Mechanisms establishing tonotopic organization in the vertebrate cochlea

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Mechanisms establishing tonotopic organization in the vertebrate cochlea

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박사과정을 시작하고서 어느덧 5년여의 시간이 지났습니다. 박사과정 동안 제게는 성장통을 겪는 시간이었고, 덕분에 많이 성장할 수 있는 좋은 시간들이었습니다. 이 곳에서 배운 것을 통해 앞으로의 일도 기대가 됩니다. 그리고, 학위과정 동안 많은 분들의 지지와 응원이 있었기에 성장하고 앞으로 나갈 수 있었습니다. 저의 등을 밀어주고 함께 동행해주신 분들에게 감사의 말씀을 전하고 싶습니다.

제가 박사 학위 과정 동안 연구논문이 나오기까지, 많은 열정을 쏟아 부어주시며 가르쳐 주시고 방향을 잡아주신 복진웅 교수님께 감사 드립니다. 그리고 박사과정의 시작에 함께 하자고 손 내밀어 주시고, 조언도 아끼지 않으셨던 손은진 교수님께도 감사 드립니다. 학위논문이 나오기까지 아낌없는 조언과 지도를 해주신 김철훈 교수님, 정호성 교수님, 김성헌 교수님께 감사 드립니다.

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2017년 6월 마 지 현 올림

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ABSTRACT

Mechanisms establishing tonotopic organization in the vertebrate cochlea

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(Directed by Professor Jinwoong Bok)

The auditory organ is able to discriminate the sound frequencies. Sound frequency discrimination starts at the basilar papilla in birds and the organ of Corti in mammals. These auditory organs tonotopically organized with a basal end (proximal region) that detects high frequencies and an apical end (distal region) that senses low frequencies. Sonic Hedgehog (Shh) secreted from the notochord and floor plate is crucial for cochlear duct development in species. Early otocyst is required high level of Shh signaling secreted in ventral midline source at apex but not at base in chicken and mouse. In this study, gain-of-function experiment involving of Shh-soaked beads in chicken embryo in ovo and in a continuously activated Shh signaling pathway mouse model, demonstrated the specification of the positional identity of the cochlear duct in these animal species. However, region specified regulator genes are not conserved between the both species. In chicken, expression of these altered

genes, led to morphological and physiological characteristic alterations in hair cells. These features included tonotopic properties, such as the total number of stereocilia per hair cell and gene expression of an inward rectifier potassium channel (IRK), *Kcnj2*, which is an inherent characteristics of apical hair cells in the basilar papilla. In addition, this study suggests that Shh from the ventral midline, is required for the tonotopic organization during the developing cochlear duct in avian and mammal species.

Key words : Basilar papilla, cochlear, hair cell, inner ear, tonotopic organization

Mechanisms establishing tonotopic organization in the vertebrate cochlea

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I. INTRODUCTION

Sound frequency discrimination is well developed in the vertebrate inner ear. Frequency discrimination ability is very important to live the in natural environment and communicate with members of society. The sound wave from the external source is received by the outer ear, transmitted through the middle ear and finally enters the inner ear in the form of mechanical vibrations. These sound waves are converted into nerve impulses in the inner ear and are transmitted to the brain. The inner ear is divided into two parts: the vestibular apparatus, for detecting angular acceleration and the auditory apparatus for the sense of hearing. The hearing process is characterized by its unique tonotopic organization in the inner ear. The tonotopic organization contributes to elaborating the spatial arrangement of numerous cellular components in the

cochlear duct. The cochlear hair cells, the mechanosensory transducers in the organ of Corti, have different morphological and electrophysiological characteristics based on their location along the longitudinal axis of the cochlear duct, which enables them to respond to distinct sound frequencies.[1] The spiral ganglion neurons show unique electrophysiological properties along the tonotopic axis.[2,3] Mechanical properties of the basilar membrane and the tectorial membrane also vary along the longitudinal axis of the cochlear duct.[4-6]

Several signaling molecules are involved in the early development cellular determination and patterning in the cochlear duct.[7,8] Previous studies have shown that the deletion or inactivation of Sonic Hedgehog (Shh) signals inhibit the outgrowth of the cochlear duct, the ventral part of the inner ear in mouse and chicken embryos.[9,10] Secreted Shh ligands originate from the notochord and floor plate of the neural tube and establish the regional identity and pattern of cell types generated in the ventral neural tube by graded Shh morphogen. During cochlear duct elongation, in the early development of the inner ear, the Shh gradient shows different mechanisms. Opposing gradients of Gli activators (GliA) and repressors (Gli3R) reciprocally act along the dorsoventral axis, such that the apical region requires a high level of Shh with GliA, and the basal region requires a low level of Shh with GliR.[11] It is likely that Shh influences cochlear development through both direct effects on the inner ear structures and indirect effects on the patterning of surrounding tissues.[7,12] One of the questions that could be drawn from previous studies, is whether the Shh signaling is sufficient to determine the tonotopic characteristics within the cochlear duct. Because the apical and basal regions are require

different levels of Gli activators and Gli repressors. Hence the positional identity may be altered by changing the normal, graded levels of Shh signals. Furthermore, recent studies have revealed that bone morphogenic protein-7 (Bmp7) and retinoic acid (RA) regulate the tonotopic pattern of chicken cochlea.[13,14] These signaling molecules have shown a gradient level from the distal to proximal regions along the tonotopic axis. Bmp7 and RA signaling pathways positively regulate the tonotopic organization along the distal to proximal regions of the chicken cochlear, at embryonic day (E) 6.5 to 7, when hair cell differentiation starts.

This study investigated the hypothesis that the graded pattern of Shh signaling along the longitudinal axis along the cochlea, confers unique positional identities of the cochlear duct, contributing to the establishment of tonotopy in mouse and chicken embryos. Thues, the current experiment was designed to up-regulate the Shh signaling and disrupt its gradient in the developing cochlear duct. Inner ear specific conditional mutation of *Smoothened* (*Smo*), the Shh receptor, yielded constitutively activated *Smo* in the developing mouse cochlea, and Shh-soaked beads were implanted in the otocysts of chicken embryos to up-regulate Shh signaling in the inner ear.

In this study, the Shh signaling was spatially and temporally up-regulated along the longitudinal axis of the cochlea to investigate whether graded Shh levels confer unique positional identities along the cochlear duct, contributing to the establishment of tonotopy in the developing mouse and chick embryos.

The contents of this dissertation were published in 2015 in *PNAS*, under the title. “Conserved role of Sonic Hedgehog in tonotopic organization of the avian basilar papilla and mammalian cochlea”

II. MATERIALS AND METHODS

1. Mice management

All the mice used in this research were managed in a temperature controlled room (22± 2°C) under artificial illumination (lights on from 08:00 to 20:00), at 50±10% relative humidity, and noise of below 60 dB, with access to food and water. Their health was checked daily. The *Tg(Pax2-cre)1Akg/Mmmc* mice were termed $Pax2^{Cre}$ mice (Mutant Mouse Regional Research Center) and $Gt(ROSA)26Sor^{tm1(Smo/EYFP)Amc/J}$ mice termed Smo^{M2} mice (Jackson Laboratory). $Foxg1^{Cre}; Shh^{lox/-}$ mice were produced as previously described.[15]

2. Microsurgery of bead implantation into chicken otocyst

Fertilized eggs (Alchan Farm and CJ Freshian) were incubated at 37°C in a humidified chamber (80%). Affi-Gel blue gel agarose beads (Bio-Rad: 100 mesh) were soaked in 1μg/μl Shh or Bmp7 protein solutions for 1hr (R&D Systems). Then, bead implantation was performed as previously described.[16] Briefly, a slit was made on the right side of the chicken otocyst, using a tungsten needle, and then, the ligand-soaked beads were inserted into the otocyst through the slit by using the tip of forceps. Bead implantation was performed in the same otocyst for three sequential days E2.5 (1 bead), E3.5 (3 beads), and E4.5 (5 beads) [Hamburger-Hamilton (HH) stages 17-18, 21-22, and 24-25, respectively]. As controls, embryos implanted with BSA-soaked beads, sham operated, or

untreated otocysts were used for the same experiments as the Shh-implanted embryo. After surgery, embryos were further incubated and harvested at E6.5 (HH stages 29-30), E9 (HH stage 35), E13 (HH stage 39), or E16 (HH stages 42-43) for *in-situ* hybridization, phalloidin staining, or scanning electron microscopy (SEM).

3. *In - situ* hybridization

Harvested embryos were fixed in 4% paraformaldehyde in phosphate-buffer saline (PBS). For dehydration, embryos were immersed in 30% sucrose and embedded in optimal temperature (OCT) compound (Tissue-Tek). The frozen specimen was sectioned at 12μm thickness in a cryotome chamber at –20°C onto coated superfrost slides and stored at –80°C. These sectioned tissue slides were dried at room temperature, and post-fixed. Hybridization was performed in pre-hybridization solution. Each bag contained four slides and 10 ml of hybridization solution with a probe.

4. Phalloidin staining and width measurement of the stereociliary bundles

Dissected basilar papilla was stained with Alexa Fluor 488 phalloidin (Invitrogen). Stained basilar papilla was mounted by Prolong Gold anti-fade (ThermoFisher Scientific) and was covered with coverslip (EMS, glass thickness #0) on the coated slide glass. Images were capture at 5, 25, 50, 75% and 95%

positions from the basal end of the basilar papilla using a confocal microscope (Zeiss LSM700). Rectangles (150 × 75 μm) were drawn along superior edge of the basilar papilla and then 30 hair cells were randomly selected from each rectangle. The width of the stereociliary bundle was obtained using the ImageJ program (National Institutes of Health).

5. Paint-fill injection

Mice at E14.5 were harvested and fixed overnight in Bodian's fixative. Specimens were dehydrated in ethanol and then cleared in methyl salicylate. The inner ears were visualized by injecting commercial correction liquid in methyl salicylate (1:800) into the lumen of the inner ear. The micropipette was inserted on the lateral surface of the inner ear.

6. SEM

Dissected basilar papillae were fixed in pre-fixative solution, composed of 2.5% (wt/vol) glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer (pH7.4) at room temperature for 2hr. These specimens were fixed in fixative solution containing 2mM $CaCl_2$ with 2.5% (wt/vol) glutaraldehyde in 0.1M sodium cacodylate buffer and 3.5% (wt/vol) sucrose at 4^oC overnight. After fixation, specimens were post-fixed using the 1% osmium tetroxide (OsO_4) / 0.1% thiocarbohydrazide (OTOTO) protocol.[17] The specimens were then dehydrated using a graded series of ethanol (20, 40, 60, 70, 80, 90, 95

and 100%), dried using a critical point dryer (HCP-2; Hitachi), attached to a stub, and coated with platinum using a sputter coater (E1030; Hitachi). The coated specimens were mounted on a stub holder and viewed using a cold field emission scanning electron microscope (S-4300; Hitachi) operated at 15kV.

III. RESULTS

1. Ectopically enhanced Shh in the chicken otocyst exhibits the features of the apical region in the proximal basilar papilla

A. Shh soaked bead implanted ears disrupt the gradient gene pattern

Previous studies reported that chicken hair cells display different stereocilia formation along their position on the basilar papilla.[18,19] During development the basilar papilla, it receives graded levels of Shh signaling from the notochord and floor plate.[9,11] *GLI-Kruppel (Gli1)* and *Patched1 (Ptch1)* are Shh downstream target genes,[20,21] which show a gradient expression, regulated by graded Shh signaling. These two genes show a ventral to dorsal gradient expression in otocysts. Thus, to investigate the ectopic effect of altered Shh signaling on the acquisition of tonotopy, the inner ears of chicken embryos were examined after the Shh-soaked beads were inserted into the developing otocyst. Compared to the control ears on the contralateral side, the inner ear structures of the Shh soaked bead implanted ears were subjected to ectopic Shh signals. In Shh soaked bead implanted ears, the Shh gradient was disturbed at E2.5 to E4.5. The *Gli1* and *Ptch1* expression domains were extended in Shh-implanted basilar papilla relative to the controls (Figure 1. E, F, red arrowhead) at E5.5. The induced Shh signaling up-regulated the Shh level over the otocyst stage in the next consecutive days.

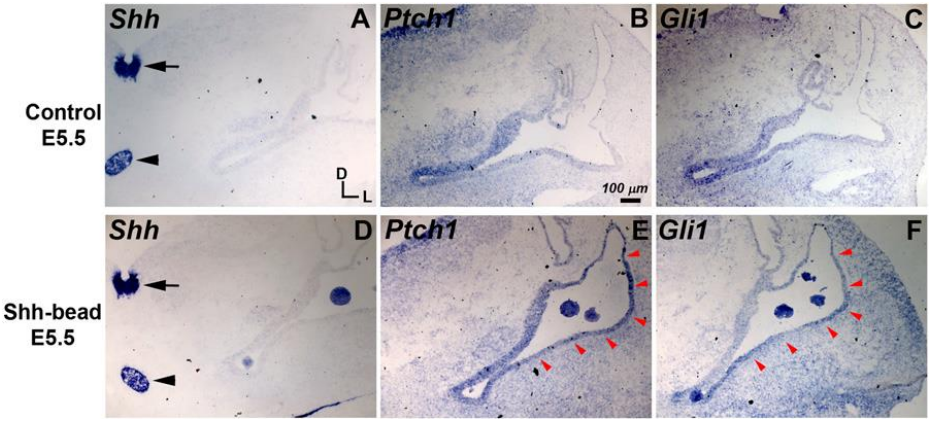

Figure 1. *Ptch1* and *Gli1* were strongly expressed in Shh bead implanted chicken otocyst at E5.5. In control and Shh-soaked bead implanted ears, Shh transcripts are expressed in the notochord (arrowheads, A and D) and floor plate (arrows). *Ptch1* and *Gli1* are strongly expressed in the ventral-to-dorsal gradient (B and C) in control inner ears. These patterns of gradient expressed genes are disrupted in Shh-soaked bead implanted ears. *Ptch1* and *Gli1* are expressed in the entire otocyst including the dorsal region (red arrowheads, E and F).

B. Stereocilia morphology is changed in Shh soaked bead implanted ears

Previous studies on the gain-of Shh function in mice, have shown malformed vestibular apparatus and the Shh-soaked bead implanted chicken inner ears also show similar phenotypes.[10,11] Hence, we asked whether the characteristics of the hair bundles are changed in the developing basilar papilla. So, we examined the stereocilia phenotypes along the tonotopic axis. Phalloidin staining revealed that similar stereocilia morphology was observed between E9 and E11 (Figure 2. A–F). The basilar papilla is a sickle-shaped structure consisting of two types of hair cells: the tall hair cells are located on the superior side, and have a relatively constant surface area along the tonotopic axis, whereas short hair cells, which are located on the inferior side, have a highly variable surface area depending on their position (Figure 3. E).[18] In our analysis, we concentrated on the bundle of tall hair cells (Figure 3. A). After E11, hair bundles develop at different rates exhibiting varying width and length along the basilar papilla. At E16, the basilar papilla was shorter and thicker in the Shh soaked bead implanted ears than the control ears (Figure 3. E and F). At E9, the hair bundle width was similar along the tonotopic axis but from E13, the hair bundle showed a gradient whereby the hair bundles had increased width in the basal region and decreased width in the apical region (Figure 4. A). This morphological gradient was observed at E13 onwards. The width of stereocilia in the hair bundle of basal region was wide; however, the hair bundle of apical region was narrow width in controls. We were able to precisely discriminate the

basal to apical gradient of hair bundle morphology at E16 (Figure 4. A, dark blue line). For the controls, the stereocilia width in the hair bundle of the basal region as wide, the hair bundle of the apical region was narrow in width. The width of stereocilia was quantified in the hair bundles comparing both control and Shh-soaked bead treated ears (Figure 4. B). At E16, the basal region of Shh-treated ears exhibited a similar width to the hair bundle in the middle region of the controls. In contrast, the width of stereocilia in Shh-treated basal hair cells was notably narrower than the width of stereocilia in control basal hair cells (Figure 4. B, asterisk). This result suggests that a high level of Shh in the otocyst had altered the basal identity into an apical identity.

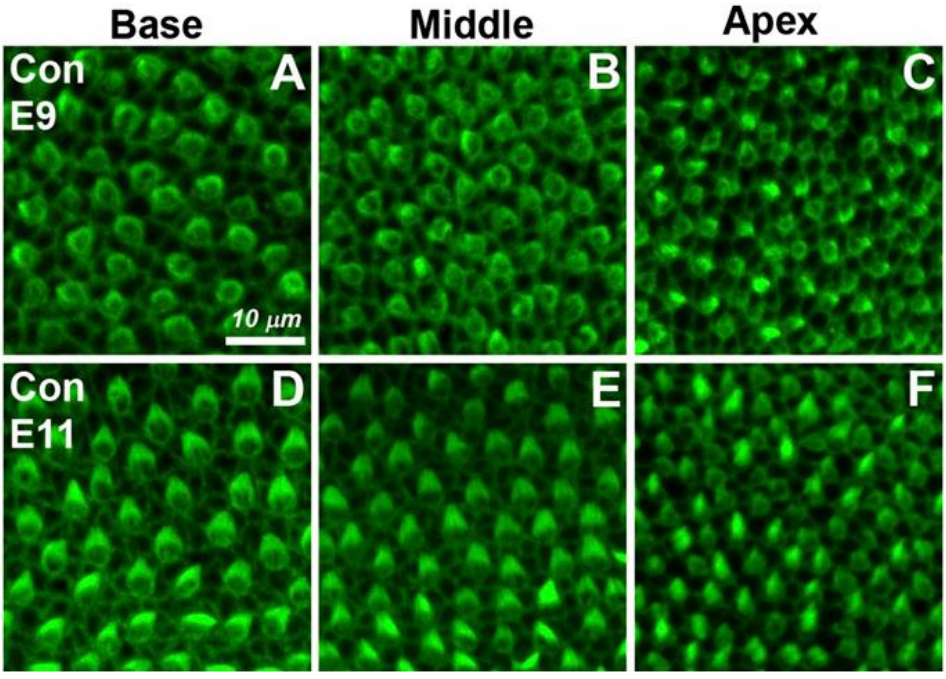

Figure 2. Stereocilia morphology was similar along the tonotopic axis in basilar papilla at E9 and E11. The stereociliary bundles were immature. The developing basilar papilla showed a similar pattern at E9 (A–C) and E11 (D–F).

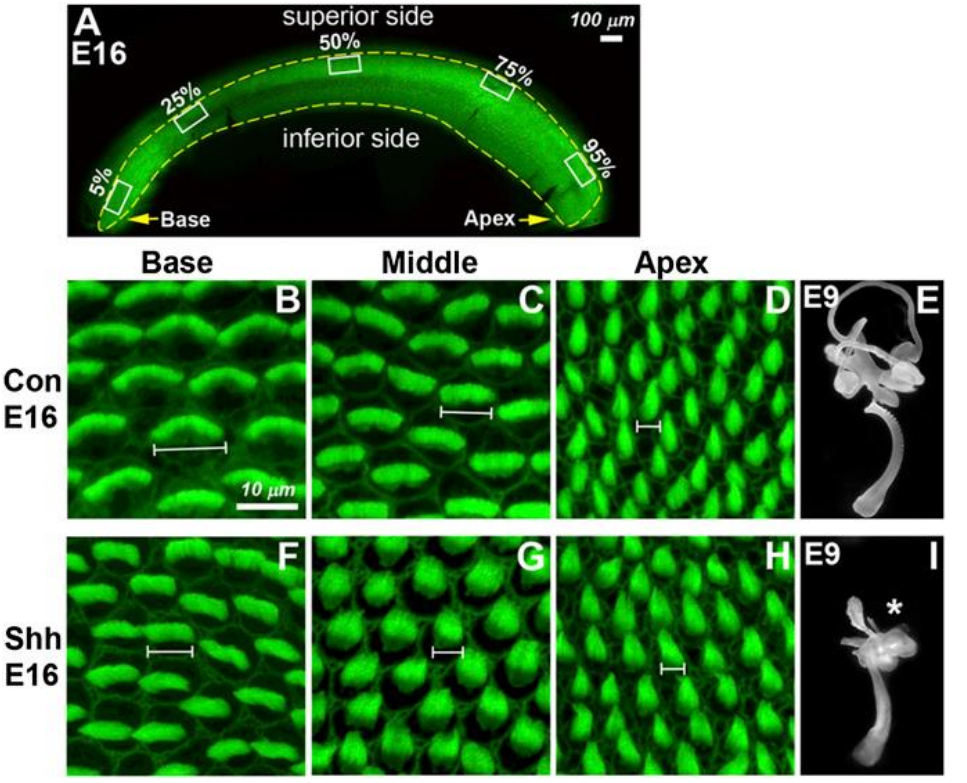

Figure 3. Shh-soaked bead implanted inner ears show an altered stereocilia width, and the anatomical structures were abnormal. (A) Phalloidin-stained E16 basilar papilla. In each region (distance from the basal end at 5, 25, 50, 75, and 95 %) the width of the stereociliary bundle on the superior side was measured. For the base, 5 % region correspond each basal region, 50 % is middle, and 95 % is apex. The width of the stereociliary bundle in each region (base, middle, and apex); control E16 (B–D), and Shh-soaked bead implanted E16 ears (F–H). (E) Paint-fill injected inner ear in control at E9, (I) Shh-soaked bead implanted inner ear shows an abolished vestibule region (white asterisk) and shortened basilar papilla.

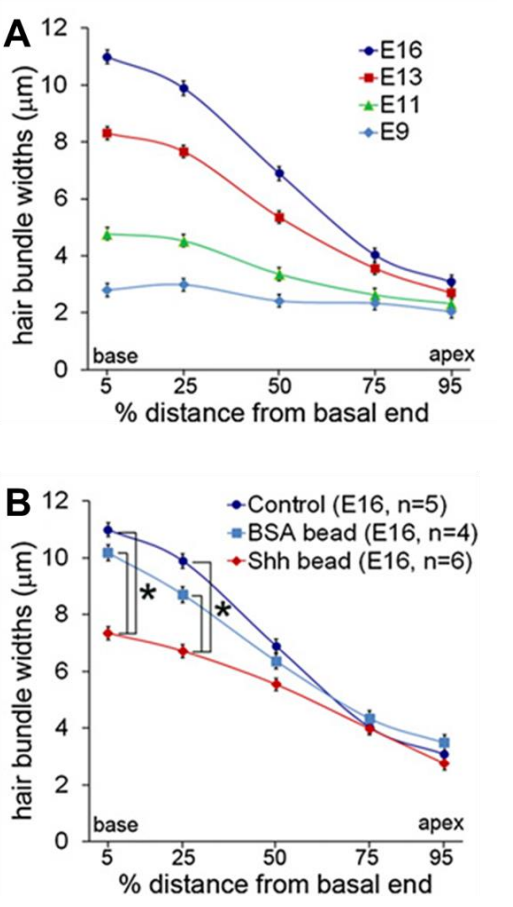

Figure 4. Hair bundle width of developing basilar papilla in control and Shh-soaked bead implanted ears at E16. The hair bundle width is measured along the tonotopic axis relative to the distance from the basal end. (A) The stereociliary bundle width at E9 (light blue line), E11 (green line), E13 (red line), and E16 (dark blue line). (B) In the Shh-soaked bead implanted ears, the hair bundle width of basal region shows a significant decrease, that it is similar to the middle region in the control. (*$P < 0.0001$)

C. Shh-soaked bead implanted ears show an apical phenotype

However, the resulting patterns of the bundle widths in Shh-soaked bead implanted ears at E16 were comparable to those of control ears at E13 (Figure 4. A). The narrow bundle widths of the Shh-bead-implanted ears raise a possibility that the results obtained by the Shh-soaked bead implantation could attribute to a developmental delay rather than a change in the tonotopic identity. Therefore, to distinguish between the two possibilities, we examined another tonotopic characteristic feature which is established earlier than E13. The total number of stereocilia per hair cell that display a gradual decrease along the tonotopic axis towards the apex was shown to be determined at E10 or earlier and was due to a developmental delay, but not due to a tonotopic change; the stereocilia number of the basal hair cells would be similar to that of controls.[19] We performed the SEM to observe the total number of stereociliary bundles per hair cell. The total number of stereocilia was similar in each region for the control at E13 and E16 (Figure 5. A–F) ; with over 200 stereocilia in the basal region, around 100 stereocilia in the middle region and 50 stereocilia in the apex (Figure 6). Furthermore, the stereocilia numbers in the middle region of the control hair bundles were similar to the basal region of the Shh treated ears (Figure 5. G–I). Also the number of stereocilia in the basal region was dramatically reduced in Shh-treated ears, compared to the number of stereocilia in the middle region of the controls. This result demonstrates that the altered phenotypes in the stereocilia of the Shh-treated ears are due to the shifts that occurred in the tonotopic identity. Collectively, these results suggest that

ectopically activated Shh in the developing otocysts, adapt the tonotopic characteristic features of apical hair cells typically exhibited by the basal hair cells of the basilar papilla.

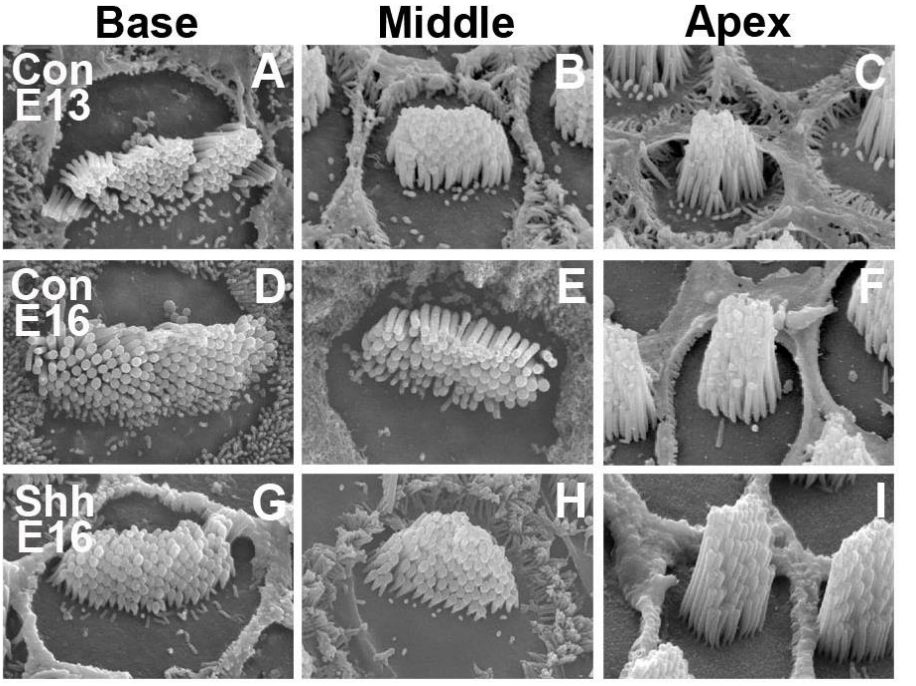

Figure 5. Scanning electron micrographs of the hair bundle. (A–C) Control hair bundle at E13. (D–F) At E16, the stereociliary bundle shows the number and the length of stereocilia. (G–H) The stereociliary bundle number was distinctly reduced in the basal and middle regions. (I) The number of stereocilia in Shh-soaked bead implanted ears was not changed when compared with the controls.

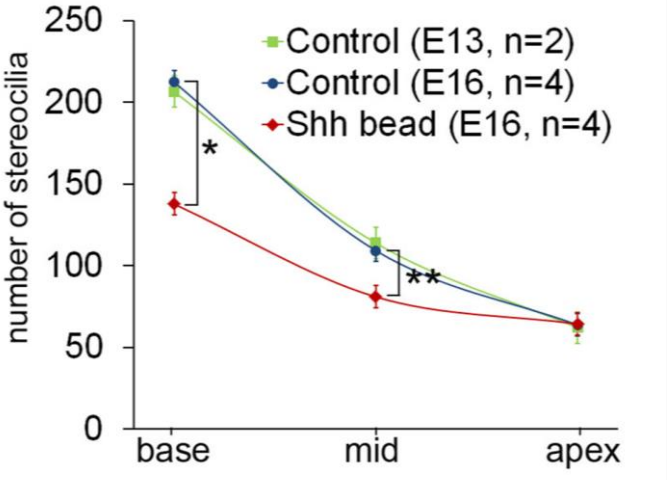

Figure 6. The total number of stereocilia bundles per hair cell along the tonotopic axis in the basilar papilla. The number of stereocilia shows similar gradient along the longitudinal axis in control ears at E13 and E16 (green and blue line, respectively, $P > 0.9999$). Shh-soaked bead implanted ears display a significant reduction in the number of stereocilia compared to the control (*$P < 0.001$, **$P < 0.05$).

D. The Shh signal is crucial for regulating Bmp7 expression in the developing basilar papilla

Recent studies revealed that the apical to basal *Bmp7* gradient expression determines the tonotopic organization in the explants of basilar papilla.[13] We investigated whether Shh-treated ears regulate *Bmp7* expression which determines the tonotopic organization, or if ectopic *Bmp7* signaling can regulate *Ptch1* expression in the basilar papilla. In the control, the *Bmp7* expression pattern showed a strong gradient expression in the apical region, wherease the expression was weak in the basal region of the basilar papilla at E9 (Figure 7. A1–A3). At E9, the expression of *Bmp7* in the basal region of Shh-soaked bead implanted basilar papilla was increased compared to that of control basilar papilla (Figure 7. B1–B3). *Bmp7* expression was strongly expressed in the middle and basal region in Shh-bead-treated ears (Figure 7. B1 and B2, red arrow). On the contrary, Bmp7-soaked bead implanted ears showed no change in *Ptch1* expression (Figure 7. D1–D3). Consequently, the graded Shh signaling regulates the expression pattern of *Bmp7*, by mediating the specification of positional identity in the basilar papilla.

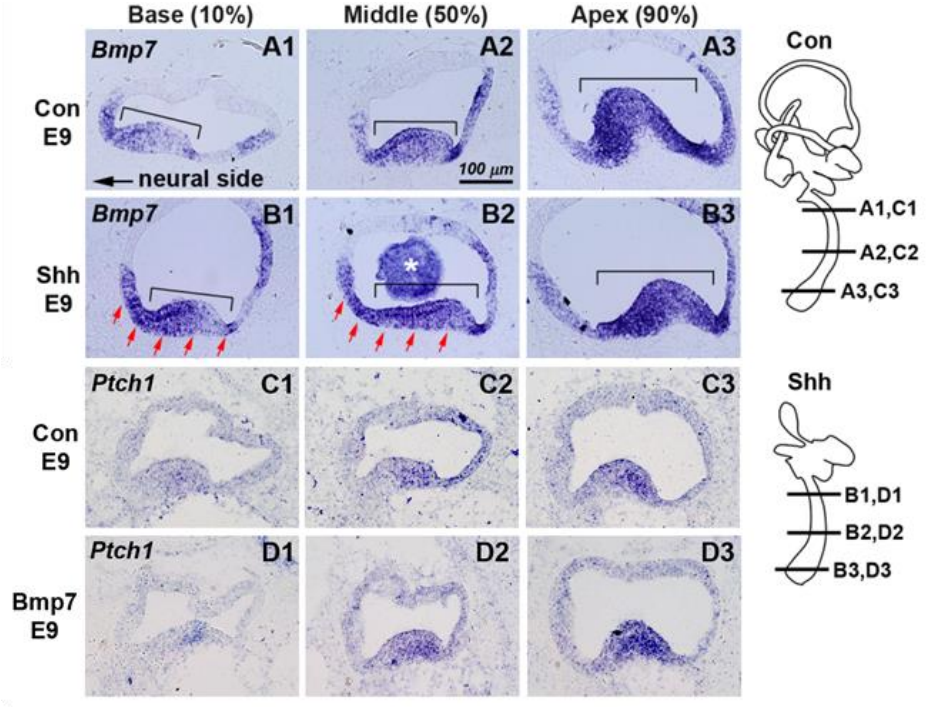

Figure 7. *Bmp7* expression pattern was expanded in the Shh-soaked bead implanted ears but *Ptch1* expression was not expanded in Bmp7-soaked bead implanted ears. (A1–A3) *Bmp7* expression shows a strong gradient in the apex that weakens toward the base. (B1–B3) Expanded *Bmp7* expression was shown in Shh-treated basilar papilla (B1 and B2, red arrow). (C1–C3) *Ptch1* was expressed strongly in the apical region and weakly in the basal region of basilar papilla at E9. (D1–D3) At E9, *Ptch1* expression shows a strong gradient in apex and weak gradient in base at E9.

2. A high A high level of Shh induced apical region properties the in basal region.

Then, we examined whether a high spatio-temporal Shh level can change the expression of genes, determining the physiological characteristics of hair cells along the tonotopic axis. Previously, it has been reported that grdients of *Calbindin* expression occure in the basilar papilla of chicken's inner ear. Its expression in the basal region is stronger than in the apical region. Importantly the topological gradient of *Calbindin* stabilizes the developmental tonotopic gradient of calcium channel expression.[22] Compared to control ears, the intensity of *Calbindin* expression was limited to the basal region of Shh-treated basilar papilla (Figure 8. A1–D3). The expression of *Calbindin* was decreased in the middle region (Figure 8. B2). Similarly, the *Calbindin* expression was also reduced in the middle region of Bmp7-treated ears (Figure 8. D2). Together, these results demonstrate that a Shh level suppresses the *Calbindin* expression indirectly through the Bmp7 molecule. Moreover, *Kcnj2*, encoding IRK1 an inward rectifier potassium channel, was strongly expressed in the apical region of control basilar papilla (Figure 8. E1–E3). In Shh-soaked bead implanted basilar papilla, *Kcnj2* expression was increased towards the middle region (Figure 8. F2). Thus, these results suggest that enhanced Shh signaling positively alters the physiological features along the tonotopic axis.

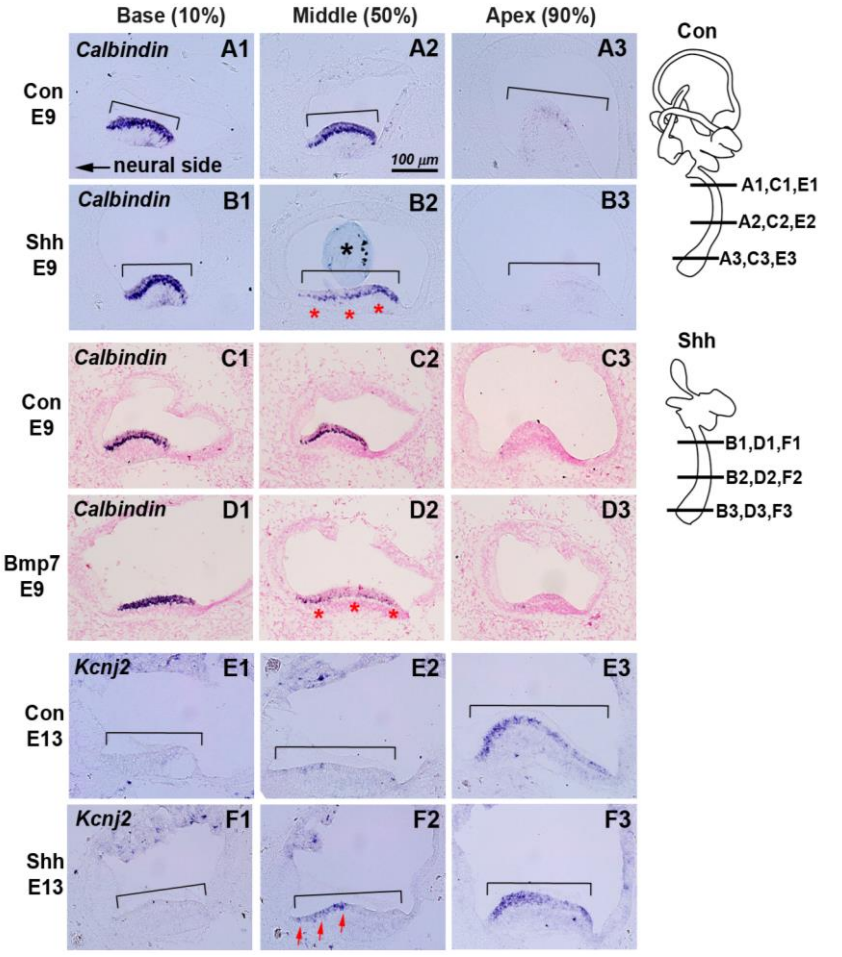

Figure 8. Expression of genes determining physiological characteristics in Shh and Bmp7 soaked bead implanted eaers at E9. (A1–A3, C1–C3) *Calbindin* is strongly expressed in the basal and middle region. (B1–B3, D1–D3) The expression pattern of *Calbindin* significantly decreases in Shh (black asterisk, B2) and Bmp7-soaked bead treated basilar papilla (red asterisk, B2, D2). (E1–E3) *Kcnj2* is strongly expressed in apex strongly. (F1–F3) Shh-bead- implanted ears show expanded expression of *Kcnj2* (red arrow F2).

3. Expression patterns of region-specific genes maintained during the development of the cochlear duct from the cochlear primordium

In order to investigate the specified apical and basal regions of the cochlea, we identified the early developmentally expressed genes that are specifically expressed in the apical and basal cochlear regions. Previous studies revealed that several regional-specific genes including *follistatin* (*Fst*), *ephrin B2* (*Efnb2*), *and msh homeobox 1* (*Msx*1) are expressed in the apical region and *alpha–2–macroglobulin* (*A2m*) and *inhibin beta–A* (*Inhba*) are expressed in the basal region.[11,23] *Fst* is known to exhibit graded expression in the apical region to the basal region from P0 to P8.[23] However, the temporal expression pattern of *Fst* during the early cochlear duct development is not fully known. So, we examined the temporal expression pattern of *Fst* together with other genes including *Efnb2*, *Msx1*, *A2m* and *Inhba*. Spatial expression of regional genes, supports the characteristics of regional identity. *Fst*, *Efnb2*, and *Msx1* began their expression in the cochlear duct at E10.5 onwards (Figure 9. A–D). Apical-specific expressed genes continued their graded expression patterns from the apical region to the basal region, till E18.5. *A2m* and *Inhba* graded expression patterns were highly restricted to the basal region from E11.5 to E18.5 (Figure. 10, 11). Expression of *A2m* began to appear at E11.5 (Figure 9. E) and *Inhba* appeared in the basal region at E12.5 or E13.5 onwards (Figure 10. B). These genes were able to discriminate the regional identity of the cochlear duct throughout its development (Figure 10. F).

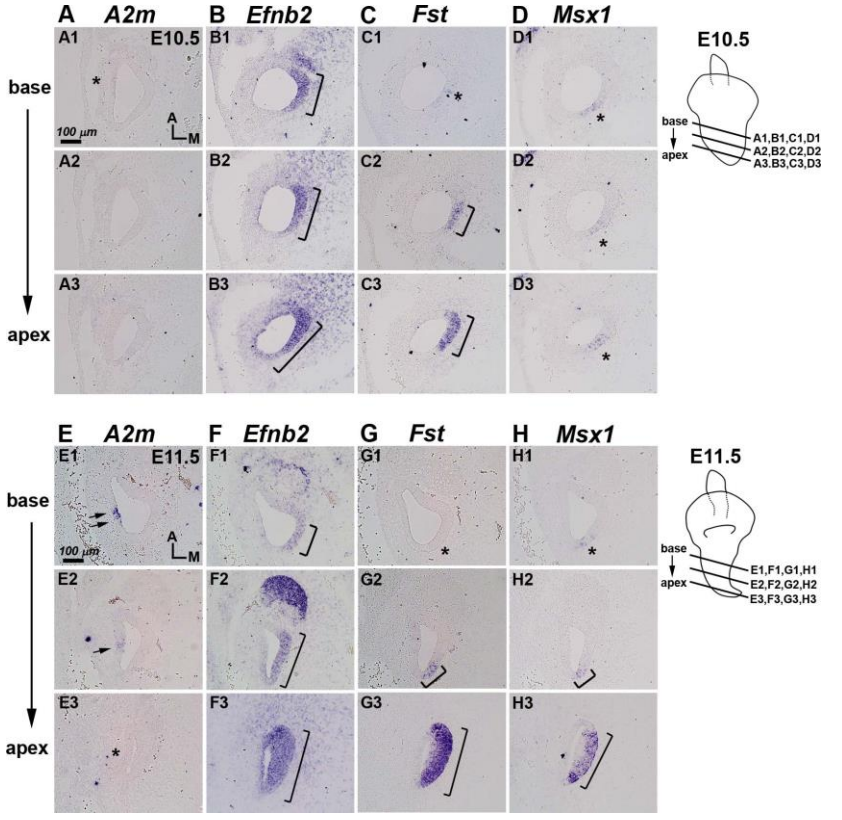

Figure 9. Expression pattern of region-specific genes in the cochlear primordium at E10.5 and E11.5. (A1–A3) *A2m* was the expressed in the cochlear duct at E10.5. (B1–B3) *Efnb2* is broadly and strongly expressed at E10.5. (C1–C3) *Fst* expression is slightly narrowed compared to *Efnb2* in the cochlear primordium. (D1–D3) *Msx1* expression is not evident (asterisk). (E1–E3) *A2m* expression begins in the basal region (black arrows) at E11.5. (F1–F3) *Efnb2* expression is more restricted in the apical region (brackets). (G1–G3) *Fst* is strongly expressed in the apical region (brackets), (H1–H3) *Msx1* expression is restricted in the apical region (brackets). Brackets show expression region. Asterisks indicate a low level of expression.

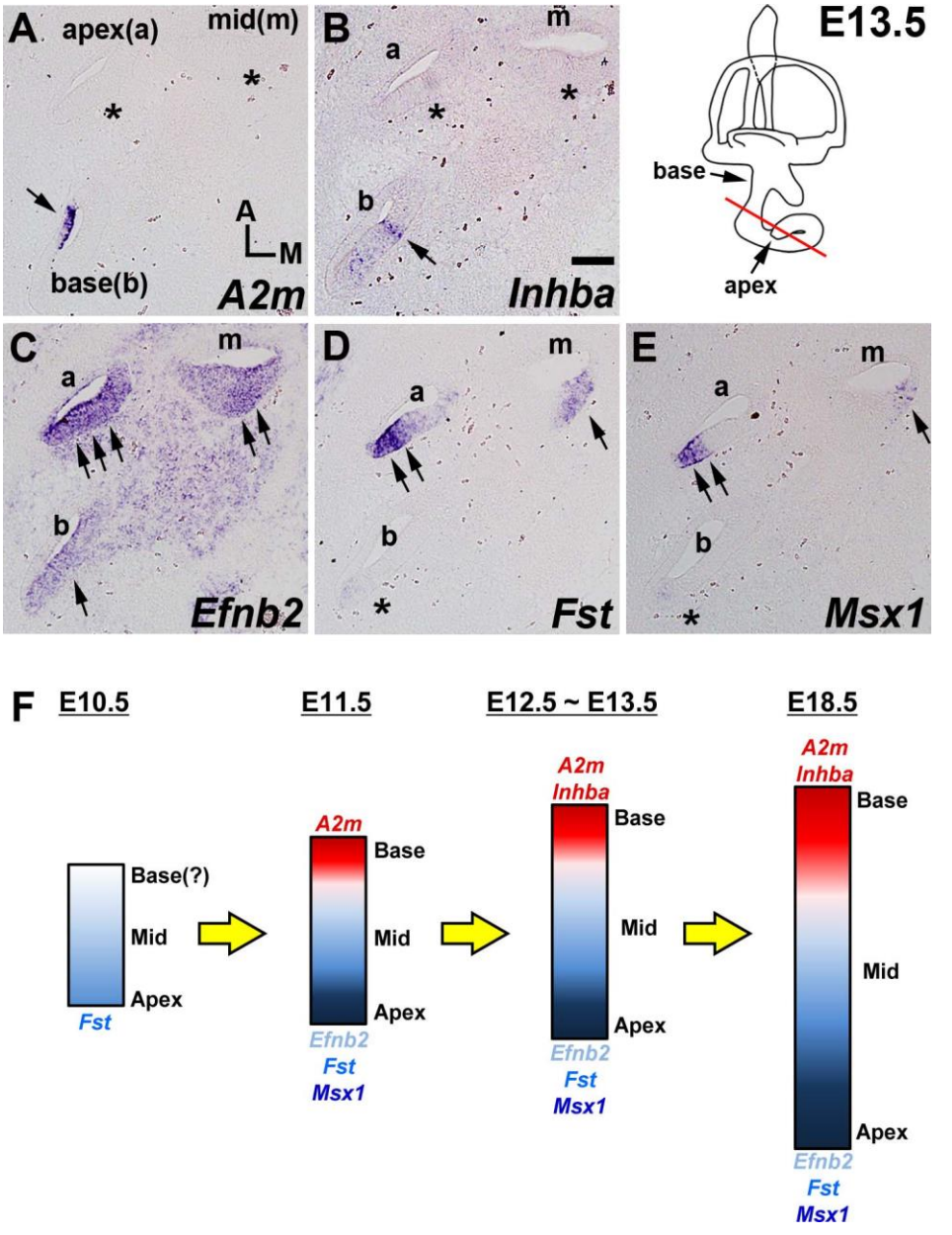

Figure 10. *A2m* and *Inhba* are expressed in the basal region, while *Enfb2*, *Fst*, and *Msx1* are strongly expressed in the apical region. (A, B) *A2m* and *Inhba* show expression only in the basal region (black arrow). (C–E) *Efnb2*, *Fst*,

and *Msx1* show a weakening gradient expression pattern from the apex to the base; black arrows exhibit the intensity. (F) Schematic diagram indicating the regional markers during cochlear duct development.

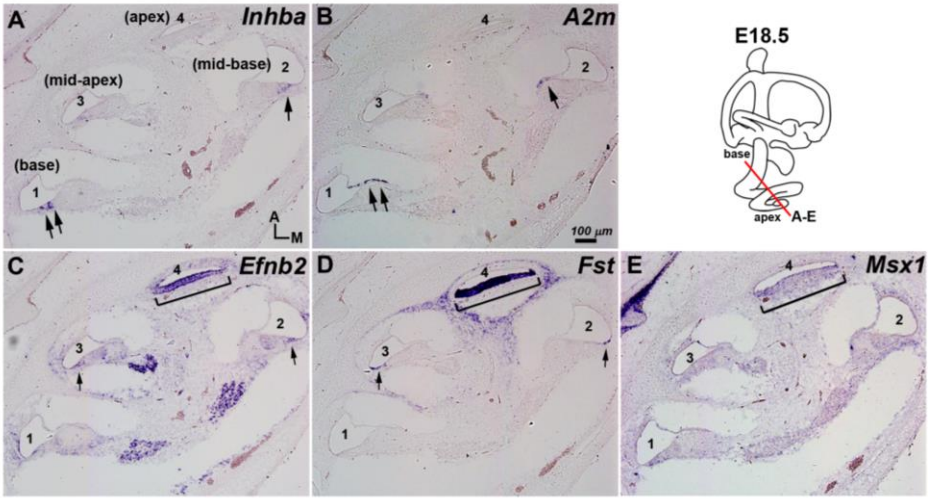

Figure 11. Differentially expressed regional markers maintained their expression levels till E18.5. (A) *Inhba* is expressed in the basal turn of the sensory region. (B) *A2m* expression shows the gradient level in the Reissner's membrane in the basal region of the cochlear duct. (A, B) Black arrows display the expression intensity in the basal region. (C, D) *Efnb2*, and *Fst* were strongly expressed in an apical-to-basal gradient (black arrow). (E) *Msx1* shows restricted expression in apical region (bracket).

4. Ectopic up-regulation of Shh signaling shows the apical region of the mouse cochlear duct.

Previous studies showed that the Shh signaling gradient is required for mouse cochlear duct development.[10,11] *Gli2/Gli3* compound mutants, demonstrated that Shh signaling specified a high level of Shh in the apical region, whereas the basal region is specified by a low level of Shh signaling.[11] Therefore, to test whether altering Shh signaling can change the regional identity of the cochlear duct, we generated a conditional mutant mouse model, in which the *Smo*, an important mediator of Shh signaling, is constitutively active in the otic placode stage using the $Pax2^{Cre/+}$ mice ($Pax2^{Cre/+}$; $Smo^{M2/+}$ mice).[24,25] Unfortunately, $Pax2^{Cre/+}$; $Smo^{M2/+}$ mutants are embryonic lethal at E14.5. These mutant inner ears were extremely deformed, with a lack of semi-circular canals and a shortened cochlear duct (Figure 12. B, B') compared to the control (Figure 12. A and A'). The expression pattern of *Ptch1* which is readout of the Shh signaling pathway, showed a graded level that was strong in the apex and weak in the base (Figure 12. C, black arrow). Ectopic up-regulated Shh signaling in $Pax2^{Cre/+}$; $Smo^{M2/+}$ mutant ears, expressed *Ptch1* in the entire cochlear duct compared to $Smo^{M2/+}$ controls (Figure 12. G, red arrow). The expression pattern of apical region-specific expressed genes, *Efnb2, Fst* and *Msx1* expanded in the entire cochlear duct epithelium of $Pax2^{Cre/+}$; $Smo^{M2/+}$ mice (Figure 12. H–J, red arrow). On the contrary, the marker genes of the basal region, *A2m* and *Inhba* were expressed in the basal turn in the cochlear duct (Figure 13. A and B). These basal region-specific expressed genes were completely down-regulated in

Pax2$^{Cre/+}$*; Smo*$^{M2/+}$ mice (Figure 13. C and D). The ventral otic marker, *Otx2*, and prosensory marker, *Sox2* did not change their expression by ectopic Shh signaling molecules (Figure 14. A–D). Therefore, constitutively active Shh signaling is sufficient to broaden the apical properties in the entire cochlear duct.

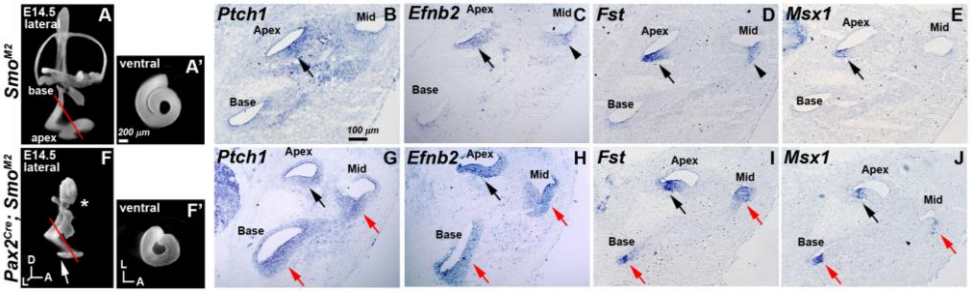

Figure 12. Apical region-specific genes are expanded in *Pax2*$^{Cre/+}$; *Smo*$^{M2/+}$. (A, A') *Smo*$^{M2/+}$ lateral (A) and ventral view of paint-fill (A'). (F, F') Anatomical structure of in *Pax2*$^{Cre/+;}$ *Smo*$^{M2/+}$ mice cochlea, shows malformation of the vestibule region (F, asterisk) and a shortened cochlear duct (F' ventral view). (B) *Ptch1* expression is graded strong in the apex and weak in the base level in the *Smo*$^{M2/+}$ cochlear duct. (C–E) Apex regional marker genes are gradient expressed in the *Smo*$^{M2/+}$ cochlear duct. (G–J) *Ptch1*, *Efnb2*, *Fst* and Msx1 show an expanded expression pattern in the entire mutant cochlear duct.

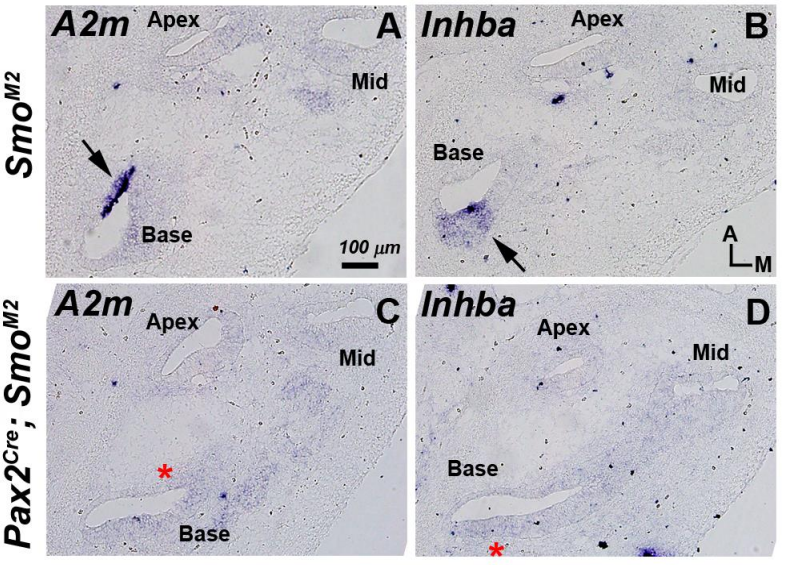

Figure 13. *A2m* and *Inhba* expression are absent from *Pax2*$^{Cre/+}$; *Smo*$^{M2/+}$ ears at E14.5. (A, B) A2m and Inhba are expressed in the basal region of the cochlear epithelium (black arrow). (C, D) The expression level was evidently decreased in *Pax2*$^{Cre/+;}$ *Smo*$^{M2/+}$ mutant ears (red asterisk).

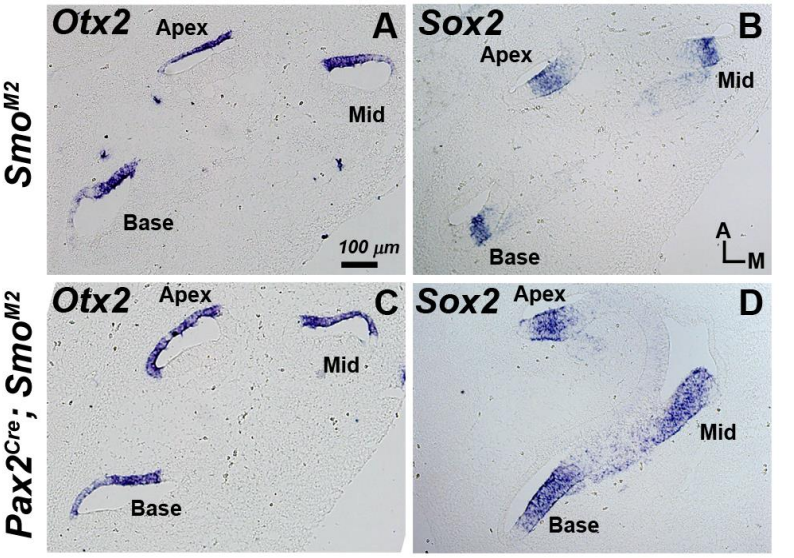

Figure 14. *Otx2* and *Sox2* expression pattern in the cochlear duct at E14.5. (A, C) The ventral regional marker gene (*Otx2*) expression in the epithelial cells of the ventral region for control and *Pax2*$^{Cre/+;}$ *Smo*$^{M2/+}$ mutant ears. (B, D) *Sox2* is a prosensory regional marker with an expression pattern maintained in the prosensory region of the control and *Pax2*$^{Cre/+;}$ *Smo*$^{M2/+}$ mutant cochlear duct.

5. *Bmp7* and *Bmp4* are negatively regulated by Shh signaling in the mouse cochlear duct

In the avian model, *Bmp7* was identified as the major downstream regulator of Shh signaling. The *Bmp7* expression pattern demonstrated an apex to base gradient intensity in the basilar papilla. We questioned whether the *Bmp7* gradient expression pattern is maintained in mammalian the cochlear duct. Furthermore, whether the graded expression pattern of *Bmp7* is affected in the disrupted Shh signaling pathway in the cochlear duct of mice. The *Bmp7* transcript did not display a gradient expression in the control cochlear duct at E14.5. The *Bmp4* gene, another Bmp family member, showed a slightly weakened expression in the apical region at E14.5 in the control (Figure 15. B, black arrow). In $Pax2^{Cre/+}$; $Smo^{M2/+}$ mutant mice, the *Bmp7* expression was significantly reduced in the entire cochlear duct. In addition, *Bmp4* expression almost disappeared in the mutant cochlear duct (Figure 15. D, red asterisk). These results suggest that Bmp family members, *Bmp7* and *Bmp4*, were negatively regulated downstream targets of Shh signaling in mice.

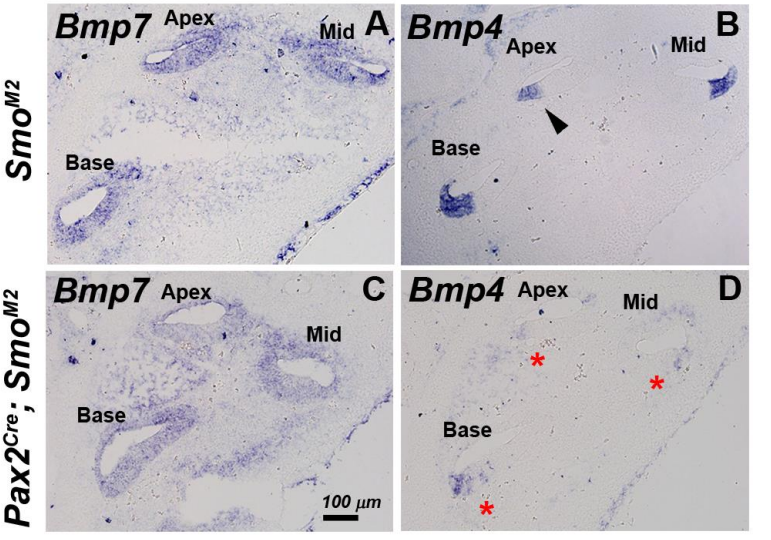

Figure 15. *Bmp7* and *Bmp4* expressions are reduced in *Pax2*$^{Cre/+}$; *Smo*$^{M2/+}$ ears. In control ears, *Bmp7* and Bmp4 mRNA expression are evident in cochlear epithelium; (A) *Bmp7* is broadly expressed in the cochlear epithelium. (B) *Bmp4* is expressed in the lateral epithelial ridge and shows a slightly weak expression in the apical region (black arrowhead). In *Pax2*$^{Cre/+;}$ *Smo*$^{M2/+}$ mutant ears, (C) *Bmp7* expression level is significantly reduced. (D) Bmp4 expression is down- regulated in the lateral epithelium (red asterisk).

6. The early ventral midline Shh source is enough to specify the positional identity in the cochlear duct

Shh signaling from the ventral midline of the neural tube, is required during the early stage of cochlear development. After E11.75, spiral ganglion neurons, which are delaminated from the otic epithelium, act as the second (late) Shh source.[15,26] Thus, we asked if Shh signals secreted from the notochord and floor plate, are sufficient to assign the positional identity to the developing cochlea. Accordingly, we used *Foxg1*$^{Cre/+}$*; Shh*$^{lox/-}$ mutant mice to delete the Shh signal in the spiral ganglia neurons. In these mutants, the early ventral midline Shh source was not affected. The *Foxg1*$^{Cre/+}$*; Shh*$^{lox/-}$ mutants had a shortened cochlear duct, yet *Msx1* expression was observed at the tip of the shortened cochlea.[11] In addition, other apical markers, *Fst* and *Efnb2*, were also expressed in the apical region of the *Foxg1*$^{Cre/+}$*; Shh*$^{lox/-}$ mutant cochlear ducts. These results suggest that the apical cochlear identity was specified in Shh conditional knock-outs. Moreover, the basally expressed genes *A2m* and *Inhba*, were also expressed normally in the shortened cochlear duct of the *Foxg1*$^{Cre/+}$*; Shh*$^{lox/-}$ mutants. These results suggest that the ventral midline source of Shh signaling is sufficient to organize the tonotopic axis identity in the cochlear duct.

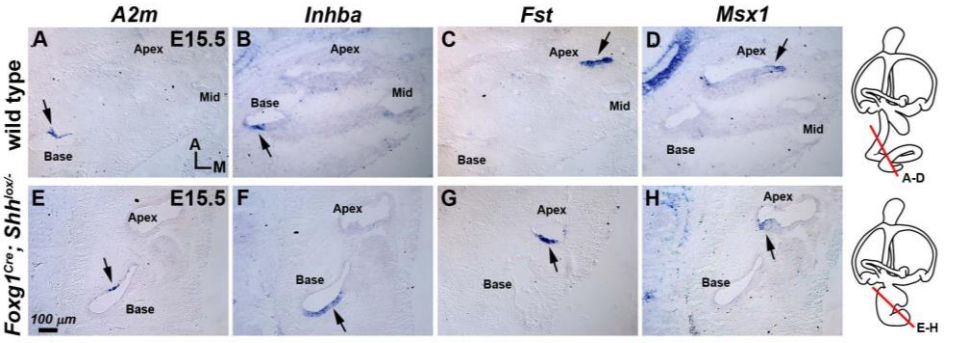

Figure 16. Ganglionic Shh source does not affect the specification of positional identity. (A - D) The region-specific expression genes are expressed in specific regions at E15.5. (A, B) *A2m* and *Inhba* are expressed in the basal region (black arrow). (C, D) *Fst* and *Msx1* are expressed in the apical region. (E–H) *Foxg1*$^{Cre/+;}$ *Shh*$^{lox/-}$ mutant mouse ears show a shortened cochlear duct. Also, the region-specific genes are expressed in their specific region, *A2m* and *Inhba* are expressed in the base (E, F black arrow), and *Fst* and *Msx1* are expressed in the apical turn in mutant ears (G, H black arrow). A, anterior; M, medial.

IV. DISCUSSION

1. Gradient Shh signaling is required to establish the positional identity of the cochlear duct along the tonotopic axis.

These results present evidence that graded Shh signaling confers the tonotopic axis identity to the cochlear primordium. We identified the expression of several regional-specific markers in the apical and basal regions of the cochlea, which discriminate the positional properties. The Shh signaling was secreted from the notochord and floor plate in a graded level at the early stage of otocyst development upstream of *Bmp7*, which established the tonotopic organization in chicken (Figure 17. A). By implanting Shh-soaked beads into chicken otocyst at an early developmental stage (E2.5 to E4.5), the Shh signaling disrupted otocyst, showed the apical region phenotype in the basal region at E16, in the basilar papilla. The regional-specific genes were expanded (*Bmp7*) or restricted (*Calbindin* and *Kcnj2*) in the basilar papilla (Figure 17. B). The positional identity, specified in the cochlear primordium as early as E11.5, reliably designated the relative positions along the tonotopic axis during the cochlear development (Figure 17. C). In mutant, $Pax2^{Cre}; Smo^{M2/+}$ mice apical properties were acquired in the entire cochlear duct, revealing that a high level of Shh was sufficient to change the positional identity in the otocyst (Figure 17. D). On the contrary, previous studies show that *Gli3Δ699* and $Gli2^{-/-}; Gli3^{-/-}$ mutants fail to mediate high levels of the Shh signal, so that the apical identity is lost.[11] Shh signaling is required for the initial patterning of the tonotopic axis in the cochlear

primordium that is mainly provided by the ventral midline sources, the floor plate and notochord, and then Shh signaling from the second source, the spiral ganglion, regulates the pre-patterned elongation of the in cochlear primordium(Figure 17. E).[15] Our results also demonstrated that the role of Shh in the tonotopic patterning, is conserved in the chicken basilar papilla, in which ectopic Shh activation resulted in the adaptation of apical hair cells characteristics by the hair cell in the basal region and lost their basal identity.

2. TGF-β/BMP signaling is downstream mediator of the Shh signaling pathway in the avian but not in the mammalian inner ear.

It is currently unclear how the Shh gradient exerts its function on conferring positional identity to the developing cochlea. Our gene expression analyses, however, suggested a possible involvement of TGF-β/BMP signaling, at least, in the mouse cochlear duct. *Inhba* is encoded by a subunit of the activin member. The TGF-β superfamily is strongly expressed in the base. *Fst*, a TGF-β/BMP antagonist is expressed in a graded pattern, i.e., strongest in the apex and gradually weakend towards the base.[23] In addition, the expression levels of *Bmp4* and *Bmp7* were weak in the apical end of the cochlear duct. Shh signaling suppresses *Inhba* and activates *Fst* in the developing cochlea. Thus, it is possible that an opposing gradient of activin/BMP signaling is established by the Shh gradient. Previous studies reported that *Fst* is directly activated by the major transcriptional activator of Shh signaling in epidermal cells.[27] Furthermore, activin was shown to inhibit the Shh pathway via *Gli3* repressors in neural

precursors, suggesting an antagonistic relationship between the two signaling pathways.[28] Whether the activin/BMP gradient is involved in mediating the Shh signaling, and if so, how the developing cochlea integrates these signaling inputs over time and space requires further investigation.

Even though the Shh gradient is a primary regulator required for the initial patterning of the tonotopic axis in both mammals and birds, several differences were observed in the cochlea and basilar papilla suggesting that the downstream regulatory mechanisms may evolve differently. In the mice cochlear duct, *A2m* is expressed in the basal region, but, it is strongly expressed in the apex of the chicken basilar papilla.[29] In addition, *Calbindin* is strongly expressed in the apical cochlear duct of guinea pigs and gerbils. However, its expression is stronger in the basal region than apical region of the chicken basilar papilla.[22,30,31] Furthermore, the direction of hair cell differentiation along the tonotopic axis, is different between mammals and avians, such that hair cells differentiate in a base-to-apex direction in the mouse cochlea, but in an apex-to-base direction in the chicken basilar papilla.[32-34] It will be interesting to investigate whether the positional information imparted by the same Shh signaling is controlled by different downstream mechanisms in the mammalian cochlear duct and avian basilar papilla.

3. Shh signaling is temporally required for tonotopic organization in the early stage of cochlea development.

The roles of Shh gradient in specifying positional identities have been

demonstrated in other organs, such as the neural tube and limb digits. Neural tubes specify a variety of neuronal subtypes along the dorso-ventral axis, and limb digit positional identities along the antero-posterior axis.[35,36] The Shh signal from the notochord acts as the first source and then, the floor plate acts as a second source in specifying the neuronal subtypes of the ventral neural tube. It is suggested that the neuronal specification occurs early due to initial exposure to the graded Shh signal emanating from the notochord, which is reinforced by the second wave of Shh gradient from the floor plate.[36,37] Initially, the limb digit specification is determined by the Shh source, zone of polarizing activity, whereas, the Shh signal is steadily required to generate the necessary amount of cartilage progenitor cells.[38] In addition to the Shh signaling, temporal exposure to fibroblast growth factor (FGF) signaling is sufficient to specify the entire limb bud outgrowth along the proximo-distal axis but the specific limb digits need to continue the FGF expression along the proximo-distal axis.[39,40] Therefore, it seems to be a common theme for morphogen-mediated specification of positional identity indicating that the early graded morphogen gives the positional information to the primordial cells. It prefigures the characteristic spatial organization in the mature structures which are substantiated by the prolonged exposure of the graded morphogen signaling. Results from this and previous studies demonstrate a similar temporal sequence in the tonotopic patterning of the cochlea. Initial exposure to the Shh gradient from the ventral midline is sufficient to specify the entire cochlear duct along the tonotopic axis. Moreover, Shh signaling from the spiral ganglion neuron is continuously required for cochlear duct elongation. The pre-patterned primordium into a tonotopically organized

mature cochlea. It is still unclear how the level and time of Shh exposure are integrated into the cochlear duct during the tonotopic organization, as previously described in the neural tube and limb digits.

Collectively, our results from this and previous studies demonstrated the multiple and distinct roles of Shh signaling during cochlear development. The initial exposure of the otocyst to a gradient Shh signaling provided from the ventral midline that establishes the tonotopic organization along the longitudinal axis, distinguishes the otocyst into a dorsal vestibular structure and ventral cochlear duct cell fates and also, provides a positional identity in the cochlear primordial cells.

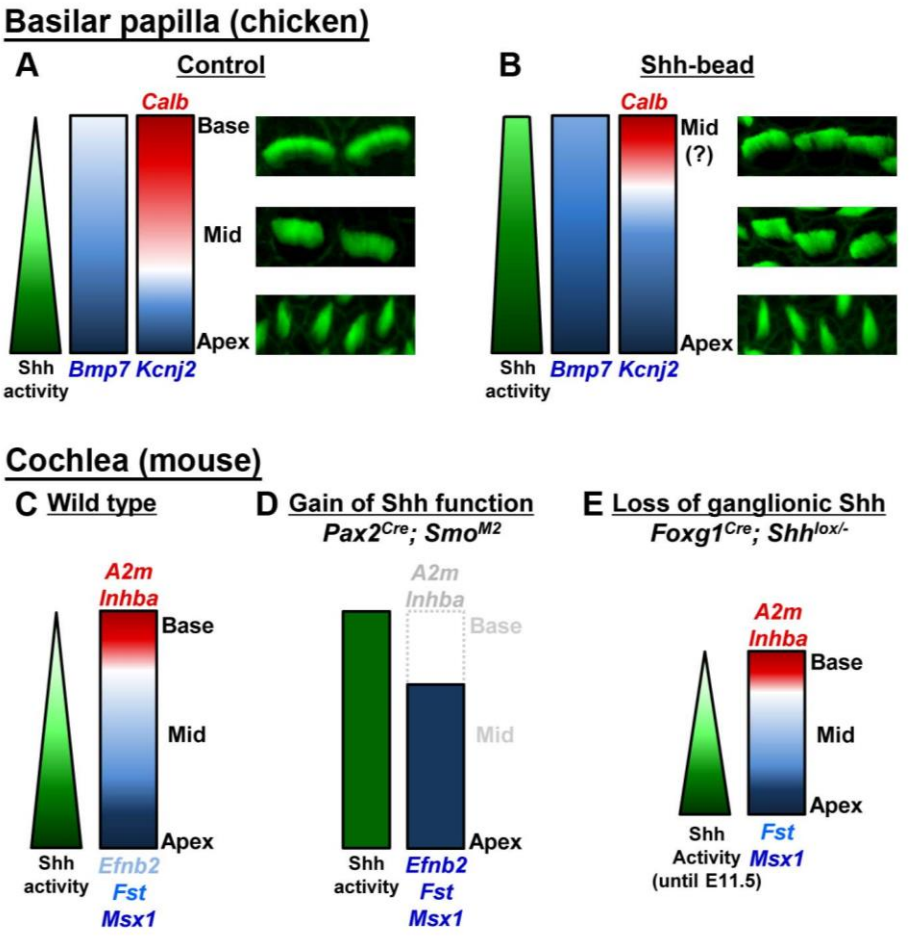

Figure 17. Summary of tonotopic organization in cochlea of avian and mammal species. In control basilar papilla, graded Shh signaling induces an apical-to-basal gradient of *Bmp7* expression, which is an intrinsic factor for establishing the tonotopic axis in the developing basilar papilla. *Calbindin* (*Calb*) is gradient expressed in basal-to-middle region, but *Kcnj2* expressed is restricted to the apical region. The stereociliary bundle phenotype has a gradient morphology, wide at the base and narrows toward the apex. (B) In Shh-soaked bead implanted basilar papilla, apical region expression genes, *Bmp7* and *Kcnj2*,

show an expanded expression region, whereas, *Calb* expression is reduced in the basal region. Furthermore, the hair bundle phenotype is changed to an apical region-like phenotype. (C) In wild type mice, Shh activity shows an apex-to-base gradient that induces the regional-specific genes in the developing cochlear duct (*A2m* and Inhba in the base, *Efnb2*, *Fst* and *Msx1* in the apex). (D) Ectopically enhanced high-level Shh signaling regulates the regional marker genes, such that *A2m* and Inhba are absent, whereas *Efnb2*, *Fst*, and *Msx1* expand their expression range in the entire cochlear duct. (E) When there is a loss of the ganglionic Shh source, the midline Shh activity is sufficient to establish the positional identity from the base to the apex in a shortened cochlear duct.

V. CONCLUSION

In conclusion, our results suggested that establishment of extrinsic sources of the Shh signaling gradient, confers the positional identity to the developing cochlear both in avian and mammal species. An ectopic enhanced Shh level is sufficient to change the phenotype of the basal region of the stereociliary bundle to an apical region phenotypes, and the expression of region-specific genes and functional-specific genes shift the expression pattern in the basilar papilla. Region-specific expression genes were expressed in the early primordium in the inner ear and these gene expression patterns were maintained during embryonic development of the mammalian cochlea.

In avians but not in mammal, the Shh gradient establishes the intrinsic *Bmp7* gradient in the cochlear epithelium, which patterns the tonotopy of the basilar papilla. The downstream target genes of Shh for the tonotopic organization are different in avian and mammal species. In avian species, *Bmp7* is crucial as the major mediator that determines the tonotopic axis. However, BMP signaling is not a positive regulator of Shh signaling.

The ventral midline source of Shh is sufficient to establish the tonotopic organization in the cochlea. Shh signaling-removed spiral ganglion neurons in $Foxg1^{Cre}; Shh^{lox/-}$ mutants, show normal patterned apex to base region in the cochlear duct. Early secreted Shh signaling from the midline source is important to specify the tonotopic identity.

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ABSTRACT(IN KOREAN)

척추동물 와우의 tonotopic organization의 형성 기전

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마지현

청각기관은 소리주파수를 구별할 수 있다. 소리의 구별은 포유류의 와우의 Corti 기관과 조류의 내이유모세포에서 시작된다. 포유류와 조류 두 종의 청각 기관의 해부학적으로 바깥쪽에 위치한 와우와 내이유모세포의 토노토피 시스템에 의해 반응되는데, 기저부(proximal)에서는 고주파를 인지하고, 반대로 첨부 (distal)에서는 저주파를 감지하게 된다. 척색과 바닥판으로부터 분비되는 소닉헤지호그 (Shh)는 포유류와 조류의 와우 발달에 중요한 역할을 하는 것으로 잘 알려져 있다. 포유류와 조류의 초기 이소낭은 배측 중앙선에서 분비되는 높은 레벨의 Shh 신호전달물질이 첨부에서는 많이 필요하게 되는 반면, 기저부에서는 필요하지 않다. 이 연구에서는 닭 모델에서 Shh 신호전달 물질이 스며든 구슬을 조류의

배아에 이식하는 기법을 이용하였으며, 그리고 마우스 모델에서는 계속적으로 높은 활성을 보이는 Shh 신호전달 기법의 기능 획득 실험으로 조류와 포유류 와우의 위치 결정을 증명하였다. 반면에, 위치를 결정하는 조절유전자는 두 종에서 보전되지 않는다. 조류의 경우, 위치 결정 조절 유전자들의 변화로 인해 형태학적, 생리학적 특징들이 바뀌었는데, 형태학적인 것에는 총 부동섬모 개수와 조류의 내이유모세포에 본질적 특성을 가지는 내향성 칼륨 이온 채널 (IRK), *Kcnj2*의 유전자 발현이 바뀌었다. 이 연구를 통해서 배측 중앙선을 따라 분비되는 Shh 이 조류와 포유류의 와우 발달 동안 tonotopic organization을 형성한다는 것을 제안한다.

핵심되는 말 : 내이, 내이유모세포, 와우, 유모세포, tonotopic organization

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