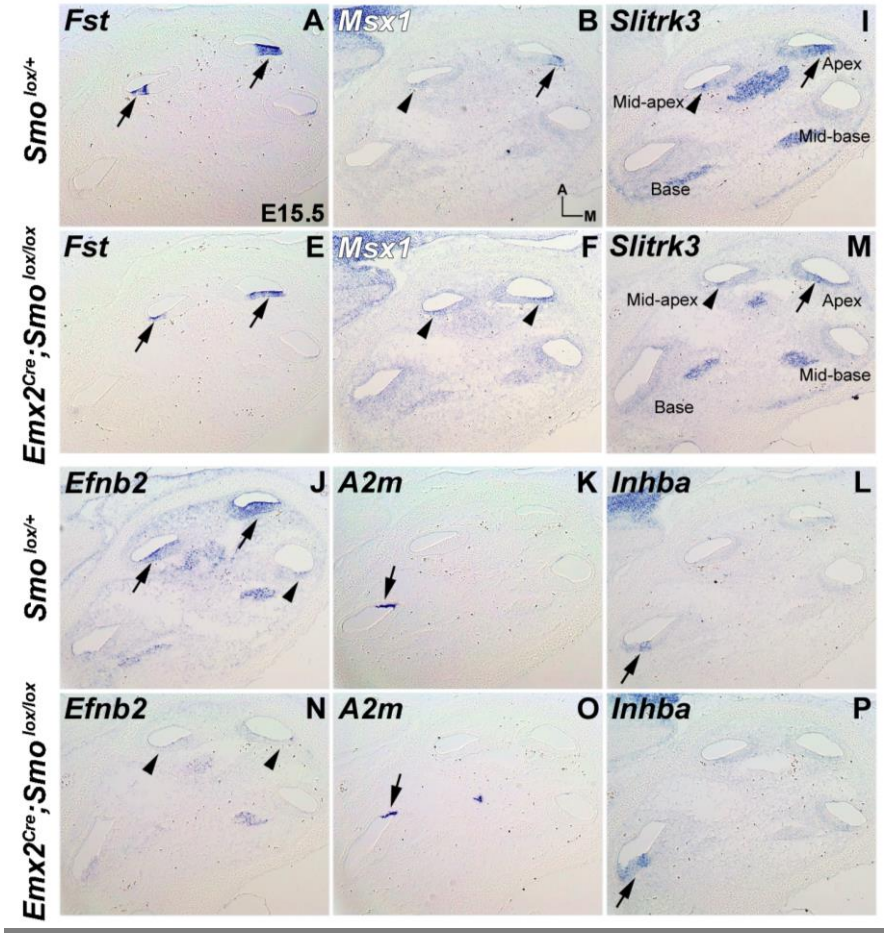


Figure 5. FST cooperates with SHH activity to maintain apical cochlear identity. *Fst* is highly expressed in apical cochlea in control (A). The expression pattern is maintained in Smo cKO (E). *Msx1* expression, however, is downregulated at E15.5 (Fig. 5B, F). Similarly, expression of *Sltirk3* and *Efnb2* is downregulated in Smo cKO (I, M, J, N). Basal identity is remained in Smo cKO according to the A2m and Inhba expression (K-L, O-P).

6. Fst is required for the maintenance of apical cochlear identity by expanding the cell population of apical cochlea

We observed that Fst is necessary to maintain the apical cochlear identity and is required for promoting the apical cochlear identity. We aimed to figure out the mechanism of which Fst maintains and promotes the apical cochlear identity during development. We noted that the most restricted apical gene, *Msx1* starts to be downregulated from E12.5 in Fst KO. To observe the *Msx1* expressing cells in detail, we labeled the cells that express *Msx1* using $Msx1^{CreERT2/+}$ and $tdtomato^{lox/lox}$ mice. The $Msx1^{CreERT2/+}$; $tdtomato^{lox/lox}$; $Fst^{+/-}$ mice were bred with $tdtomato^{lox/lox}$; $Fst^{+/-}$ mouse line, and tamoxifen was injected to pregnant female at E12.5, the embryos were harvested at E18.5 (Fig. 6A). The *Msx1* expressing cells were labeled by tdtomato positive cells (red), mostly localizing at the lateral side of apical cochlea (Fig. 6B, B', arrows). Also, similar to the *in situ* hybridization result in previous figure, the *Msx1* expressing cells were significantly reduced in Fst KO (Fig. 6C, C', arrowheads). This result is further confirmed by the tdtomato fluorescence quantification using ZEN blue program. The apical-most 10-15% of cochlear duct was missing exhibiting similar tomato fluorescence level to that of mid-basal cochlea (Fig. 5D).

According to the previous results, Msx1 regulates the cell proliferation and differentiation in dental mesenchymal cells and is required for the cell proliferation in limb development by regulating Fgf9/18 signaling and activating the MAPK signaling [33,34]. We thus asked whether the reduction of *Msx1* expressing cells in lateral region of cochlear duct leads to the reduced cell proliferation. We injected EdU to pregnant females at E12.5 and the embryos were harvested at E18.5. Edu staining was followed by the Myo7a antibody staining to mark the sensory hair cells. The cochlear duct was divided by five regions, 0% from basal end, and the Edu positive cells were counted in LER region where having same width as in the 3 rows of OHC in each cochlear region. Consistent to our expectation, the Edu positive cells were remarkably reduced in mid-apical turn, showing similar decreasing pattern in

mid-turn (Fig. 6F-H, J-L, yellow bracket, M). Cochlear length was examined which shows slightly shortened cochlear duct (~10%) in Fst KO cochlea (Fig. 6O) possibly due to the reduced cell proliferation in LER region. The reduced proliferating cells in LER region provoked us to examine the cell proliferation within the sensory region. Similar with the proliferation in LER region, the percentage of double labeled cell was decreased in mid and mid-apical region possibly due to the premature cell cycle exit (Fig. 6F-H, J-L, N). Note that the reduction is not observed in the apical end, since the cell cycle exit within the sensory region is already completed at E12.5 when EdU was incorporated. Together, Fst is necessary to maintain the Msx1 expression in LER region of apical-most cochlear duct, which enables the proper cell proliferation to specify and maintain the apical-most cochlear population.

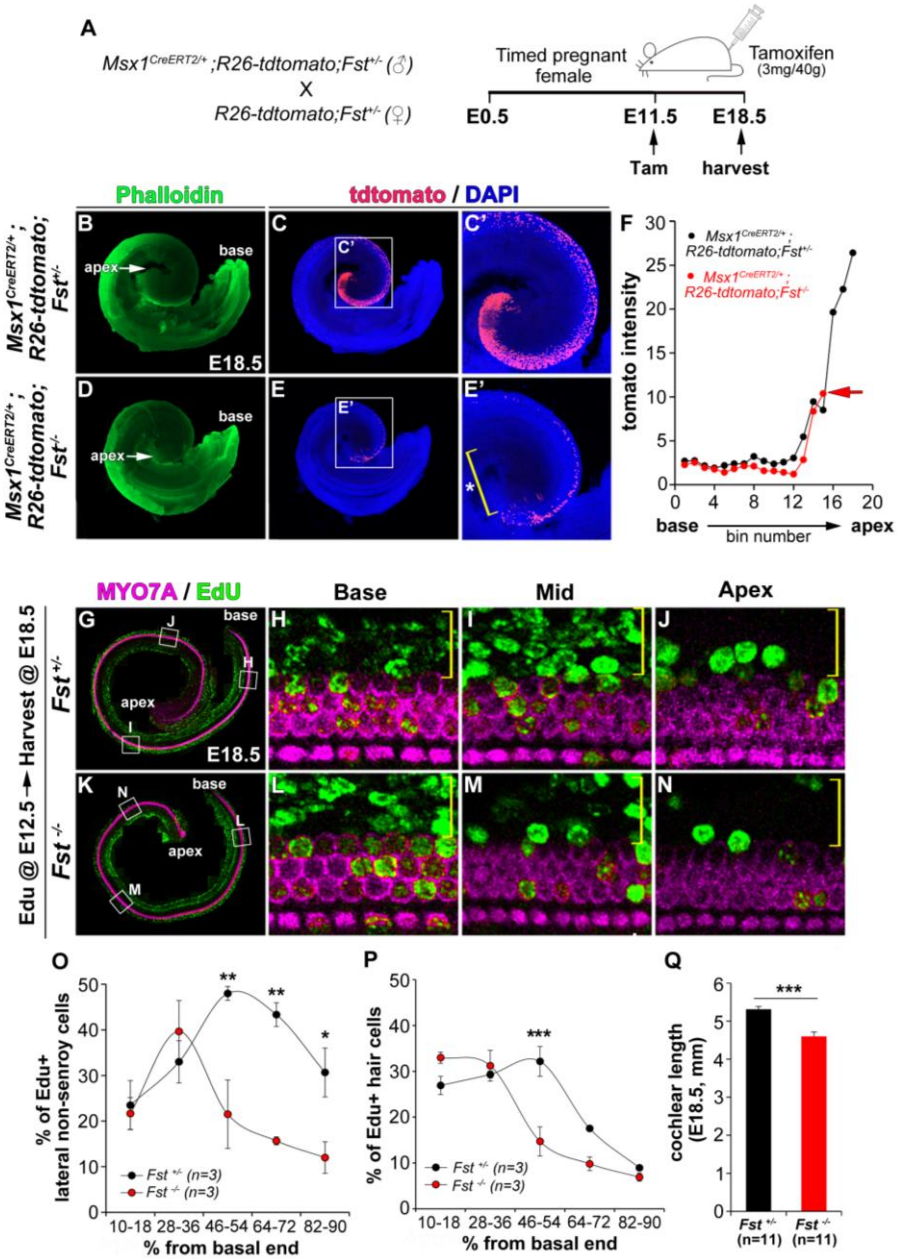


Figure 6. Fst plays a role in the expansion of apical cochlear cell population. The *Msx1* expressing cells at E11.5 was labeled by using *Msx1*$^{CreERT2/+}$ mouse line. When the cochlear duct was examined at E18.5, the Msx1 expressing cells are pooled in lateral region of apical cochlea (C, C'). In

Fst KO, the Msx1 expressing cells are significantly downregulated in apical cochlea (E, E'), also exhibiting shortened cochlear duct. The decrease of Msx1 expressing cells is clearly shown in quantitative analysis of its signal intensity, along with the shortened cochlear duct (F). (G-N) At E12.5, EdU reagent was injected three times to pregnant female, the cochlear duct was stained with Myo7a and EdU reagent at E18.5 to examine the cell proliferation ability. The proliferation cells, stained both with Myo7a Ab (magenta) and EdU reagents (green), are downregulated in Fst KO apical turn both in lateral region (K-N, yellow bracket, O) and sensory region (K-N, P).

7. The reduced proliferation in sensory region leads to the premature hair cell differentiation

In Recent study showed that overexpression of Fst leads to delayed hair cell differentiation due to the delayed cell cycle exit [25]. In addition, in previous figure, we observed that the proliferation in sensory region is reduced. We thus asked whether the premature hair cell differentiation occurs in Fst KO. The status of hair cell differentiation was examined by phalloidin staining at E15.5 and E18.5. Although the stereocilia bundle are still developing, one row of inner hair cells and three rows of outer hair cells are apparently observed in base and middle cochlear duct (Fig. 7B-C). In apical turn of control, the cuticular condensation of hair cell rows are undergoing (Fig. 7D). In Fst KO, by contrast, one row of inner hair cell and three rows of outer hair cells are observed in apical cochlea (Fig. 7H, yellow arrowheads). At E18.5, the hair cell rows are better organized than those of E15.5, the stereocilia hair bundles are evident in base, middle cochlea (Fig. 7J-K). Immature hair bundles are found in apical cochlea (Fig. 7L). In Fst KO, hair cell rows are more tightly organized in apical turn, and these cells have more morphologically developed stereocilia (Fig. 7P). These results show that the hair cell differentiation occurs prematurely in Fst KO due to the premature cell cycle exit.

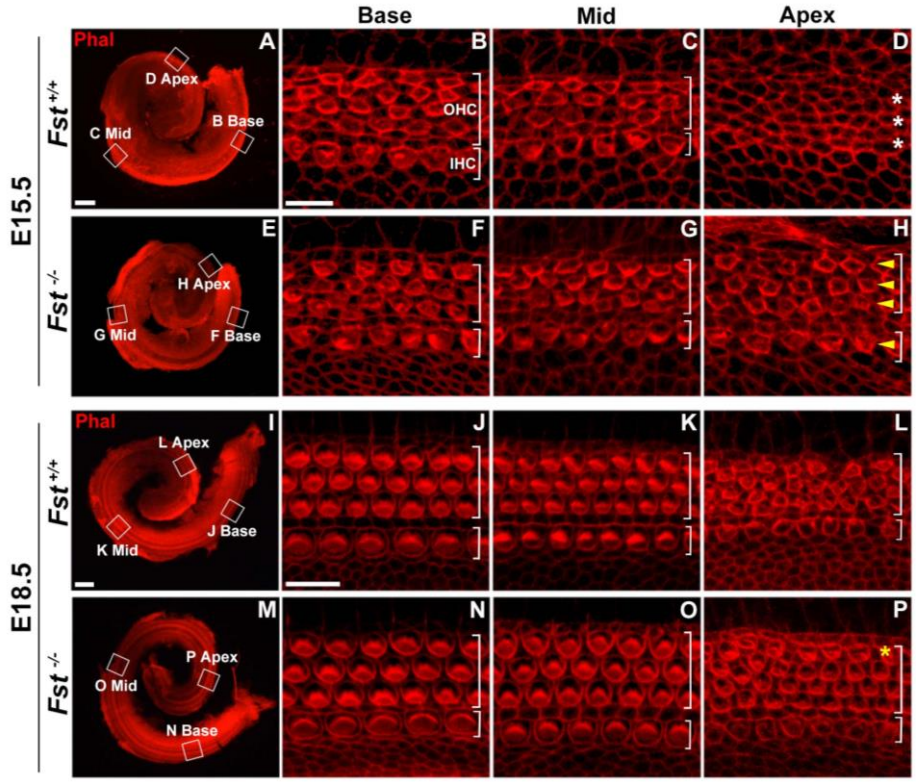


Figure 7. FST controls cell proliferation of both sensory and non-sensory cochlear cells. (A-D) At E15.5, the hair cell differentiation is observed in basal and middle cochlea, but not in apex (asterisks). (E-H) In Fst KO, differentiating hair cells are observed in base, middle cochlea, and hair bundles of apex start to be formed in inner hair cell, the cuticular condensation near outer hair cells are more mature than in control (arrowheads). (J-L) At E18.5, morphologically mature hair cell and hair bundles are observed through base and middle cochlea. The cuticular condensation is in progress in control apex, and the hair bundle seems started to be formed. (M-P) Fst KO exhibits more arranged cuticular condensation and the organization of 3-4 rows of outer hair cells than control (P, asterisk).

8. The counter relationship between Fst and Activin A is not essential in tonotopic organization

Recent study also showed that the Inhba gene, which encodes the Activin A subunit Inhibin βA promotes the hair cell differentiation [25]. Moreover, the delayed hair cell differentiation by Fst overexpression is partially rescued by the induction of Activin A. This suggests that the counter gradient of Activin A and Fst is important for the proper timing of hair cell differentiation. To investigate whether the counter relationship between Activin A and Fst is also conserved in tonotopic organization, we examined the expression of regional markers in Inhba KO mice. Even though the deletion of *Inhba* was confirmed, *A2m*, one of the basal cochlear markers is expressed in basal cochlear duct (Fig. 8C-D, G-H) which implies that the basal cochlear identity is specified. The apical cochlear markers *Msx1*, *Fst* are also normally expressed in apical cochlear duct (Fig. 8 A-B, E-F). To confirm whether the normal regional identity is sincere phenotype, or the different mouse line caused different phenotype, we analyzed whether delayed hair cell differentiation is recapitulated in Inhba KO mice. At E18.5, rows of hair cells are organized by cochlear middle region, showing immature organization in apical cochlear turn. (Fig. 8J-L). However, in Inhba KO, the ectopic IHCs are observed in basal region along with the immature hair bundle morphology, consistent with the previous report [25] (Fig. 8N-P). Together, even though the hair cell differentiation is delayed in Inhba KO, the positional identity was not affected. These results suggest that other ligand rather than Activin A relating with Fst is more important for the tonotopic organization of the developing mouse cochlea.

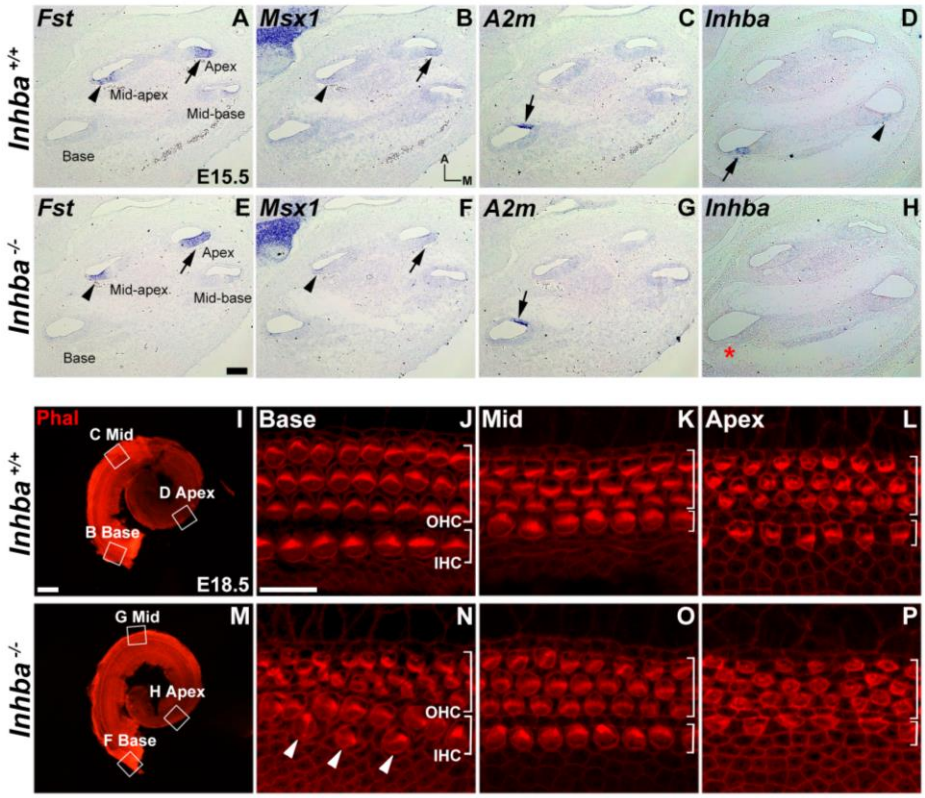


Figure 8. The counter gradient of activin is not essential for the regional cochlear identity. (A-D) In control, the apical markers *Fst* and *Msx1* is preferentially expressed in apical cochlea (A-B), whereas the basal markers *A2m* and *Inhba* are expressed in basal cochlea (C-D). In Inhba KO, these region-specific expression patterns are not altered (E-H). (I-L) At E18.5, the hair cell differentiation is occurred in base and middle cochlea, hair cells in apical cochlea are less mature than those of base and middle cochlea. In Inhba KO, the ectopic inner hair cells are formed in basal cochlea (N, arrowheads), the morphology of hair cell and hair bundle seem to slightly immature than control (K-L, O-P).

9. Loss of apical identity leads to the morphological change of tonotopically patterned stereocilia

We then asked whether the loss of apical regional identity in the embryonic cochlea of Fst KO mice leads to the changes in various tonotopic characteristics of mature cochlea. We investigated the morphologies of stereocilia that are known to have morphological gradient from base to apex. Since Fst KOs are neonatal lethal, we generated ear specific Fst conditional KO (cKO) mice by crossing Pax2Cre [22] with Fst floxed [23] mice. Fortunately, cKO mice are viable until at least three months. In control, the lengths of the stereocilia are nicely graded which have short stereocilia in base and become longer towards the apex (Fig. 9G-I, S; black dots). Interestingly, the lengths of stereocilia are significantly shorter in Fst cKO except for the most basal end (Fig. 9J-L, S; red dots). The angles of stereocilia which normally show the widest angle in the base and gradually become narrower towards the apex in control (Fig. 9M-O, T; black dots), are significantly wider in Fst cKOs (Fig. 9P-R, T; red dots). Stereociliary numbers which decreases as it goes from base to apex are also increased in Fst cKOs. These results suggest that the loss of apical cochlear identity in the embryonic cochlea results in the changes in tonotopic characteristics of stereociliary morphologies in mature cochlea.

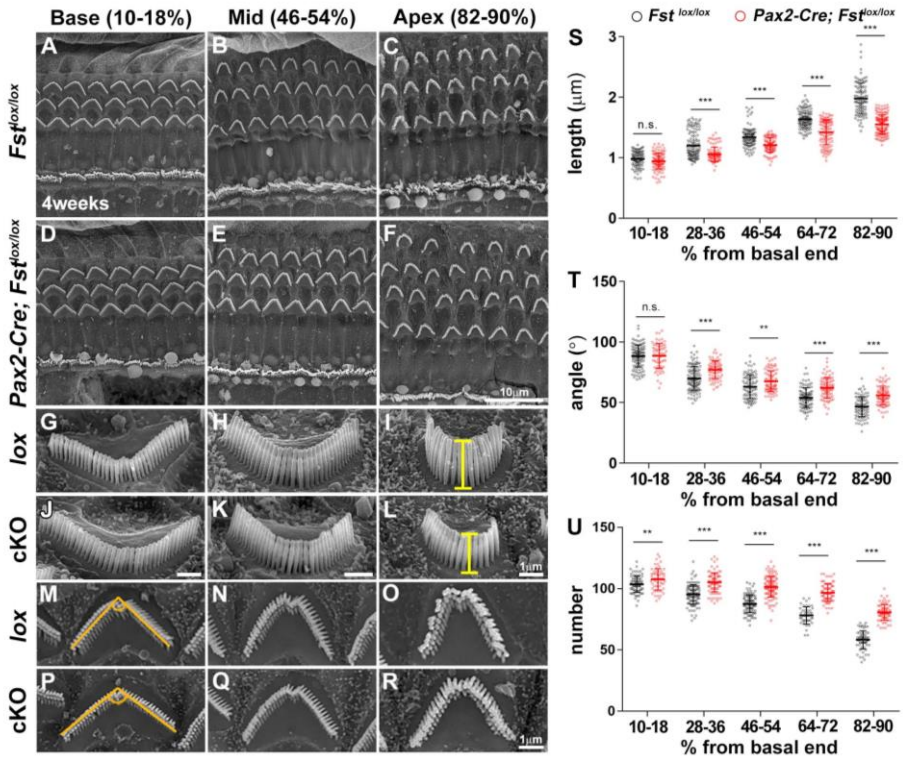


Figure 9. Loss of apical identity leads to the morphological change of tonotopically patterned stereocilia. (A-F) The overall normal hair bundle morphologies are observed from basal to apical cochlea at 4weeks. Except for the extra-rows in apical turn of Fst cKO, no hair cell degeneration and other defects are not observed. (G-L) To measure the length of stereocilia, high magnified pictures of each outer hair cell are shown. The increasing stereociliary length is observed both in control and Fst cKO. However, the lengths of stereocilia of Fst cKO are shortened in almost all cochlear region except for the basal end (G-L, yellow line, S). Similarly, the gradual decreasing stereociliary angles are observed in both genotypes, showing increasing pattern in Fst cKO (M-R, orange line, T). The number of stereocilia per outer hair cell gradually decreases along the tonotopic axis in control and Fst cKO, stereocilia of Fst cKO shows increased numbers of stereocilia (M-R, U).

10. Smo cKO exhibits similar morphological change in tonotopically patterned stereocilia

We showed that the loss Fst leads to the morphological change of tonotopically patterned stereocilia. We next asked whether the Smo cKO which is driven by the deletion of Smoothened shows similar morphological change as Fst cKO. Due to the decreasing cKO mouse viability, we analyzed the morphology of P15 cochlea, after the hearing onset. In control, the increasing stereociliary length from base to apex is observed (Fig. 10 G-I, S: black dots). Decreasing stereociliary angle and number along the basal to apical axis are also clearly observed (Fig. 10 M-O, U: black dots). In Smo cKO, like those of Fst cKO, the stereociliary length are decreased, angles and the number are also increased (Fig. 10 J-L, P-R, S, T, U; red dots). Note that the rows of outer hair cells in apical cochlea are mostly 2 rows which is also renowned by the previous study at P7 and 4 weeks [32] (Fig. S6 F). Possible reasons were suggested that the cre recombination was only sufficient for the late differentiating epithelium, cochlear apex and lateral compartments. These similar morphological changes in Smo cKO further supports that the loss of apical cochlear identity during early embryonic period in turn leads to the morphological alterations in tonotopically graded stereocilia in mature cochlea.

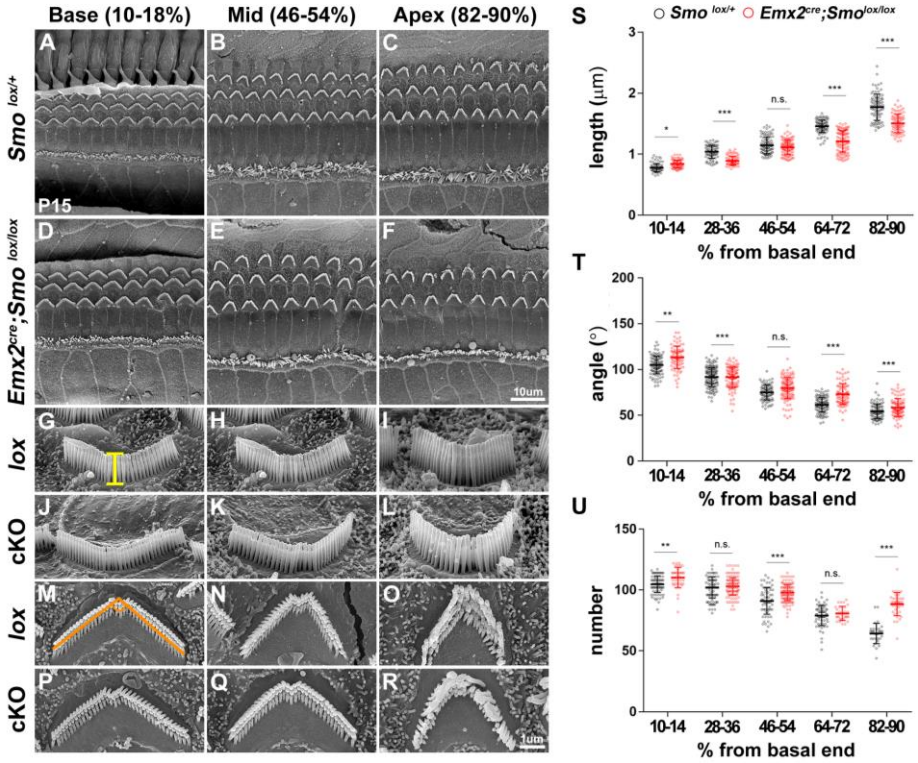


Figure 10. Smo cKO exhibits similar morphological change in tonotopically patterned stereocilia. (A-F) The overall normal hair bundle morphologies are observed from basal to apical cochlea at P15. Except for the two-rows in apical turn of Smo cKO, no hair cell degeneration and other defects are not observed. (G-L) To measure the length of stereocilia, high magnified pictures of each outer hair cell are shown. The increasing stereociliary length is observed both in control and Smo cKO. However, the lengths of stereocilia of Smo cKO are shortened in almost all cochlear region (G-L, yellow line, S). Similarly, the gradual decreasing stereociliary angles are observed in both genotypes, showing increasing pattern in Smo cKO (M-R, orange line, T). The number of stereocilia per outer hair cell gradually decreases along the tonotopic axis in control and Smo cKO, stereocilia of Smo cKO shows increased numbers of stereocilia (M-R, U).

11. Loss of apical identity leads to the change of graded gene expression patterns

We next asked whether the changes in the tonotopic properties of stereociliary lengths, angles and numbers are accompanied by partial gene expression changes. To answer this question, 4 weeks of control and Fst cKO cochlear duct were divided into base, mid and apex and pooled to be sequenced. Three replicates from three locations of two genotypes generated total 18 sequencing data with 101 bp-long reads, ranging from 72.9 million reads to 95.9 (mean 82.0 million). We then analyzed the RNA-seq results using Principal Component Analysis (PCA) using FPKM values of all genes. Three replicates for each control and cKO group are clustered together closely. Moreover, gene expression patterns of base, middle and apex are distinctly separated from each other in control (Fig. 11B; blue circles). Interestingly, apex of Fst cKO shows better clustering with middle region than any other sample groups in Fst cKO (Fig. 11B; pink circles), representing the gene expression pattern of cKO's apex became more like that of middle. Among these dataset, we focused on the genes that have increasing (Up(1)) or decreasing expression gradient (Down (2)) from base to apex. In Fst cKO, we found several genes which slope of expression gradient become smaller than control (Up 1.1 or Down 2.1) (Fig. 11C). Heap map shows 128 up-gradient genes and 269 down-gradient genes that have 1.5>fold change in Fst cKO (Fig. 11D). To gain insight which genes or pathways are enriched in Up gradient or Down gradient, we examined the GO analysis using DAVID. Interestingly, in Up gradient gene set, GO terms closely related to inner ear or inner ear function such as stereocilium, sensory perception of sound is enriched. By contrast, GO terms related overall development such as tissue development, organism development is enriched in Down gradient gene set. These results indicate that genes that are fundamental for inner ear function of frequency discrimination are possible to have base to apical increasing expression gradient.

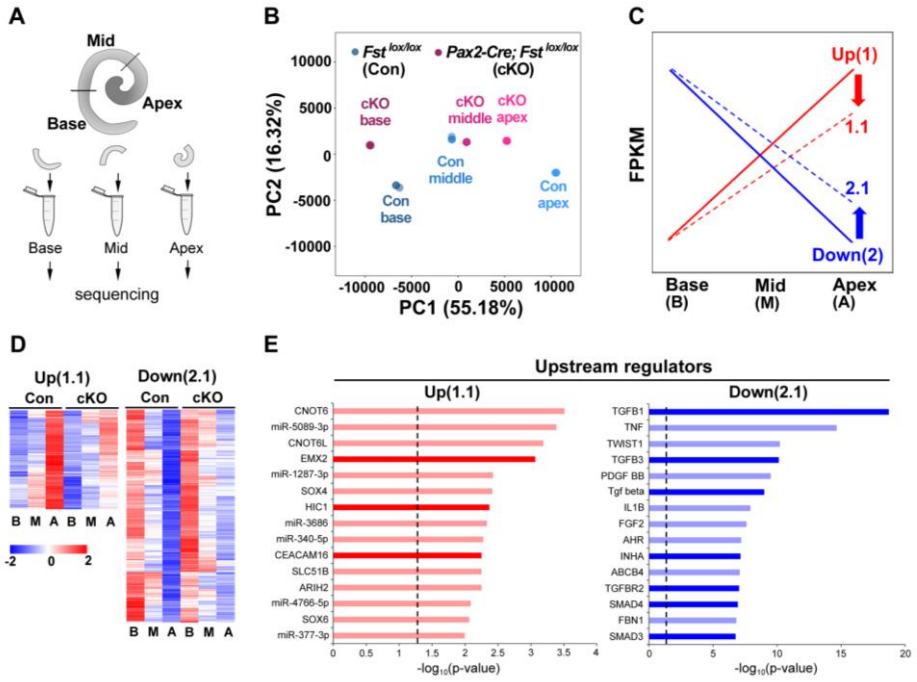


Figure 11. Loss of apical identity leads to the change of graded gene expression patterns. (A) The sample preparation for the RNA-sequencing. 4weeks of cochlea was divided in to three regions: base, mid, and apex. Tissues from three cochlear region was collected and pooled and sequenced, respectively. (B) The RNA-seq results was analyzed using principal component analysis using FPKM values of all genes. Three replicates for each sample group are clustered together closely, gene expression patterns of base, mid, and apex are distinctly separated from each other (control: blue dots, Fst cKO: pink dots). (C) The analysis scheme of RNA-seq is drawn. The genes that showing basal to apical increasing gradient is categorized as Up (1). Among genes from (1), the genes that show reduced increasing inclination are categorized as 1.1 (Detailed categorization criteria is written in M&M section). The gene that showing basal to apical decreasing gradient is categorized as Down (2). Among genes from (2), genes that show reduced decreasing inclination are categorized as 2.1.

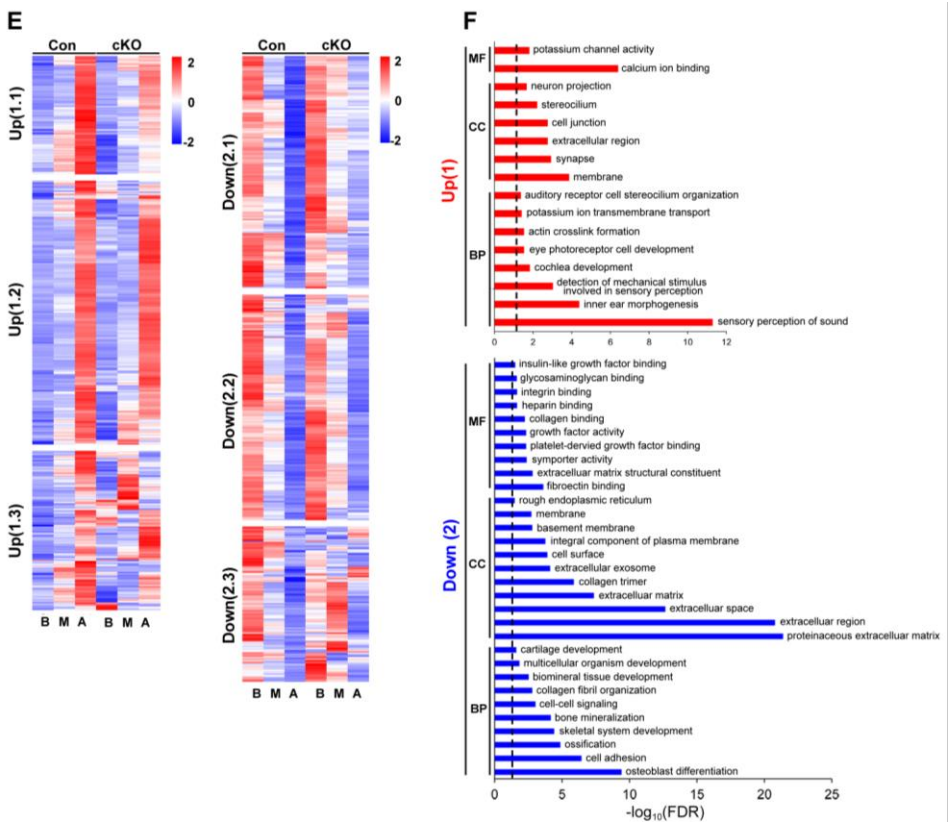


Figure 12. Heat map and GO enrichment analysis on genes showing graded expression levels along the tonotopic axis. (D) The heat map shows the increasing (Up 1.1, 1.2, 1.3) or decreasing (Down 2.1, 2.2, 2.3) gene expression patterns of control and Fst cKO. (E) When Up gradient gene set was compared with Down gradient gene set, ineer ear related GO terms such as channel activity, ion binding, stereocilium are enriched in Up gradient gene set (red bars). By contrast, GO terms such as multicellular organism development, cell-cell signaling are enriched in Down gradient gene set (blue bars).

12. Loss of apical identity leads to the low frequency specific hearing impairment

We wondered whether these morphological, gene expression changes lead to the functional alteration ultimately. If the apical identity is lost within the cochlear duct, low frequency sound which is sensitively conferred in apical cochlea would be affected. We first examined the hearing sensitivity using Auditory Brainstem Response (ABR) analysis. We observed that there are significant threshold shifts specifically in the low frequencies such as 4 to 8 kHz ranges (Fig. 12A, asterisks). To determine whether the OHC function is also deteriorated, we performed Distortion Product Otoacoustic Emission (DPOAE) analysis. Consistent with ABR results, there were significant decrease in DPOAE threshold in 6kHz but not in higher frequencies (Fig. 12B, asterisk). We also followed the hearing ability at 8, 12weeks to confirm whether low frequency specific hearing impairment is maintained or is progressive. We observed the continuous low frequency specific hearing impairment both at 8 and 12 weeks (Fig. 12 C-D, E-F) which suggest that the loss of apical identity during embryonic period leads to the consistent low frequency specific hearing loss. To further analyze the hearing ability of Fst cKO, we examined the amplitude of Wave I which is not significantly altered in Fst cKO, both in 6 and 18kHz (Fig. 13 A-C). We further performed DPOAE Input-Output (I-O) function analysis. While significant decrease is observed in 6 kHz and 8kHz (Fig. 13 D, E), Dp level is comparable with control in 10kHz (Fig. 12F). However, from 18kHz to 30kHz, slight reduction in Dp threshold was observed (Fig. 13 G-I). These results suggest that even though the Dp threshold does not exhibit statistically significant difference in higher frequencies, the amplification ability towards the low-decibel sound is reduced in Fst cKO. To confirm that the neuronal innervation is intact in Fst cKO, hair cells and type II neurons were stained by Myo7a and Tuj1, respectively. Also, to check whether the mechanotransduction channels are functional, we treated FM1-43 dye to live P5 cochlea. Type II neurons are properly innervated to outer hair cells along the basal to apical axis (Fig. 14 A-H) and

mechanotransduction is normally in progress (Fig. 14 I-P). These results confirmed that threshold shifts in Fst cKO are not due to abnormal neuronal innervation or hair cell function. Together, the loss of apical cochlear identity during embryonic period is directly connected to the functional impairment that low frequency hearing ability is undermined.

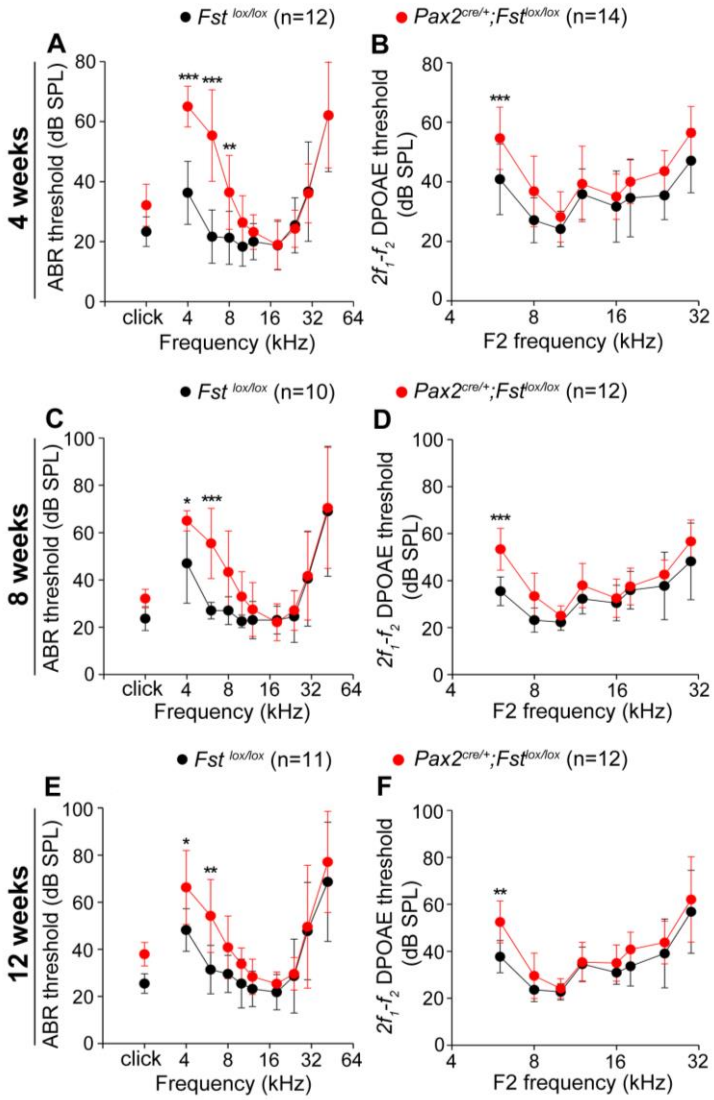


Figure 13. Loss of apical identity leads to the low frequency specific hearing impairment. (A) The hearing ability was examined by ABR analysis. The Fst cKO mice exhibits low frequency (4~8kHz) specific hearing impairment. (B) The outer hair cell function was examined by using DPOAE analysis. Dp threshold is significantly shifted in low frequency (6kHz). The low frequency specific hearing impairment is maintained at 8 and 12 weeks (C-D, E-F).

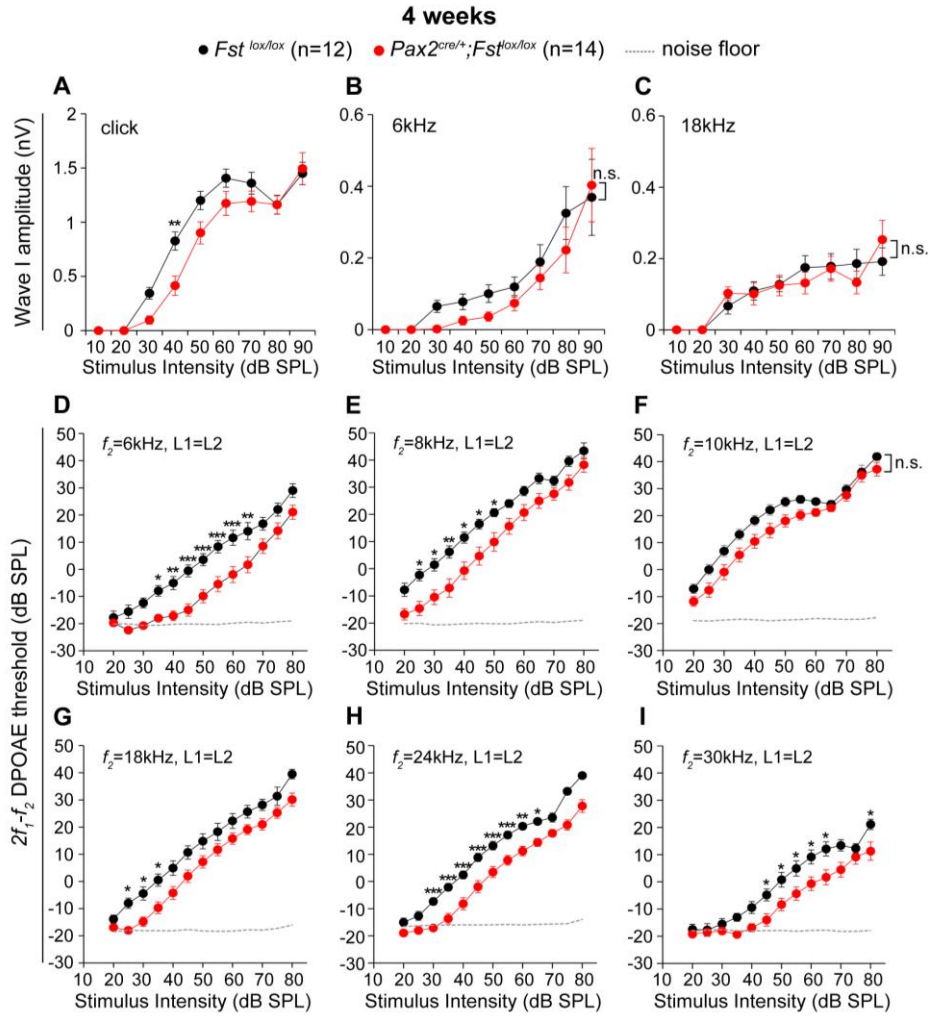


Figure 14. Loss of apical identity leads to the deteriorated outer hair cell amplification in low frequency (A) The Wave I amplitude was examined by ABR I/O analysis. The amplitude of Fst cKO was comparable in click, 6, 18kHz (Fig. 13 A-C). Dp input-output analysis shows that significant decreased Dp amplitude in mid-high frequency (18kHz). Slight decrease in Dp threshold is also observed in mid-high frequencies (18~30kHz) (G-I).

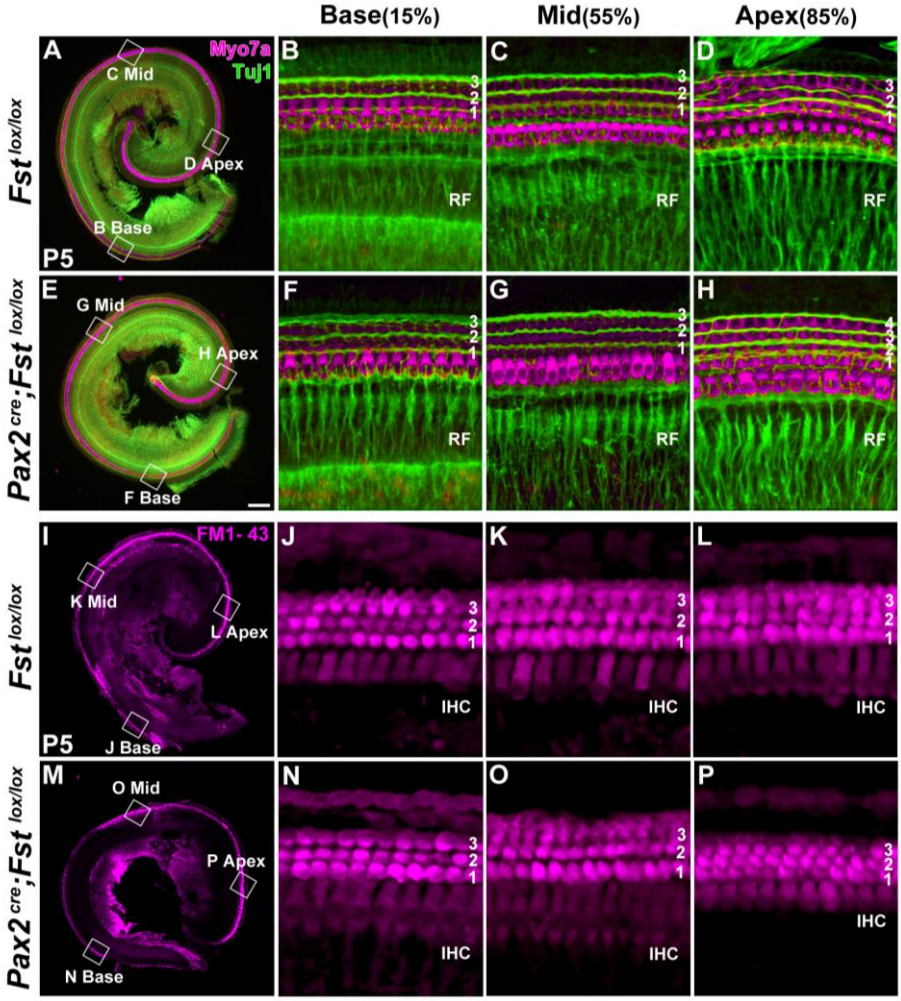


Figure 15. Neuronal innervation and mechanotransduction function are normal in Fst cKO (A-D) When the hair cells and neuronal innervation is stained with Myo7a (magenta), Tuj1 (green), respectively, the neuronal innervation is clearly observed in inner, three rows of outer hair cells at P5. In Fst cKO, innervation patterns are not different from controls (E-H) Neurons are innervated to the ectopic fourth row of outer hair cells. (I-L) The mechanostransduction function was examined by the FM1-43 dye. FM1-43 dye was incorporated in all three cochlear turns. In Fst cKO also, the dye incorporation was observed in base to apical cochlea (M-P).

13. Loss of Shh signaling leads to the overall threshold shifts along with the malformation of midde ear

Since we observed similar morphological changes both in Fst cKO and Smo cKO, we wondered whether the low frequency specific hearing loss is also observed in Smo cKO. Surprisingly, the hearing ability of Smo cKO was severe than our expectation, exhibiting threshold shifts in all tested frequencies of ABR and DPOAE (Fig. 15 A, C). Although the magnitude of threshold shifts is bigger near the low frequencies (4~12kHz) than the higher frequencies (18~42kHz) (Fig. 15B), the threshold shifts are statistically significant in all tested frequencies. The hearing phenotype of Smo cKO was partially covered by the previous study [32] but the exact reason for the hearing loss was not examined. We thus thought that there would be additional cause for the severe hearing loss. In previous report, the deletion of smoothened in neural crest derived cells (*Wnt1*$^{cre/+}$*; Smo*$^{lox/lox}$) directs the loss of initial condensation of malleus-incus of middle ear ossicles [29]. We thus hypothesized that if the Emx2 cre recombination is observed in middle ear ossicles, the middle ear defect can occur in Smo cKO. To validate our idea, we observed the middle ear of *Emx2*$^{Cre/+}$*; tdtomatolox*$^{/+}$ at P0. As expected, the tdtomato positive cells are observed in part of malleus and stapes and whole incus (Fig. 15 D, J, white arrows). Next, we compared the expression of *Ptch1* to mark the middle ossicles. Compared to controls, the *Ptch1* expression in incus region is completely loss (Fig. 15 E, K, red asterisk). Further, we dissected out the middle ear ossicles and stained with Alcian blue to observe the middle ear morphology in detail. Consistently, we found that the incus is severely malformed, easily detached form the malleus, and because of the malformation of incus, the stapedial head is also affected (Fig. 15 F-I, L-O, red asterisks). Together, as well as the threshold shifts in low frequency ranges are observed in Smo cKO, the maldevelopment of middle ear ossicles made the hearing phenotype more severe than that of Fst cKO.

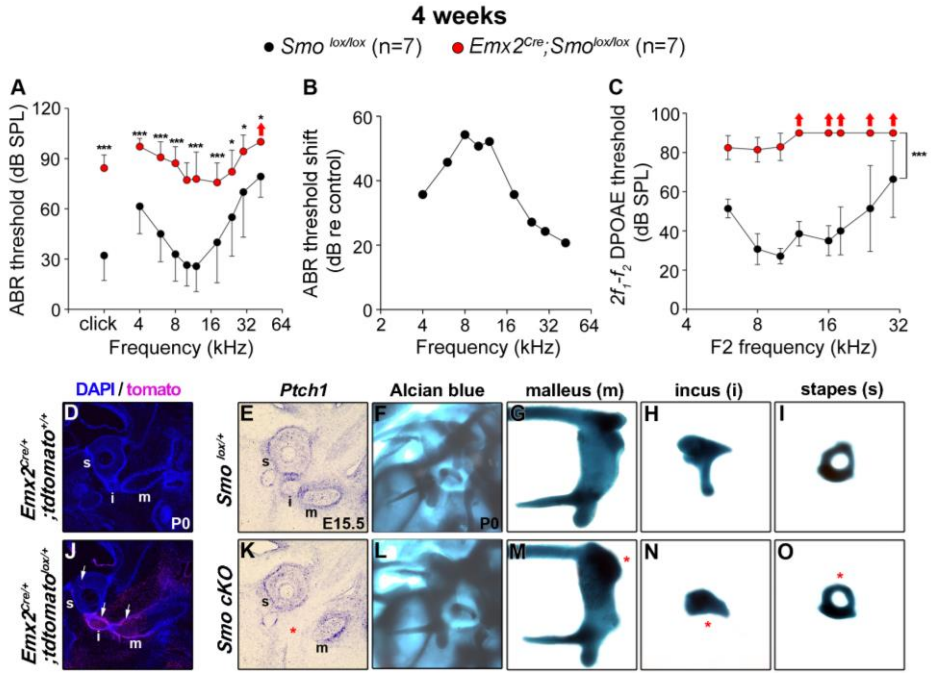


Figure 16. Loss of Shh signaling leads to the overall threshold shifts along with the malformation of middle ear. ABR analysis shows the overall threshold shifts in all tested frequencies (A, asterisks). When the threshold shifts are compared according to the tested frequencies, the magnitude of shifts are bigger in relatively lower frequencies (B, 4~16kHz). Dp thresholds are also shifted in all tested frequencies (C). The Emx2cre recombination is visualized by crossing with *tdtomato*$^{lox/lox}$ mice. The Emx2cre recombination is observed in middle ear ossicle region (D, J, arrows). The possibility of malformed middle ear ossicles is confirmed by *Ptch1* in situ, *Ptch1* expression is not observed in incus region of Smo cKO (E, K, asterisks). (F-I) The middle ear ossicles were stained by alcian blue dye. In control, all middle ear ossicles were normal. In Smo cKO, the outstanding defect was observed in incus (N), because of the malformed incus, the malleus-incus joint, and the stapedial head was also affected (M, O).

14. The continuous gradient of Fst during postnatal stages for proper tonotopic organization

We showed that the loss of apical identity during embryonic period leads to the morphological, gene expression changes in adult mature cochlea and indeed leads to the low frequency specific hearing impairment. However, since we used Fst conventional Knock-out, inner ear specific conditional Knock-out whose cre-recombination starts from around E8.5, and Fst TG mice that doxycycline administered from E10.5-E15.5, the manipulation was started from relatively early stage in cochlea. According to our RNA-seq results, at P28, most genes that we have used as embryonic apical cochlear markers such as *Msx1*, *Slitrk3* are expressed in low level, *Efnb2* does not show base-to-apex increasing gradient. However, *Fst* continues to have base-to-apex increasing expression gradient at P28 (Fig 11, RNA-seq data). Thus, we reasoned that the Fst gradient might have fundamental role not only during early embryonic stage but also in adult cochlea. To examine, human FST gene was overexpressed by inducing the doxycycline to newborn pups from P0 to P9 and the stereociliary morphology and hearing function was analyzed at 4 weeks (Fig. 16 A-B). Interestingly, in contrast to Fst cKO mice, overexpression of FST results in threshold shifts more in the high frequency ranges such as from 16 to 32 kHz (Fig. 16P). Although the magnitude of significance differs from cochlear region, the lengths and angles of Fst TG were slightly longer and wider (Fig. 16L-N, K, O; blue dots). These results suggest that disrupted Fst expression gradient during postnatal stages obstructs normal hair cell morphology throughout the cochlear duct.

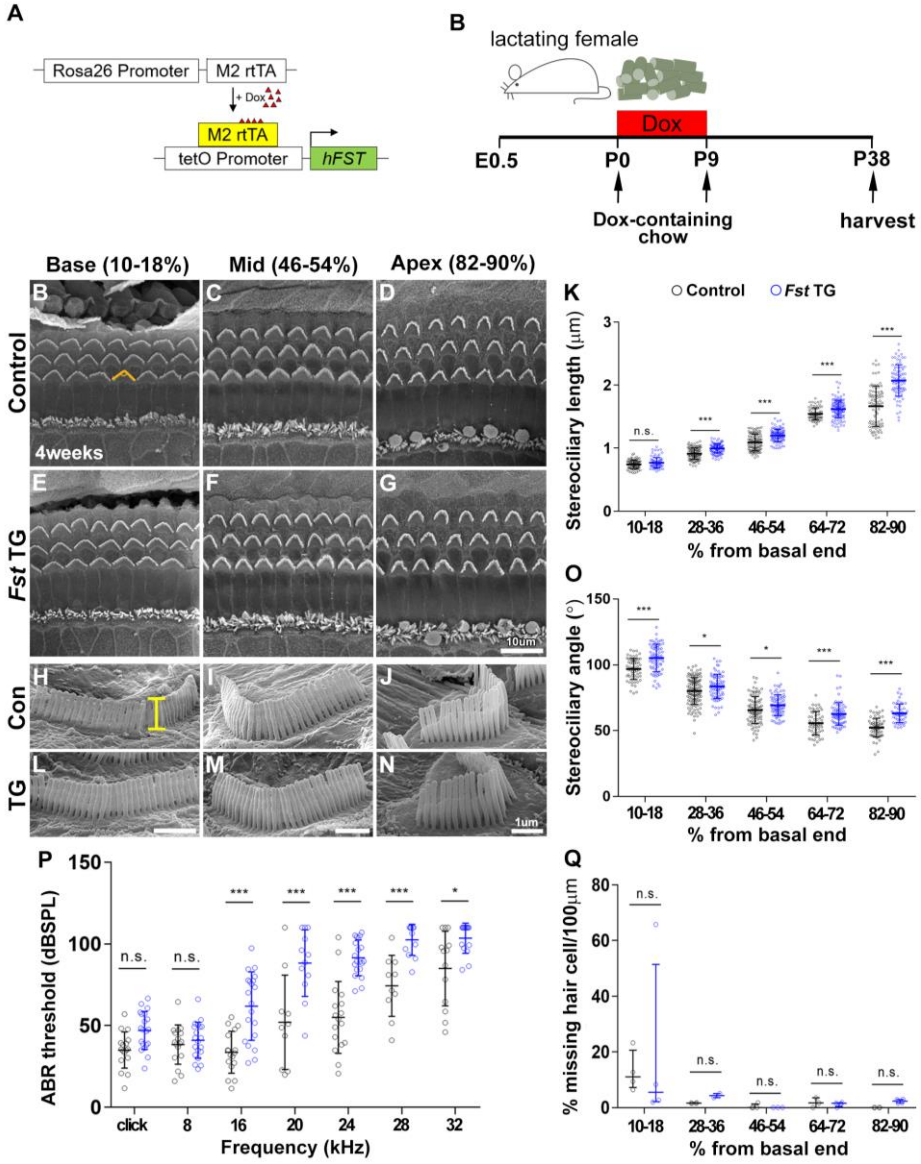


Figure 17. The continuous gradient of Fst during postnatal stages for proper tonotopic organization. (A-B) Inducible FST transgenic mouse model scheme. FST was overexpressed in whole embryo by crossing the M2rtTA mouse line to an inducible Follistatin (FST) transgenic mouse line, where human FST (hFST) is overexpressed under the control of a Tet-On promoter. M2rtTA tg/+; FST tg/+ mice overexpress hFST ubiquitously upon

doxycycline administration. Doxycycline (dox) was treated to lactating female by dox-containing chow from P0 to P9. The pups were harvested at P38. The overall normal hair bundle morphologies are observed from basal to apical cochlea at 4 weeks. Significant hair cell degeneration and other defects are not observed in Fst TG mice (B-G). (H-N) To measure the length of stereocilia, high magnified pictures of each outer hair cell are shown. The increasing stereociliary length is observed both in control and Fst TG. However, the lengths of stereocilia of Fst TG are slightly longer in almost all cochlear region (H-N, K). Similarly, the gradual decreasing stereociliary angles are observed in both genotypes, showing increasing pattern in Fst TG (O).

IV. DISCUSSION

We report a key role for Fst in tonotopic organization by expanding and maintaining the apical cochlear identity in mouse cochlea. The loss of apical identity during early developmental periods entirely affects the morphological, gene expression, even function in adult mature cochlea. The morphologies of stereocilia length, angle and number are slightly shifted towards basal region together with some extent of change in graded gene expression pattern. Finally, we showed that the failure of maintenance of apical regional identity is account for the low-frequency specific hearing loss for the first time.

1. Possibility of sufficiency of Fst for promoting the apical cochlear identity

In our study, we provisionally concluded that the Fst itself is not sufficient for promoting the apical cochlear identity based on the expression patterns of apical markers at E15.5 (Fig. 2). It was unexpected result because the previous report showed that the Bmp7 is sufficient for promoting the stereociliary morphology of apical cochlea in chicken basilar papilla [17]. The basilar papilla in this study was isolated at E6.5 when the *Bmp7* shows proximal to distal increasing expression gradient along the basilar papilla. And the exogenous Bmp7 was treated in culture media for 6 days. We, on the other hand, used the Fst TG mice to overexpress *Fst* by giving doxycycline contained chow to pregnant female from E10.5 to E15.5. We expect that the overexpressing efficiency of treating chow might be lower than treating exogenous protein directly into the culture medium. Though it is not clearly known how much it will take for doxycycline to take actions in mice organ, it might take at least 1 day to switch the plasma concentration throughout the animal's body [35]. It was not feasible for us to give the doxycycline chow earlier because the overexpression of Fst gene is not restricted to inner ear in our system. Fst is known for one of the antagonists of TGF-beta superfamily such as Activins and Bmps, and these ligands has several fundamental roles in

embryonic development. Thus, if the doxycycline chow is treated earlier than E10.5, the normal development of embryos would be severely obscured. If we could overexpress Fst from earlier stage and for longer period, it is possible that the apical cochlear markers are expanded towards the basal cochlear. The slightly upregulated expression of *Slitrk3* and *Efnb2* in Fst TG E15.5 embryo (Fig. 2) in basal cochlear epithelium propose the possibility of apical promotion. In addition, the fact that mid-high frequency hearing impairment occurs in doxycycline treated mice from P0 to P9 (Fig. 16), it supports the hypothesis that Fst could be sufficient for the promoting the apical cochlear identity.

2. Long term effect of Fst gradient in tonotopic organization

Fst expression gradient initiates as soon as cochlear primordium emerges from ventral side of the otocyst at E10.5 in mice [6]. It maintains in the developing cochlea throughout embryonic and postnatal periods as well as in the mature cochlea at least up to 4 weeks. Even though Shh is crucial for establishing Fst gradient at the early stage, it is unlikely to act to maintain its graded expression continuously. First, when the second source of Shh (from spiral ganglion neurons) are genetically removed, *Fst* gradient is maintained [3] until E15.5. Second, when the Shh signaling is deleted relatively late stage (~E14.5), Fst expression is comparable with control (Fig. 5) at E15.5 and its expression is maintained until E17.5 (data not shown). Third, Shh gradient is no longer present in the neonatal cochlea [36]. Therefore, what maintains Fst expression gradient in mature cochlea is currently unclear. When Fst is overexpressed throughout the whole body from P0 to P9, the tonotopic characteristics of hair bundle morphologies of apical cochlea are expanded toward the basal cochlea. More importantly, the mid-high frequency hearing ability is attenuated. These results possibly imply the importance of Fst gradient in postnatal stages as well as in embryonic stages for the tonotopic organization. Since Fst is well-known for the antagonist of TGF-beta superfamily ligands such as Inhba and Bmp4 which shows the opposite

expression gradient with Fst at postnatal stages [37], the ectopically upregulated Fst might inhibit these ligands to be normally expressed. Recently, it was shown that Bmp signaling related gene such as Bmp2, Bmp7, Bmpr1 are highly expressed in postnatal spiral ganglia [38]. $Bmp4^{+/-}$ mice exhibit hearing impairment possibly due to the reduced neuronal processes [39]. Moreover, recent study showed that Bmp4 has the potential to promote the survival and preserve the structure of spiral ganglion neurons (SGN) [40]. Taken together, the base to apex increasing gradient of Fst is essential for the tonotopic organization from early embryonic stages to postnatal stages to maintain tonotopically organized mature cochlea. Future studies in finding direct target of Fst would enrich the understanding of how tonotopy is established in mammalian cochlea.

3. Lateral part of cochlear apex is the proliferating spot

We reasoned that the gradual loss of *Msx1* expression in Fst KO causes the loss of apical cochlear identity due to the failure of cell expansion in apical LER (Lesser Epithelial Ridge) region. According to the previous studies, Msx1 is shown to regulate the cell proliferation and differentiation in dental mesenchymal cells and be required for the cell proliferation in limb development by regulating Fgf9/18 signaling and activating the MAPK signaling [33,34]. Moreover, it was shown that the YAP which is involved in hippo signaling as a transcriptional activator is necessary for cochlear cell proliferation, especially around LER region. In YAP ckO mice, the cell proliferation labeled by EdU is dramatically reduced around apical LER region where p27kip1 domain is ectopically expanded towards the LER region at E13.5. It was also shown that the cochlear length and subsequent total number of hair cells are reduced in YAP cKO at E18.5 [41]. Together, though absence of Yap has more severe phenotype than that of the absence of Fst because of its larger expression domain throughout the cochlear duct, the proper proliferation in LER region seems to be fundamental to expand the cell population and elongate along the cochlear axis.

4. Relationship between organ of Corti's ultrastructure and hearing ability

In Fst KO and cKO, extra rows which seem to be the effect of increased Bmp4 [42] in OHC were evident in apical cochlea (~18% from apical end). Despite the ectopic OHCs in apical cochlea, it is unlikely that these ectopic OHCs deteriorate the low frequency hearing ability. We confirmed that the mechanotransduction channel and Type II neuronal innervation are comparable with controls in fourth row of OHCs (Fig. 14). Furthermore, in previous studies, mice lacking Dfna5 show a diverging number of cochlear fourth row of outer hair cells but there is no difference in hearing threshold [43]. In Huwe1 KO, even though the extra IHCs were formed, ABR and DPOAE thresholds were remained normal at P30 [44]. When supernumerary IHCs and OHCs were formed at the same time in p130 KO mice, the hearing ability which is proved by ABR and DPOAE is not significantly different compared to control [45]. These previous observations suggest that the slight alterations in rows of cochlear hair cells have no obvious effects on its function.

In Fst cKO, because of the loss of apical identity during the development, the tallest sterociliary length resembled those of in middle cochlea. According to the previous study [46], the length of stereocilia correlates with the hearing efficiency. The peak efficiency shifts to higher frequencies for shorter stereocilia whereas to lower frequencies for longer stereocilia. Further, the peak power output occurred at higher frequencies for shorter stereocilia while the magnitude of power output decreases with stereocilia length. Thus, the alteration in morphological tonotopic gradient such as stereociliary length, angle and numbers could be one of the reasons of low-frequency hearing impairment. Basilar membrane (BM) which is the basement membrane of Oragn of Corti (OoC) is regarded as the starting point of the tonotopic organization. George von Bekesy showed that an acoustical stimulus initiates a travelling wave in the cochlea that propagates from the base toward the apex of the basilar membrane. The points responding to high frequencies are at the base of the basilar membrane where it is stiffer and narrower, the points

responding to low frequencies are at the apex where it is smoother and wider [7-9], giving rise to a topographical mapping of Tonotopy. Previous publications show that Emilin2 which is localized in BM ground substance has base to apex increasing expression gradient [11,47]. More recently, it was shown that the Emilin2 KO mice display broadened mechanical and neural frequency tuning with irregular peaks shifted to frequencies below characteristic frequency (CF) at a given location on the BM. Emilin2 KO mice also exhibit threshold shifts in wide range of frequencies; click, 8, 16, 32 kHz [48]. We expect that the reduced mechanical stiffness of BM leads to the reduced longitudinal coupling and cause sharper frequency tuning with hearing threshold shifts [49]. However, in our RNA-seq results, the base to apex increasing gradient of Emilin2 was recapitulated in control and the slope of increasing gradient of cKO was not differ from control (fold change 0.9). We assume that the mechanical BM stiffness would be maintained in our mouse model which does not lead to the overall threshold shifts in ABR.

5. Signaling cascade to establish apical regional identity in the developing cochlea

It has been demonstrated that the Shh signaling gradient, higher in apex and gradually lower toward base along the developing cochlea, paves the roadmap for the tonotopic axis by conferring regional identities along the cochlear duct [3]. It has been shown that Fst expression is positively regulated by Shh signaling so that expression gradient of Fst, higher in apex and gradually lower toward base, is established in the developing cochlea as soon as the cochlear primordium emerges from the otocyst [3,6]. Thus, we hypothesize that Fst could be the downstream mediator for Shh signaling gradient to confer regional identity to the developing cochlea. *Msx1*, which is specifically expressed in the apical tip of the developing cochlea and thus has been used as a regional marker for apex of the cochlea, is abolished in various mutants showing reduced Shh activity or Gli activator function and is promoted in Shh hypermorph mutant suggesting that strong Shh/Gli activity is required for

apical cochlear patterning [30]. When Fst is deleted in the developing cochlea in Fst KO, *Msx1* expression is completely downregulated suggesting that apical identity is lost in the absence of Fst whereas Shh signaling is unaffected based on the expression patterns of Shh target genes such as *Gli1* and *Ptch1*. In addition, in Shh hypermorph without Fst, *Msx1* expression is not fully promoted toward the basal end of the cochlea. These results indicate that Shh-activated Msx1 expression in the apical cochlea is mediated by Fst, suggesting that Fst gradient reinforces the apical patterning that has been initiated by Shh gradient. To summarize the expression patterns of apical cochlear markers in Fst KO ($Fst^{-/-}$), PS ($Pax2^{Cre/+}$; $Smo^{M2/+}$), PSF ($Pax2^{Cre/+}$; $Smo^{M2/+}$;$Fst^{lox/lox}$) mutants, Smo cKO ($Emx2^{Cre/+}$;$Smo^{lox/lox}$), it is suggested that the initial apex specification is done by Shh signaling at E11.5. From E11.5 to E12.5, the dominant Shh signaling switched from the midline source to spiral ganglion source, Fst should take over the role of Shh signaling to expand and maintain the apical identity.

V. CONCLUSION

We examined the region-specific gene expression patterns, apical cell lineages in developing cochlea, and hair bundle morphologies and auditory functions in mature cochlea. Our results reveal that SHH signaling gradient initially confers the apical regional identity along the cochlear primordium and Fst works in combination with SHH signaling. We showed that Fst is required to maintain and expand the apical cochlear region by controlling the cell proliferation in both sensory and non-sensory, especially in lateral domain of cochlear apex.

In the absence of FST function, apical cochlear identity is lost during development due to the failure of apical cell population expansion, leads to alterations of stereociliary morphologies, gene expression profiles of mature cochlea. These eventually affects the auditory hearing function, causing low-frequency hearing impairment.

Together, we show for the first time that the regional identity that is disrupted during the development of single organ affects the function of the mature organ.

REFERENCES

1. Tilney LG, Saunders JC. Actin filaments, stereocilia, and hair cells of the bird cochlea. I. Length, number, width, and distribution of stereocilia of each hair cell are related to the position of the hair cell on the cochlea. J Cell Biol 1983;96:807-21.

2. Lelli A, Asai Y, Forge A, Holt JR, Geleoc GS. Tonotopic gradient in the developmental acquisition of sensory transduction in outer hair cells of the mouse cochlea. J Neurophysiol 2009;101:2961-73.

3. Son EJ, Ma JH, Ankamreddy H, Shin JO, Choi JY, Wu DK, et al. Conserved role of Sonic Hedgehog in tonotopic organization of the avian basilar papilla and mammalian cochlea. Proc Natl Acad Sci U S A 2015;112:3746-51.

4. Basch ML, Brown RM, 2nd, Jen HI, Groves AK. Where hearing starts: the development of the mammalian cochlea. J Anat 2016;228:233-54.

5. Lim DJ. Functional structure of the organ of Corti: a review. Hear Res 1986;22:117-46.

6. Son EJ, Wu L, Yoon H, Kim S, Choi JY, Bok J. Developmental gene expression profiling along the tonotopic axis of the mouse cochlea. PLoS One 2012;7:e40735.

7. Ehret Gn, Frankenreiter M. Quantitative analysis of cochlear structures in the house mouse in relation to mechanisms of acoustical information processing. Journal of Comparative Physiology ? A 1977;122:65-85.

8. Keiler S, Richter CP. Cochlear dimensions obtained in hemicochleae of four different strains of mice: CBA/CaJ, 129/CD1, 129/SvEv and C57BL/6J. Hear Res 2001;162:91-104.

9. Morell M, Lenoir M, Shadwick RE, Jauniaux T, Dabin W, Begeman L, et al. Ultrastructure of the Odontocete organ of Corti: scanning and transmission electron microscopy. J Comp Neurol 2015;523:431-48.

10. Zajic G, Schacht J. Comparison of isolated outer hair cells from five mammalian species. Hear Res 1987;26:249-56.

11. Yoshimura H, Takumi Y, Nishio SY, Suzuki N, Iwasa Y, Usami S. Deafness gene expression patterns in the mouse cochlea found by microarray analysis. PLoS One 2014;9:e92547.

12. Beisel KW, Rocha-Sanchez SM, Morris KA, Nie L, Feng F, Kachar B, et al. Differential expression of KCNQ4 in inner hair cells and sensory neurons is the basis of progressive high-frequency hearing loss. J Neurosci 2005;25:9285-93.

13. Fettiplace R, Nam JH. Tonotopy in calcium homeostasis and vulnerability of cochlear hair cells. Hear Res 2019;376:11-21.

14. Imamura S, Adams JC. Immunolocalization of peptide 19 and other calcium-binding proteins in the guinea pig cochlea. Anat Embryol (Berl) 1996;194:407-18.

15. Langer P, Grunder S, Rusch A. Expression of Ca2+-activated BK channel mRNA and its splice variants in the rat cochlea. J Comp Neurol 2003;455:198-209.

16. Wersinger E, McLean WJ, Fuchs PA, Pyott SJ. BK channels mediate cholinergic inhibition of high frequency cochlear hair cells. PLoS One 2010;5:e13836.

17. Mann ZF, Thiede BR, Chang W, Shin JB, May-Simera HL, Lovett M, et al. A gradient of Bmp7 specifies the tonotopic axis in the developing inner ear. Nat Commun 2014;5:3839.

18. Thiede BR, Mann ZF, Chang W, Ku YC, Son YK, Lovett M, et al. Retinoic acid signalling regulates the development of tonotopically patterned hair cells in the chicken cochlea. Nat Commun 2014;5:3840.

19. Moon KH, Ma JH, Min H, Koo H, Kim H, Ko HW, et al. Dysregulation of sonic hedgehog signaling causes hearing loss in ciliopathy mouse models. Elife 2020;9.

20. Muthu V, Rohacek AM, Yao Y, Rakowiecki SM, Brown AS, Zhao YT, et al. Genomic architecture of Shh-dependent cochlear morphogenesis. Development 2019;146.

21. Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A. Multiple defects and perinatal death in mice deficient in follistatin. Nature 1995;374:360-3.

22. Ohyama T, Groves AK. Generation of Pax2-Cre mice by modification of a Pax2 bacterial artificial chromosome. Genesis 2004;38:195-9.

23. Jorgez CJ, Klysik M, Jamin SP, Behringer RR, Matzuk MM. Granulosa cell-specific inactivation of follistatin causes female fertility defects. Mol Endocrinol 2004;18:953-67.

24. Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. Genes Dev 2004;18:937-51.

25. Prajapati-DiNubila M, Benito-Gonzalez A, Golden EJ, Zhang S, Doetzlhofer A. A counter gradient of Activin A and follistatin instructs the timing of hair cell differentiation in the murine cochlea. Elife 2019;8.

26. Roby YA, Bushey MA, Cheng LE, Kulaga HM, Lee SJ, Reed RR. Zfp423/OAZ mutation reveals the importance of Olf/EBF transcription activity in olfactory neuronal maturation. J Neurosci 2012;32:13679-88a.

27. Hochedlinger K, Yamada Y, Beard C, Jaenisch R. Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell 2005;121:465-77.

28. Morsli H, Choo D, Ryan A, Johnson R, Wu DK. Development of the mouse inner ear and origin of its sensory organs. J Neurosci 1998;18:3327-35.

29. Ankamreddy H, Min H, Kim JY, Yang X, Cho ES, Kim UK, et al. Region-specific endodermal signals direct neural crest cells to form the three middle ear ossicles. Development 2019;146.

30. Bok J, Dolson DK, Hill P, Ruther U, Epstein DJ, Wu DK. Opposing gradients of Gli repressor and activators mediate Shh signaling along the dorsoventral axis of the inner ear. Development 2007;134:1713-22.

31. Brown AS, Epstein DJ. Otic ablation of smoothened reveals direct and indirect requirements for Hedgehog signaling in inner ear development. Development 2011;138:3967-76.

32. Tateya T, Imayoshi I, Tateya I, Hamaguchi K, Torii H, Ito J, et al. Hedgehog signaling regulates prosensory cell properties during the basal-to-apical wave of hair cell differentiation in the mammalian cochlea. Development 2013;140:3848-57.

33. Feng XY, Zhao YM, Wang WJ, Ge LH. Msx1 regulates proliferation and differentiation of mouse dental mesenchymal cells in culture. Eur J Oral Sci 2013;121:412-20.

34. Yang Y, Zhu X, Jia X, Hou W, Zhou G, Ma Z, et al. Phosphorylation of Msx1 promotes cell proliferation through the Fgf9/18-MAPK signaling pathway during embryonic limb development. Nucleic Acids Research 2020;48:11452-67.

35. Gengenbacher M, Zimmerman MD, Sarathy JP, Kaya F, Wang H, Mina M, et al. Tissue Distribution of Doxycycline in Animal Models of Tuberculosis. 2020;64:e02479-19.

36. Liu Z, Owen T, Zhang L, Zuo J. Dynamic expression pattern of Sonic hedgehog in developing cochlear spiral ganglion neurons. Dev Dyn 2010;239:1674-83.

37. Waldhaus J, Durruthy-Durruthy R, Heller S. Quantitative High-Resolution Cellular Map of the Organ of Corti. Cell Rep 2015;11:1385-99.

38. Lu CC, Appler JM, Houseman EA, Goodrich LV. Developmental profiling of spiral ganglion neurons reveals insights into auditory circuit assembly. J Neurosci 2011;31:10903-18.

39. Blauwkamp MN, Beyer LA, Kabara L, Takemura K, Buck T, King WM, et al. The role of bone morphogenetic protein 4 in inner ear development and function. Hear Res 2007;225:71-9.

40. Waqas M, Sun S, Xuan C, Fang Q, Zhang X, Islam IU, et al. Bone morphogenetic protein 4 promotes the survival and preserves the structure of flow-sorted Bhlhb5+ cochlear spiral ganglion neurons in vitro. Sci Rep 2017;7:3506.

41. Gnedeva K, Wang X, McGovern MM, Barton M, Tao L, Trecek T, et al. Organ of Corti size is governed by Yap/Tead-mediated progenitor self-renewal. Proc Natl Acad Sci U S A 2020;117:13552-61.

42. Puligilla C, Feng F, Ishikawa K, Bertuzzi S, Dabdoub A, Griffith AJ, et al. Disruption of fibroblast growth factor receptor 3 signaling results in defects in cellular differentiation, neuronal patterning, and hearing impairment. Dev Dyn 2007;236:1905-17.

43. Van Laer L, Meyer NC, Malekpour M, Riazalhosseini Y, Moghannibashi M, Kahrizi K, et al. A novel DFNA5 mutation does not cause hearing loss in an Iranian family. J Hum Genet 2007;52:549-52.

44. Cheng YF, Tong M, Edge AS. Destabilization of Atoh1 by E3 Ubiquitin Ligase Huwe1 and Casein Kinase 1 Is Essential for Normal Sensory Hair Cell Development. J Biol Chem 2016;291:21096-109.

45. Rocha-Sanchez SM, Scheetz LR, Contreras M, Weston MD, Korte M, McGee J, et al. Mature mice lacking Rbl2/p130 gene have supernumerary inner ear hair cells and supporting cells. J Neurosci 2011;31:8883-93.

46. Breneman KD, Brownell WE, Rabbitt RD. Hair cell bundles: flexoelectric motors of the inner ear. PLoS One 2009;4:e5201.

47. Amma LL, Goodyear R, Faris JS, Jones I, Ng L, Richardson G, et al. An emilin family extracellular matrix protein identified in the cochlear basilar membrane. Mol Cell Neurosci 2003;23:460-72.

48. Russell IJ, Lukashkina VA, Levic S, Cho YW, Lukashkin AN, Ng L, et al. Emilin 2 promotes the mechanical gradient of the cochlear basilar membrane and resolution of frequencies in sound. Sci Adv 2020;6:eaba2634.

49. Dewey JB, Xia A, Muller U, Belyantseva IA, Applegate BE, Oghalai JS. Mammalian Auditory Hair Cell Bundle Stiffness Affects Frequency Tuning by Increasing Coupling along the Length of the Cochlea. Cell Rep 2018;23:2915-27.

ABSTRACT(IN KOREAN)

Sonic hedgehog과 follistatin 신호전달체계가 포유류 달팽이관의 토노토피 형성에 기여하는 역할

< 지도교수 복 진 웅 >

연세대학교 대학원 의과학과

구 혜 연

척추동물의 달팽이관은 저음에서 고음까지 다양한 주파수의 소리를 구별할 수 있도록 음위적으로 조직되어 있습니다. 이는 "Tonotopy"라는 현상에 의해서 가능한데, 이 단어는 그리스어에서 유래했습니다. Tono는 음조를 의미하고 topos는 장소를 의미합니다. 따라서 달팽이관의 음조는 달팽이관을 따라 다른 주파수의 소리가 처리되는 공간적 배열을 의미합니다. Tonotopic 축을 따라 형태학적, 기능적 구배를 갖는 많은 알려진 요인들이 있지만, 이 tonotopic 조직이 달팽이관 발달 동안 어떻게 처음 만들어지는지는 최근 에야 이해되기 시작했습니다. 이전 보고에 의하면, 닭과 쥐 모두에서 달팽이관의 기저에서 첨부로 가며 농도가 증가하는 Sonic Hedgehog (SHH) 신호전달물질이 존재하고, 그 농도 기울기에 의해 조절 받는 순차적 신호 캐스케이드에 의해 토노토픽 조직이 만들어진다고 밝혔습니다. 닭의 달팽이관에서는 Bmp7과 Retinoic acid (RA)가 tonotopic 조직을 매개하는 SHH의 주요 하위 전달물질인 것으로 나타났습니다. 그러나 마우스 달팽이관에서 SHH의 하류 매개체는 여전히 밝혀지지 않았습니다. 그래서 우리는 Follistatin (Fst)이 쥐의 달팽이관 정점의 정체성을 촉진하고 유지함으로써 마우스 달팽이관에서 tonotopy를 구축하기 위한 Shh 신호의 하위 매개체임을 제안합니다. Fst이 tonotopic 축을 따라 발현하는 달팽이관 위치 특이적인 유전자 발현을 보존함으로써 성숙한 달팽이관의 적절한 저주파 소리 구별 능력 및 tonotopically 패턴된 입체 섬모 형태에 필요하다는 것을 제안합니다.

핵심되는 말: 내이, 토노토피, SHH, Fst, 청력

PUBLICATION LIST

Koo, H*., J. Y. Hwang*, S. Jung, H. Park, J. Bok and J. W. Park (2021). "Position Specific Alternative Splicing and Gene Expression Profiles Along the Tonotopic Axis of Chick Cochlea." Front Mol Biosci 8: 726976. (*equal contribution)

Ankamreddy, H*., H. Koo*, Y. J. Lee and J. Bok (2020). "CXCL12 is required for stirrup-shaped stapes formation during mammalian middle ear development." Dev Dyn 249(9): 1117-1126. (*equal contribution)

Moon, K. H., J. H. Ma, H. Min, H. Koo, H. Kim, H. W. Ko and J. Bok (2020). "Dysregulation of sonic hedgehog signaling causes hearing loss in ciliopathy mouse models." Elife 9:e56551.