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Roles of hedgehog, bone morphogenetic protein 4 and CXCL12 signaling on middle ear development

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Department of Medical Science

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Directed by Professor Jinwoong Bok

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Mammalian hearing system consists of three types of ears: outer, middle and inner ear. Middle ear consists of a chain of ossicles namely malleus, incus and stapes. These middle ear ossicles transmit the sound waves from outer ear to the inner ear. Any developmental defects in the middle ear ossicle can result in conductive hearing loss. These middle ear ossicles are derived from the neural crest cells (NCCs). The NCCs from rhombomere (r) 1 and r2 and few NC cells from r3 migrate into branchial arch (BA) 1 and develop into Meckel's cartilage, malleus and incus. The NCCs from r4 and few cells from r3 and r5 will migrate into the BA2 to form stapes. It is still unclear that how the NCCs were able to find their final destinations and differentiate into the final target tissue. In order to answer this

question, we approached cre-loxp technology to delete our genes of interest either in the NCCs or pharyngeal endoderm and analyzed the gene expression patterns in controls and their conditional knockout (cko) littermates. When we delete *Smoothened* (*Smo*) gene in the NCCs using *Wnt1*^{Cre} or *Sonic hedgehog* (*Shh*), malleus-incus initial condensation was greatly reduced in the BA1 at embryonic day (E) 10.5 and the stapedial condensation is slightly reduced in the BA2 region. At E11.5, the stapedial condensation was disappeared in the *Smo*^{cko} embryos. This loss of malleus-incus condensation or the stapedial condensation was due to the failure of NC survival in the absence of hedgehog (HH) signaling confirming the HH signaling is required for the NC survival during the middle ear condensation stage. When HH signaling was constitutively activated in the NCCs, there was the increase in the condensation of both malleus-incus and stapes of *Smo*^{M2} mutants. At later stages, we observed an ectopic cartilage which appeared to be the fused and dislocated middle ear ossicles in the *Smo*^{M2} mutants.

Similarly, when we deleted *Smad4* gene in the NCCs or *bone morphogenetic protein 4* (*Bmp4*) in the pharyngeal endoderm, we observed no change in malleus-incus condensation of both *Smad4*^{cko} and *Bmp4*^{cko} at E10.5 and also observed that stapedial condensation was absent. This loss of stapedial condensation was due to lack of the NCCs in the prospective stapes region. BMP4 signaling is not essential for the NC proliferation or survival. Based on previous studies, we hypothesized that CXCL12, a chemokine signaling can act as NCC chemoattractant and endodermal *Bmp4* is required to induce the *Cxcl12* expression in the prospective stapes region. We observed that *Cxcl12* was expressed in the

prospective stapes region although *Bmp4* was deleted in the pharyngeal endoderm. This result suggests us that endodermal *Bmp4* is not required to induce *Cxcl12* expression in the prospective stapes region. When *Cxcl12* was deleted in the whole body (*Cxcl12*$^{+/-}$), we observed normal malleus and incus cartilages at E14.5; but, stapes was greatly malformed. This result confirms that *Cxcl12* is required normal stapes development, but not required to initiate the stapedial condensation.

These all results together confirm that the HH and Bmp4 signaling from the endoderm are required for the NCCs to initiate the malleus-incus and stapedial condensations, respectively.

Key words: malleus, incus, stapes, condensation, neural crest cells, endoderm, hedgehog, bone morphogenetic protein-4, CXCL12

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I. INTRODUCTION

Hearing loss affects almost 360million people (includes 9% children) across the world and 5 out of 1000 babies are born with hearing defects or may acquire the hearing defects immediately after birth (WHO statistics, 2017). The hearing loss can occur either due to the absence or malformed hearing system. The mammalian hearing apparatus consists of three types: outer ear (ear pinna and tympanic membrane), middle ear (a chain of ossicles) and inner ear (vestibular and cochlear parts). All these types of ears are together involved in normal hearing process (Fig. 1)[1]. The middle ear consists of a chain of ossicles: malleus, incus and stapes (Fig. 2)[2]. Well-defined and unique structure of these middle ear ossicles can convey and amplify the sounds more effectively and efficiently from the tympanic membrane to the inner ear[3]. Any defects in either of the middle ear ossicles or all can lead to conductive hearing loss. Some of the middle ear associated hearing loss types include absence of middle ear ossicles, stapes fixation, stapes ankylosis, and fusion of

middle ear ossicles etc. [4].

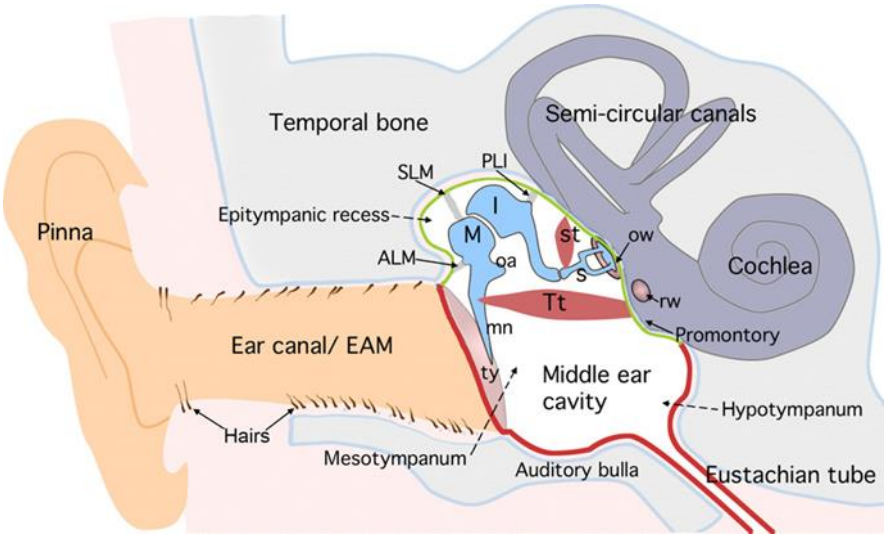

Figure 1. Mammalian hearing system consists of outer, middle and inner ear (adopted from Anathwal et al. 2015).

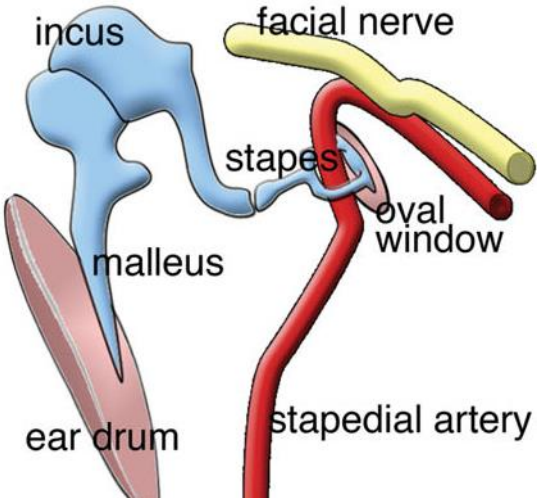

Figure 2. Middle ear consists of malleus, incus and stapes (adopted from Thomson et al. 2012).

These middle ear ossicles are derived from the neural crest cells (NCCs). Most parts of stapes are derived from the NCCs, but the stapedial footplate is derived from the mesoderm[2,5]. The NCCs from the rhombomeres (r) 1, 2 and with little contribution from 3 will populate the branchial arch (BA) 1, and form malleus, incus and Meckel's cartilage. Majority of the NCCs from r4 and with little contributions from r3 and 5 migrate into the BA2 mesenchyme and form stapes and hyoid cartilages[6]. It is still not clear that how these NCCs find their final destinations and differentiate into the tissue. As mentioned earlier, NCCs generally populate the mesenchyme of BAs and then differentiate into the tissues. Each BA is characterized with three layers: outer ectoderm, inner endoderm and middle mesoderm filled with NCCs[7]. Signals from the pharyngeal endoderm instruct the size, shape and location of the NCCs to develop into craniofacial cartilages and bones[8]. The pharyngeal endoderm houses a number of signaling molecules such as sonic hedgehog (SHH), bone morphogenetic protein-4 (BMP4), and fibroblast growth factors (FGFs) etc.[9]. Several signaling molecules such as endothelin, HH, BMP, FGF and CXCL12 (chemokine) are essential for NCCs to migrate, survive and differentiate into the craniofacial structures[10-23]. However, the roles of these signaling molecules on early development of middle ear ossicles are not known.

The mammalian HH family of secreted signaling proteins consists of Sonic HH (SHH), Indian HH (IHH), and Desert HH (DHH)[24]. Among the three, DHH is required for spermatogenesis; IHH is required for the cartilage and bone development and SHH plays crucial roles in neural tube and limb patterning etc.[24-26]. Among the three, SHH is known to have the earliest roles on craniofacial cartilage and bones development. In the absence of the HH ligand, Patched1 (Ptch1), a membrane bound receptor inhibits the activity of Smoothened (Smo), another membrane receptor. The inhibition of Smo activity results in the failure of translocation of Gli activators (transcription factors) into the nucleus and finally,

inactivates the HH signaling. In the presence of the HH ligands, either of the HH ligands can bind to Ptch1 receptor, and activates the Smo activity. This activation of Smo can result in the activation of Gli transcription factors and translocates into nucleus, and induces the expression of various downstream target genes including *Ptch1* and *Gli1* etc.[27]. Among the three ligands, SHH plays vital roles in many tissue processes such as differentiation, patterning, cell proliferation and survival, and many more[13-15,25]. SHH also plays a crucial role in hearing especially in the development of inner and middle ears. Loss of SHH ($Shh^{-/-}$) or inactivation of HH signaling in by deleting *Smo* gene in the inner ear resulted in the absence of ventral part of inner ear (cochlear duct). *Shh* can also directly establish the ventral cell fates[28,29]. Inhibiting the HH signaling in the NCCs or ablation of *Shh* in the endoderm also resulted in the loss of middle ear cartilages[13,15]. Although SHH signaling is required for NCCs, it is still not clear about the early requirement of SHH signaling for the NCCs to differentiate into the middle ear ossicles.

Bone morphogenetic proteins (BMP) is a superclass family member belongs to transforming growth factor – β (TGFβ) family. Bmp2, Bmp4 and Bmp7 etc. are included in this superclass. Binding of Bmp2/4/7 ligands to the Bmp type-II receptors (ActRIIA or ActRIIB), can activate and phosphorylate Bmp type-I receptors (Alk2/ActRIA, Alk3/BmpRIA or Alk6/BmprIB) forming a heterotetrameric complex. This activation can lead to the phosphorylation of receptor smads (R-Smad, Smad1/5/8). These phosphorylated smads cannot directly translocate into the nucleus, until they form the complex with the common smad (co-smad, Smad4). They form the complex with smad4 and translocates into the nucleus and activates the transcription[30]. During the embryogenesis, BMPs plays very important roles in maintaining cell growth, cell survival or apoptosis and differentiation[31,32]. BMP signaling is also known to have crucial roles in the NCCs migration and differentiation. Inhibiting the BMP signaling by *Xnoggin* transgenic

mice resulted in the absence of various second and more caudal BA skeletal and cartilage elements[18]. In these transgenic mice, stapes was absent in almost all *Xnoggin* transgenic mice. Heterozygous *Nogggin* mutated mice exhibited conductive hearing loss, which is due to the development of an ectopic cartilage element between the stapes and posterior wall of tympanum affecting the mobility of the stapes[33]. Loss of *Noggin* also resulted in the failure of separating the cartilage elements during the joint formation[33]. Among the members of BMP family, BMP4 is able to induce and maintain the chondrogenic identity[34]. BMP4 can also provide the positional information during the chondrogenesis[35]. Although, BMP4 is known to have crucial roles to induce the chondrogenic identity, its role on middle ear development is not clearly understood.

CXC chemokine ligand 12 (CXCL12, also known as stromal derived factor 1), a G-protein coupled family member can act as chemoattractant. CXCL12 has two specific receptors: CXCR4 and CXCR7. CXCL12 plays critical roles in various developmental processes mainly involved in hematopoiesis, heart development, craniofacial development and neurogenesis[22,36-38]. During the sympatho-adrenal specification (a NC-derivative in trunk region), BMP signaling from the dorsal aorta is required to initiate the CXCL12 expression in the para-aortic mesenchyme adjacent to the dorsal aorta. This CXCL12 in the aortic mesenchyme is able to attract the NC-derived sympato-adrenal progenitors[23]. Loss of *Cxcl12* or *Cxcr4* can lead to abnormal migration of cardiac NCs, resulting in cardiac anomalies which are similarly observed in the Digeorge syndrome (DGS) patients[39]. Deficiency of CXCL12/CXCR4 signaling also resulted in the abnormal cortical interneuron migration that can lead to the mental disorders observed in the Digeorge syndrome (DGS)[40]. As mentioned earlier, CXCL12 is required for craniofacial development and its role as a NC chemoattractant, we assumed that CXCL12 may also have the crucial roles during the development of the middle ear

ossicles, which was not reported earlier.

In this work, I examined the roles of HH, BMP4 and CXCL12 signals on the NC derived middle ear ossicular development. To examine the roles of HH and Bmp signaling, I approached Cre/Loxp strategy to inactivate HH or TGF-β signaling in the NCCs by deleting *Smo* or *Smad4* in the NCCs or by deleting endodermal *Shh* or *Bmp4* as mentioned earlier[15,41-43]. I also made *Cxcl12* knockout (KOs) mice by deleting the exon2. When HH signaling is inactivated in the NCCs or endodermal *Shh* is deleted, malleus – incus condensation was failed to form in the BA1, and the stapedial condensation was greatly reduced. I observed the expression *Bmp4* in the endoderm beneath the stapedial condensation and assumed the close relationship between the endodermal *Bmp4* and stapedial condensation. When *Smad4* was deleted in the NCCs to inactivate TGF-β signaling or *Bmp4* was deleted in the endoderm, stapes was failed to form in both the cases. Further investigations revealed that endodermal *Bmp4* signaling is essential for the NCC migration into the prospective stapes region and to initiate the stapedial condensation. However, in *Cxcl12* (*Cxcl12*$^{-/-}$) KO embryos, malleus and incus were normal; but, stapes was severely malformed (reduced) with no stapedial artery. Taking all the results together, it can be concluded that the signals from the endoderm are required for the NCCs to migrate and initiate the middle ear condensations in their respective BAs.

II. MATERIALS AND METHODS

1. Mice

All mice with genotypes *Wnt1*^{Cre}*;Smo*$^{lox/lox}$, *Wnt1*^{Cre}*;Smo*$^{M2/+}$, *Wnt1*^{Cre}*;Smad4*$^{lox/lox}$ *Foxg1*^{Cre}*;Shh*$^{lox/lox}$ *and Foxg1*^{Cre}*;Bmp4*$^{tm1/lox}$ were generated as previously described[15,41-43]. To label the NCCs derivatives, we used *ROSA26* conditional reporter line (*R26R*). To generate *Wnt1*^{Cre}*;Smad4*$^{lox/lox}$*;R26R*$^{lox/+}$, we bred *Wnt1*^{Cre}*;Smad4*$^{lox/+}$ with *Smad4*$^{lox/lox}$*;R26R*$^{lox/lox}$ [41]. *Cxcl12* knock mice were generated upon the deletion of exon2[44]. All animals were handled in accordance to the guidelines for the Care and Use of Laboratory Animals of Yonsei University College of Medicine. Mice were mated and the morning of vaginal plug formation counted as E0.5.

2. *In-situ* hybridization

Embryos were fixed overnight in 4% paraformaldehyde in DEPC treated PBS, dehydrated in 30% DEPC treated sucrose solution and mounted the embryos in OCT compound (Tissue-tek). Frozen sections were performed at 12 μm thickness using the cryotome (Thermo Scientific) onto superfrost slides (Thermo scientific) and stored at -80 °C for further experiments. Using the following antisense probes *Bmp4*[45], *Sox9*[46], *Ptch1*[47], *Shh*[25], *Acan*[48], *Ap2a* (NM_001122948.2) and *CXCL12* (NM_001012477.2), *In situ* hybridization was performed for frozen sections as previously described[45]. The micrographs of the gene expression patterns were taken using OLYMPUS BX40 and Leica DM2500 optical microscopes.

3. *LacZ* staining

Embryos for *lacZ* staining were fixed in the solution containing 2% paraformaldehyde, 2 mM MgCl2 and 5 mM EGTA for two hours at 4°C, dehydrated in 30% sucrose solution for overnight at 4°C and finally, they were

embedded in the OCT compound (Tissue-Tek). These embryos were sectioned at 12 μm thickness using cryotome (Thermo scientific) onto superfrost slides (Thermo scientific). The frozen sections were stained in the staining solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP-40, 1 mg/ml X-gal (MP Biomedicals) and 1X PBS at 37°C for overnight. The micrographs of were taken using OLYMPUS BX40 and Leica DM2500 optical microscopes.

4. Cell Proliferation (EdU staining)

Cell proliferation was performed by using Click-iT™ EdU imaging kit (C10337, Invitrogen). The pregnant mouse was injected with 10 mg/Kg body weight of EdU for three times at regular intervals of 2 hours (hrs). After 2 hrs of final injection, Embryos were fixed overnight in 4% paraformaldehyde in DEPC treated PBS, dehydrated in 30% DEPC treated sucrose solution and mounted the embryos in OCT compound (Tissue-tek). Frozen sections were performed at 12 μm thickness using the cryotome (Thermo Scientific) onto superfrost slides (Thermo scientific) and stored at -80°C for further experiments. Using the adjacent sections, *in situ* hybridization was performed for regional identification. The sections to be used for EdU staining were fixed in 4% PFA for 15 minutes at room temperature, washed with 3% bovine serum albumin (BSA), treated with 0.5% TritonX-100 in 1X PBS and finally washing was performed again with 3% BSA. Then the slides were incubated with 500 μl/slide EdU detection solution (which has to be freshly prepared) for 30 minutes. EdU detection solution consists of 430 μl of 1X Click-iT reaction buffer, 20 μl of Copper sulphate, 1.2 μl of Alexa Fluor 488 azide and 50 μl of

1X reaction buffer additive. All these components are available in the kit provided by the company. Counter staining was performed by using 5 µg/mL of Hoechst 33342 to label the nucleus. Finally, the slides were mounted with anti-fade solution.

5. Cell survival assays (TUNEL staining)

Cell survival analysis was performed by using ApopTag Plus Peroxidase *In situ* apoptosis detection kit (S7101, Millipore) as per the manufacturer instructions. Embryos were fixed overnight in 4% paraformaldehyde in DEPC treated PBS, dehydrated in 30% DEPC treated sucrose solution and mounted the embryos in OCT compound (Tissue-tek). Frozen sections were performed at 12 µm thickness using the cryotome (Thermo Scientific) onto superfrost slides (Thermo scientific) and stored at -80°C for further experiments. The sections were fixed in 1% PFA, and were permeablized in 2:1 ratio of precooled ethanol:acetic acid. To inhibit the endogenous peroxide, these slides were applied with 3% Hydrogen Peroxide (Merck). Equilibrate the sections with the equilibration buffer for 30 seconds, apply the working TdT enzyme to the sections and incubate the sections in the humidified chamber at 37℃ for 1 hr. After 1 hr stop or wash buffer was applied onto the section. Anti-digoxigenin peroxidase was applied on the section and again incubated in the humidified chamber for 30 minutes. After 30 minutes, peroxidase substrate was applied to the sections and stained for 5 mins. After the development of the color, the sections were washed with water thoroughly. Finally, the sections were treated with 1% methyl green (Sigma

Aldrich) for counter staining. Finally, the slides were mounted with synthetic mountant solution. The micrographs of were taken using OLYMPUS BX40 and Leica DM2500 optical microscopes.

III. RESULTS

1. Development of middle ear ossicles from initial condensation to cartilages

Since, there was no clear information regarding temporal development of middle ear ossicles, I studied the temporal development of middle ear ossicles from E10.5 till E15.5, the early stages from condensation to middle ear cartilages using *Sox9* (a condensation marker) up to E14.5 and *Aggrecan* (*Acan*, cartilage marker) at E15.5 along the dorso-ventral axis (normal section, Fig.3). When we section the embryo along the dorso-ventral axis, we can see both BAs together in a single section (Fig.3A). At E10.5, *Sox9* was broadly expressed in the BA1 representing malleus-incus condensation together with Meckel's cartilage (Fig.3B, M&I). *Sox9* expression in the BA2 anterior to inner ear can represent the stapedial condensation (Fig.3B, S). Still at E12.5, malleus and incus did not separate from each other and appear as a single condensation (Fig.3C). Separate malleus and incus cartilages were first observed at E13.5 i.e., three separate cartilages malleus, incus and stapes were observed from E13.5 onwards (Fig.3D). At E14.5, stapes was inserted into the oval window of the inner ear (Fig.3E). At E15.5, *Acan* was expressed in all the three separate middle ear cartilages (Fig.3F). These results indicate the middle ear condensations start at E10.5 and eventually develop into cartilage at E15.5.

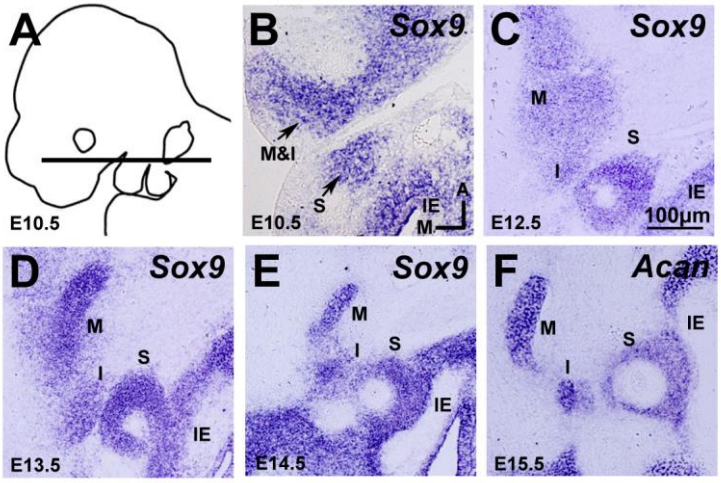

Figure 3. Temporal development of middle ear ossicles during embryogenesis. Schematic representation of E10.5 mouse embryo and line indicate the level of embryo sectioned (A). *Sox9* was expressed in the malleus, incus and stapes in the embryos at E10.5 (B), E12.5(C), E13.5 (D), and E14.5 (E). *Acan* was expressed in the malleus, incus and stapes cartilages (F). Normal sections were performed such that anterior is on top and lateral is on left side. M – malleus, I – incus, S – stapes and IE – inner ear. Scale bar: 100μm.

2. Spatial relationship of HH related genes with NC derived middle ear initial condensations

Previous studies when performed by deleting *Smo* gene in the NCCs resulted in the complete loss of middle ear cartilages[15]. These studies indicated that HH signaling is required for the NC derived middle ear cartilages. However the early role of HH signaling on middle ear development is not clearly understood. In order to clearly understand about the relationship between the NC-derived middle ear cartilages and HH signaling, I compared the expression patterns of *Sox9*, *Shh* and *Ptch1* (a downstream target gene of HH) with the *lacZ* labeled cells in the middle ear condensation regions of E10.5 mouse embryos along the anterior-posterior axis (Fig.4). To label the NCCs, I crossed *Wnt1*^{Cre} mouse with *Rosa26 Reporter* (*R26R*) mouse to obtain *Wnt1*^{Cre}*; R26R*, and performed *lacZ* staining[49,50]. When *lacZ* staining was performed, *lacZ* positive cells were observed in both the BA1 and BA2 indicating that the NCCs were populated in the BAs (Fig.4A and F, black arrows). In BA1, *Sox9* was expressed in the lateral mesenchyme adjacent to the pharyngeal endoderm which might represent the malleus-incus condensation (Fig.4B, M&I). *Shh* was expressed in the pharyngeal endoderm (Fig.4C, black arrow) and *Ptch1* was expressed gradiently in the mesenchyme adjacent to the pharyngeal endoderm (Fig.4D, black arrow). *Ptch1* expression was partially overlapping with *Sox9* expression. In BA2, *Sox9* was expressed in the mesenchyme dorsal to the pharyngeal endoderm, representing the stapedial

condensation (Fig.4G, black arrow). *Shh* was not expressed in the pharyngeal endoderm beneath the *Sox9* expression (Fig.4H, square bracket and red asterisk) and also *Ptch1* did not express in the stapedial condensation region (Fig.4I, red asterisk). Altogether, these results indicate the HH signaling is spatially and partially related with malleus-incus condensation region, but is not related with stapedial condensation.

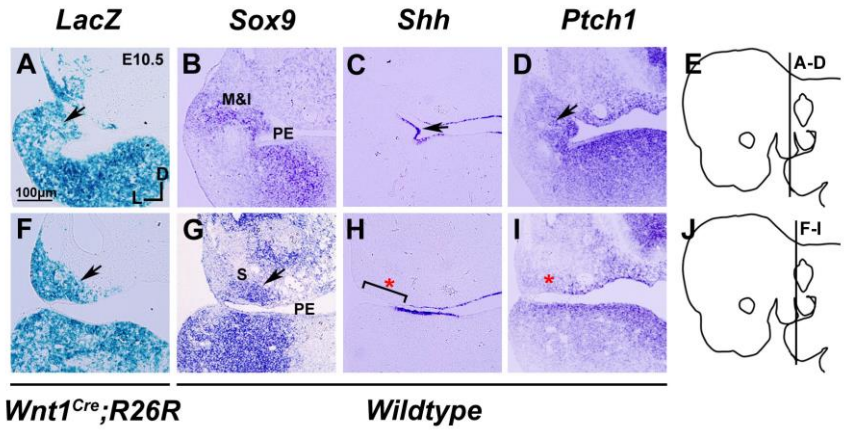

Figure 4. Spatial relationship between NC derived middle ear condensation and HH signaling. (A, F) Lineage analysis using *Wnt1^{Cre};R26R* was performed. *Sox9* was expressed in NC-derived malleus-incus (B) condensation and stapes condensation (G). *Shh* was expressed in the pharyngeal endoderm (C and H) and was not expressed in the pharyngeal endoderm under the stapes condensation (H, red asterisk and square bracket). *Ptch1* was expressed strongly in NC-mesenchyme of BA1 adjacent to pharyngeal endoderm (D, black arrow) and did not express in the lateral side of the NC-mesenchyme of BA1 (D) and did not express in the stapedial region (I). Coronal sections were performed such that dorsal is on top and lateral is on left side. Schematic representation of each level of section in E10.5 mouse embryos was shown in E and J. M – malleus, I – incus, S – stapes and PE – pharyngeal endoderm. Scale bar: 100μm.

3. HH signaling is required for NCCs to initiate malleus-incus condensation in BA1 region, but not stapedial condensation

Next, I asked whether HH signaling is required for the NCCs to initiate malleus-incus condensation. In order to answer this, I deleted *Smo* gene in the NCCs using *Wnt1*^{Cre} to inactivate the HH signaling in NCCs, as mentioned earlier[15] and compared the expression patterns of *Sox9, Ptch1* and *Shh* between controls and *Wnt1*^{Cre};*Smo*$^{lox/lox}$ (*Smo*^{cko}) embryos at E10.5 along the anterior-posterior axis. In *Smo*^{cko} embryos, *Sox9* expression in BA1 (Fig.5F, red asterisk) was greatly reduced than *Sox9* expression in the BA1 of controls (Fig.5A, M&I). *Ptch1* expression was also dramatically decreased in the mesenchyme adjacent to the pharyngeal endoderm (Fig.5G, red asterisk) and *Shh* expression was not changed in the pharyngeal endoderm of *Smo*^{cko} embryos (Fig. 5H, black arrow). These results indicate that the reduction of *Sox9* expression in the malleus-incus region is due to the lack of HH signaling in the NCCs. This suggests that HH signaling is essential for the NCCs to initiate the malleus-incus condensation. I observed that the malleus-incus condensation was absent at E11.5, suggesting that the lack of *Sox9* expression in the BA1 is not due to developmental delay. Then, I analyzed whether HH signaling is required for the NCCs to initiate the stapedial condensation using the *Smo*^{cko} embryos at E10.5. In *Smo*^{cko} embryos, *Sox9* expression in stapedial condensation region of BA2 (Fig.5O, black arrow) was slightly reduced than *Sox9* expression in the stapedial condensation of control BA2 region (Fig.5J, black arrow). *Ptch1* (Fig.5K and P) and *Shh* (Fig.5L and Q) expression were

similar in both control and *Smo*^{cko} embryos. These results suggest us that HH signaling is not necessary for the NCCs to initiate the stapedial condensation.

Although HH signaling is not necessary for NCCs to initiate the stapedial condensation, then I questioned when the stapedial condensation did disappeared. So, I analyzed *Sox9* expression in the stapedial region of both control and *Smo*^{cko} embryos at E11.5. In controls, *Sox9* was expressed in the mesenchyme between the inner ear and the pharyngeal endoderm representing stapes condensation (Fig.5M). But, in *Smo*^{cko} embryos, *Sox9* was failed to express in the mesenchyme between the inner ear and the pharyngeal endoderm suggesting the stapedial condensation was disappeared (Fig.5R, red asterisk). These results suggest that HH signaling is necessary for the development of the stapedial condensation.

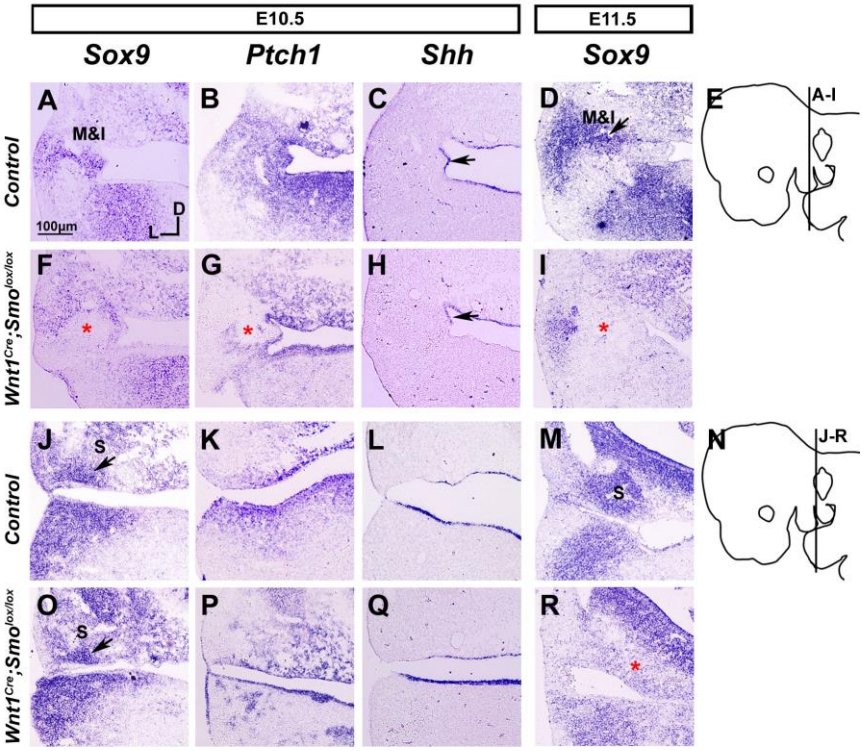

Figure 5. HH signaling is necessary to initiate th malleus-incus condensation, but not essential to initiate stapedial condensation. In *Smo*^{cko} embryos at E10.5, *Sox9* expression was greatly reduced in the malleus-incus condensation (F, red asterisk) and slightly reduced in the stapedial condensation (O, black arrow). *Ptch1* did not express in the mesenchyme of middle ear condensation regions (G and P). *Shh* is normally expressed in the pharyngeal endoderm. At E11.5, *Sox9* failed to express in the prospective middle ear condensation regions (I and R). Coronal sections were performed such that dorsal is on top and lateral is on left side. Schematic representation of each level of section in E10.5 mouse embryos was shown in E and N. M – malleus, I – incus, S – stapes and PE – pharyngeal endoderm. Scale bar: 100μm.

4. HH signaling is essential for the NCCs survival during the middle ear condensation stage.

In order to trace the reason behind the reduction of the malleus-incus and stapedial condensations in *Smo*^{cko} embryos, I performed EdU staining and TUNEL staining to examine cell proliferation and cell survival defects in *Smo*^{cko} embryos and compared with control embryos at E10.5 (Fig.6). In order to identify the regions, adjacent sections were used to analyze the *Sox9* expression pattern (Fig.6A, E, I and M). When EdU staining was performed, EdU stained cells were observed similarly in both middle ear condensation regions of both littermates (Fig.6B, F, J and N). The number of cells present within the boxed region of adjacent *Sox9* expressed tissues was quantified to analyze whether number of proliferated cells were reduced. When counting was performed, the number of EdU positive cells was similar in malleu-incus or stapedial condensations of both control and *Smo*^{cko} embryos i.e., The number of EdU proliferating cells in the boxed region of malleus-incus region are 115±11 cells/20000 um^2 area in controls and 108±8 cells/20000 um^2 in *Smo*^{cko} embryos (Fig.6D; $p>0.05$). The number of EdU proliferating cells in the stapes of control is 36± 4 cells/7500 um^2 and in *Smo*^{cko} is 34±2 cells/7500 um^2 (Fig.6L; $p>0.05$). These results indicate that HH signaling is not necessary for NCCs to proliferate during the middle ear condensation development.

Further I examined whether inactivation of HH signaling had resulted in cell survival defects that might be responsible for the loss the middle ear condensations in the BA1 and BA2 of *Smo*^{cko} embryos. To examine this, I

performed TUNEL staining according to the manufacturer's protocol. Adjacent sections were utilized for *Sox9* expression analysis in order to identify the region (Fig.6A, E, I, M). In controls, very few cells in the malleus-incus condensation were labeled with TUNEL reagents (Fig.6C, black arrowhead) and no TUNEL labeled cells in the stapedial condensation region (Fig.6K, black asterisk). In Smo^{cko} embryos, there are many TUNEL stained cells observed in both malleus-incus and stapedial condensation regions (Fig.6G and O, black arrowheads). The number of TUNEL positive cells present within the boxed region of adjacent *Sox9* expressed tissues was quantified to analyze whether number of apoptotic cells were increased. When counting was performed, the number of TUNEL positive cells was in malleus-incus and stapedial condensations of Smo^{cko} embryos were increased than controls. The number of TUNEL positive cells in the boxed region of malleus-incus region are 4±1 cells/20000 um^2 area in controls and 16±1 cells/20000 um^2 in Smo^{cko} embryos (Fig.6H; $p<0.0005$). The number of TUNEL positive cells in the stapes of control is 2±1 cells/7500 um^2 and in Smo^{cko} is 5±1 cells/7500 um^2 (Fig.6P; $p>0.05$).These results confirm that the absence of middle ear condensations is due to failure of NCCs survival in the absence of the HH signaling in the NCCs.

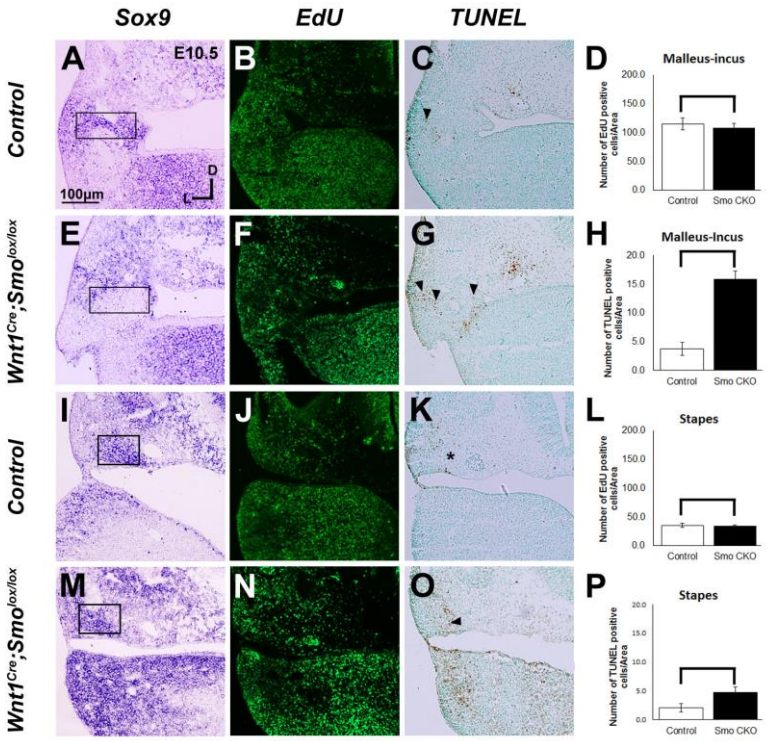

Figure 6. HH signaling is required for the NC survival, but not for cell proliferation. Cell proliferation and cell death analysis were performed in the E10.5 mouse embryos using EdU staining (B, F, J and N) and TUNEL staining (C, G, K and O). EdU stained cells were present similarly in both the genotypes middle ear regions (B, F, J and N). No significant change in the total number EdU stained cells observed in malleus-incus and stapes regions of both control and *Wnt1*^{Cre};*Smo*$^{lox/lox}$ (D and L). More number of TUNEL positive cells in the malleus-incus and stapes regions of *Wnt1*^{Cre};*Smo*$^{lox/lox}$ (G and O) were observed than control malleus-incus and stapes regions (C and K). There was a significant increase in TUNEL cells in both the regions (H and P). Black arrowheads indicate the presence of TUNEL positive cells. Coronal sections were performed such that dorsal is on top and lateral is on left side. The number proliferated and apoptotic cells were counted in the boxed area of *Sox9* expression (A, E, I and M). M – malleus, I – incus, and S – stapes. Scale bar: 100µm.

5. Endodermal *Shh* is required to initiate the malleus-incus condensation and not required to initiate the stapedial condensation

In the above, I observed that inactivation of HH signaling resulted in the loss of malleus and incus condensations and reduced stapedial condensation. But, the source of HH signaling is not known. Previous reports demonstrated that endodermal SHH signaling is necessary for the middle ear cartilages[13]. I hypothesized that the *Shh* from the pharyngeal endoderm plays a crucial role in the middle ear condensations. To examine this, I deleted *Shh* in the pharyngeal endoderm using *Foxg1*^{Cre} [51] to make *Foxg1*^{Cre};*Shh*$^{lox/lox}$ (*Shh*^{cko}) and analyzed *Sox9*, *Shh* and *Ptch1* expression patterns in the middle condensation regions at E10.5. Compared to that of the controls (Fig.7A), *Sox9* expression in malleus-incus condensation region was absent (Fig.7E, red asterisk). *Ptch1* and *Shh* did not express in the mesenchyme and pharyngeal endoderm suggesting that *Shh* in the pharyngeal endoderm was successfully deleted (Fig.7F and G, red asterisk and red arrow). These results suggest that endodermal SHH signaling is necessary for initial malleus-incus condensation. *Sox9* in the stapes region of *Shh*^{cko} embryos were greatly reduced (Fig.7L, red arrow). And no traces of *Ptch1* or *Shh* expression in the mesenchyme or pharyngeal endoderm respectively were observed (Fig.7M and N). These phenotypes were appeared to be similar to that of the phenotypes observed in the *Smo*^{cko} embryos. Henceforth, these results demonstrate that endodermal SHH signaling is necessary for the NCCs to initiate the malleus-incus condensation and not

essential for NCCs to initiate the stapedial condensation. But, endodermal SHH signaling is necessary for the development of the stapes in the later stages.

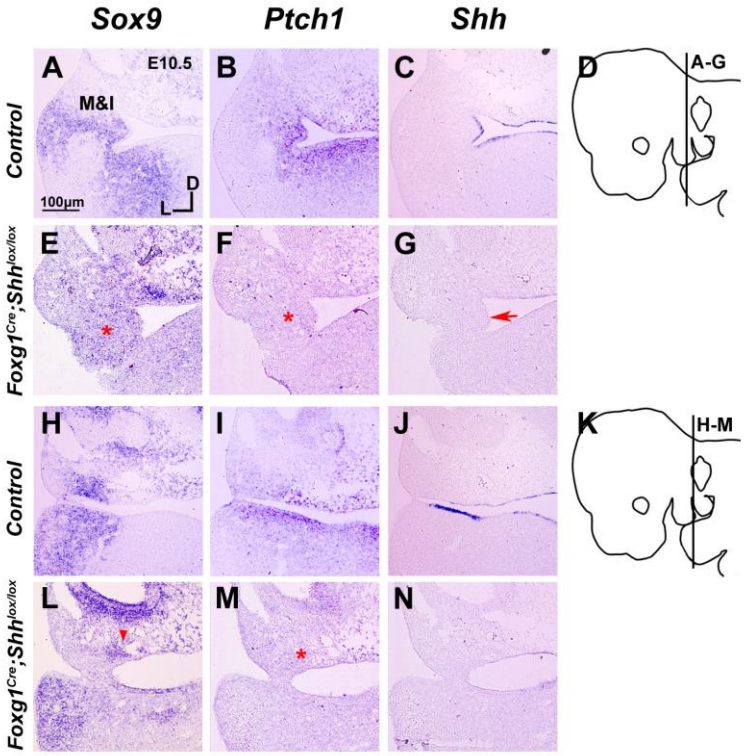

Figure 7. Loss of *Shh* in pharyngeal endoderm resulted in loss of malleus-incus condensation and reduced stapedial condensation. In *Shh*^{cko} embryos, *Sox9* did not express in the prospective malleus-incus region (A, red asterisk) and its expression was greatly reduced in the stapes region (E, red arrowhead); *Ptch1* expression was greatly reduced in the mesenchyme of both the BAs (F and M, red asterisk); *Shh* failed to express in the pharyngeal endoderm (G and N). Coronal sections were performed such that dorsal is on top and lateral is on left side. Schematic representation of each level of section in E10.5 mouse embryos was shown in D and K. M – malleus, I – incus, S – stapes and PE – pharyngeal endoderm. Scale bar: 100μm.

6. Ectopic HH signaling is sufficient to induce *Sox9* expression in middle ear condensations

Next, I questioned whether constitutively activated HH signaling can alter the stapedial condensation together with malleus-incus condensation. To examine this, I used constitutively activated HH signaling in the NCCs as previously described *Wnt1*^{Cre};*Smo*$^{M2/+}$ (*Smo*^{M2} embryos) mouse embryos[15] and compared the expression patterns of *Sox9,* and *Ptch1*. Comparing to control *Ptch1* expression in the malleus-incus and stapedial regions (Fig.8A and I), its expression in the malleus-incus and stapes regions was more broadly expressed in *Smo*^{M2} mutant embryos (Fig.8E and L, red arrowheads), suggesting that HH signaling was constitutively activated in the NCCs. *Sox9* expression in the malleus-incus region and stapes region was dramatically increased in *Smo*^{M2} mutant embryos (Fig.8F and N, red arrowheads) comparing to that of the *Sox9* expression in controls (Fig.8B and J, black arrows). When I examined the middle ear cartilages at E15.5 using *Acan* as a cartilage marker, *Acan* is expressed in the all the three separate middle ear cartilages malleus, incus and stapes of controls on the lateral and anterior side of the inner ear (Fig.9A). In *Smo*^{M2} mutant embryos, abnormal *Acan* expression was expressed on the lateral and anterior side of the inner ear, which appears to be like fused middle ear ossicles (Fig.9B, red arrowheads). This fused middle ear-like cartilage was dislocated away from the inner ear (Fig.9B, red asterisk). These results suggest that ectopic HH signaling can also alter the stapedial condensation together

with malleus-incus condensation and finally alters the normal development of the middle ear cartilages.

Since I observed the increase in middle ear condensation regions of *Smo*^{M2} mutant embryos, I assumed that this phenotype is due to increase in the cell proliferation rate as the HH signaling was constitutively active. So, I performed EdU staining to label the proliferating cells in both controls and *Smo*^{M2} mutant embryos and in situ hybridization was performed on adjacent sections using *Sox9* and *Ptch1* anti-sense probes. EdU positive cells were observed in both malleus-incus and stapedial condensations in both the littermates (Fig.8C, G, K and O). When counted the total number of proliferating cells in middle ear condensations, there was no significant change in the number of proliferating cells of both genotypes (Fig.H and P) i.e., The number of EdU proliferating cells in the boxed region of malleus-incus region are 168±3 cells/20000 um^2 area in controls and 171±4 cells/20000 um^2 in *Smo*^{M2} mutant embryos (Fig.8H; $p>0.05$). The number of EdU proliferating cells in the stapes of control is 56±2 cells/7500 um^2 and in *Smo*^{M2} mutant embryos is 57±2 cells/7500 um^2 (Fig.8P; $p>0.05$). This result confirms that the increase in the middle ear condensations is not due to the cell proliferation although HH signaling was constitutively active.

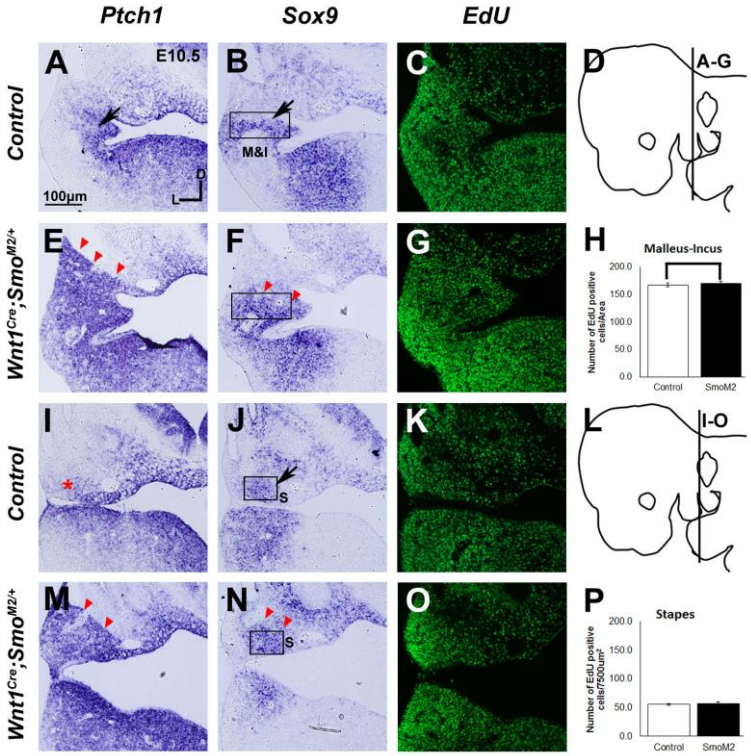

Figure 8. Constitutively activated HH signaling lead to increase in the middle ear condensation areas. In *Smo*^{M2} mutant embryos at E10.5, *Ptch1* expression was dramatically increased in the both BA1 and BA2 mesenchyme (E and M); *Sox9* expression was increased in malleus-incus and stapedial condensation regions (F and N, red arrowheads). EdU positive cells were appeared to be normal in both the BAs (G and O) and no significant increase in the number of EdU positive cells (H and P). Coronal sections were performed such that dorsal is on top and lateral is on left side. Schematic representation of each level of section in E10.5 mouse embryos was shown in D and L. M – malleus, I – incus, and S – stapes. Scale bar: 100μm.

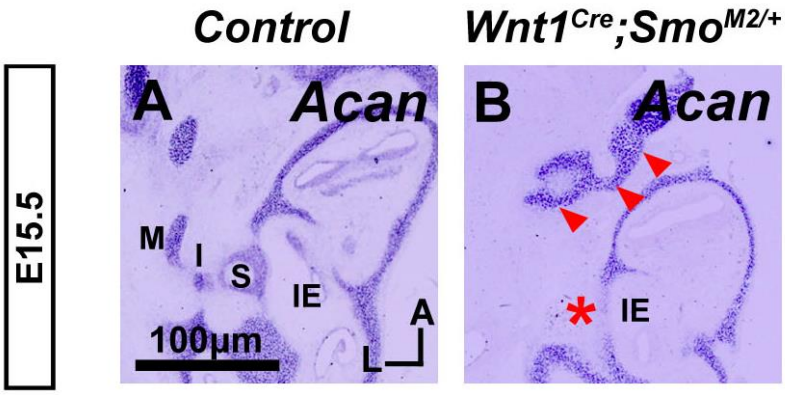

Figure 9. Constitutively activated HH signaling resulted in fused and dislocated middle ear ossicles. In control embryos at E15.5, *Acan* is expressed in malleus, incus and stapes cartilages (A). In Smo^{M2} mutant embryos, *Acan* is expressed in the fused middle ear-like cartilage (B, red arrowheads). This fused middle ear-like cartilage was dislocated away from the inner ear (B, red asterisk). Normal sections were performed such that anterior is on top and lateral is on left side. M – malleus, I – incus, S – stapes and IE – inner ear. Scale bar: 100µm.

7. Spatial relationship between endodermal *Bmp4* and middle ear condensations

In the above using *Smo*^{cko} and *Shh*^{cko} embryos, I observed the presence of reduced stapedial condensation (Fig.5O and 7L) suggesting that the NCCs were able to initiate the stapedial condensation. These results laid the possibility that some other signaling mechanisms might be responsible for NCCs to initiate the stapedial condensation. Previously, *Bmp4* expression was observed in the pharyngeal endoderm[9] and also BMP4 is alone sufficient to induce and maintain *Sox9* expression during chondrogenesis[34,35]. So, I examined the spatial expression pattern of *Bmp4* and compared with the *Sox9* and *Shh* expressions in the middle ear regions of E10.5 *wildtype* embryos along the anterior-posterior axis. In malleus-incus condensation region, *Sox9* was expressed in NC-derived mesenchyme representing malleus-incus condensation (Fig.10A, M&I), no *Bmp4* expression was observed either in the endoderm or in the mesodermal region (Fig.10B). *Shh* was expressed in the endoderm adjacent to the *Sox9* expression (Fig.10C, black arrow). In the stapedial condensation region, *Sox9* was expressed dorsal to endoderm (Fig.10E). Just beneath *Sox9* expression in the stapes region, *Bmp4* was expressed in the endoderm (Fig.10F, black arrow) and *Shh* expression was not expressed (Fig.10G, red arrow). In the most posterior BA2 region, both *Sox9* and *Bmp4* expressions were not expressed (Fig.10I and J) and *Shh* again started to express in the endoderm (Fig.10K). Altogether, these results suggest us that

endodermal *Bmp4* is closely and spatially associated with the NC-derived stapedial condensation.

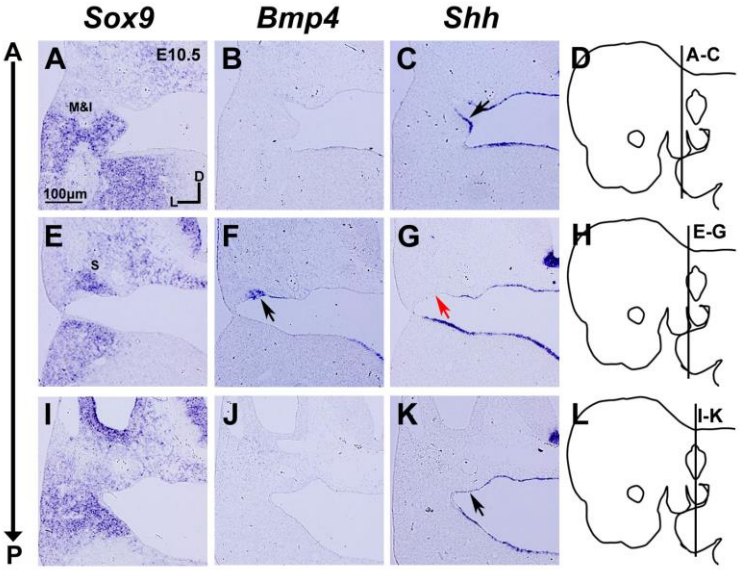

Figure 10. Endodermal *Bmp4* is closely associated with stapedial condensation. In control E10.5 embryos, *Sox9* was expressed in the malleus-incus and stapedial condensation region (A and E). *Bmp4* was strongly expressed in the pharyngeal endoderm beneath the stapedial condensation (F), but is not expressed in the malleus-incus region (B) or after passing the stapedial condensation (J). *Shh* was expressed in the pharyngeal endoderm BA1 and posterior BA2 region (C and K, black arrow). But *Shh* did not express in the pharyngeal endoderm of stapes region (G, red arrow). Coronal sections were performed such that dorsal is on top and lateral is on left side. Schematic representation of each level of section in E10.5 mouse embryos was shown in D, H and L. M – malleus, I – incus, S – stapes and IE – inner ear. Scale bar: 100μm.

8. Bmp signaling is essential for NCCs migration into the prospective stapes region and initiate the stapedial condensation

Since BMP4 belongs to transforming growth factor-β (TGF-β) family[52], I sought to inactivate BMP4 signaling in the NCCs by deleting *Smad4* gene in the NCCs using *Wnt1*^{Cre} to make *Wnt1*^{Cre};*Smad4*$^{lox/lox}$ (*Smad4*^{cko})[41,50] in order to examine the roles of Bmp signaling on NC derived middle ear condensations. To investigate the roles of Bmp signaling, I compared the expression patterns of *Bmp4* and *Sox9* in controls and *Smad4*^{cko} embryos at E10.5. *Bmp4* did not express in the BA1 endoderm of the both littermates (Fig.11A and E, red arrow). Also, similar *Sox9* expression was observed in the BA1 mesenchyme of both littermates (Fig.11B and F, black arrows). In the stapes region, *Bmp4* was expressed normally in the pharyngeal endoderm of both control and *Smad4*^{cko} (Fig.11I and M, black arrow). *Sox9* did not express in the stapes region above the endodermal *Bmp4* (Fig.11J and N, black arrow and red asterisk, respectively) indicating that stapedial condensation was failed to condense. Since, *Smad4*^{cko} embryos were embryonic lethal at E12.5[41], I was able to analyze the expression patterns of *Sox9* in the malleus-incus condensation till E12.5 only. At E12.5, in controls, single condensation for malleus-incus was observed laterally and anteriorly (Fig.11L, M&I) and a round shaped stapes (Fig.11L, S). However, in *Smad4*^{cko} embryos, *Sox9* in the prospective stapes region did not express indicating no stapes formation at all developmental stages (Fig.11P, red asterisk). These results demonstrate that Smad4 mediated

BMP signaling is required for NCCs to initiate the stapedial condensation, but not for malleus-incus condensations.

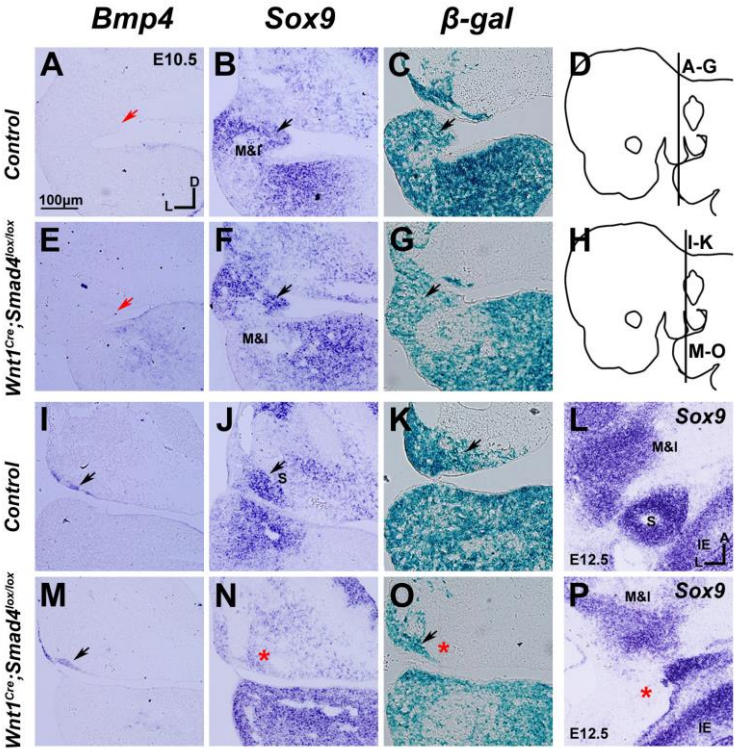

Figure 11. Inactivation of Bmp signaling resulted in the loss of stapedial condensation. In *Smad4*^{cko} embryos at E10.5, *Bmp4* expression was similar with that in controls (A, E, I and M). *Sox9* expression in malleus-incus condensation remains unchanged (F) and *Sox9* failed to express in the stapes region (N). NCCs were normally migrated to the respective BAs in *Wnt1*^{Cre}*;R26R;Smad4*$^{lox/lox}$ embryos (G and O, black arrow). But, the NCCs in stapes region were absent (O, red asterisk). Coronal sections were performed such that dorsal is on top and lateral is on left side. At E12.5, *Sox9* was expressed in the malleus-incus and stapes region of control embryos (L), but in *Smad4*^{cko} embryos, *Sox9* was normally expressed in malleus-incus condensations and was not expressed in the stapes region (P, red asterisk). Normal sections were performed such that anterior on top and lateral on left side. Schematic representation of each level of section in E10.5 mouse embryos was shown in D and H. M – malleus, I – incus, S – stapes and IE – inner ear. Scale bar: 100μm.

The absence of NC-derived stapedial condensation in *Smad4*^{cko} embryos could either due to the defects in the NC migration, NC proliferation or NC survival. So, I examined these events using *lacZ* staining to analyze NCC migration defects, EdU staining for cell proliferation and TUNEL for cell death assay. In order to analyze the NCC migration at E10.5, we made the *Smad4*^{cko} together with *R26R* mice as described previously and performed *lacZ* staining[41]. In the control and *Smad4*^{cko} littermates having *reporter* alleles, NCCs were migrated into the malleus-incus regions normally (Fig.11C and G, black arrows), suggesting that NCC migration remains unaffected in malleus-incus region. A group of NCCs were observed dorsally to that of pharyngeal endoderm in the control which might differentiate into the stapedial condensation (Fig.11K, black arrow). But in *Smad4*^{cko}, a group of cells were present laterally, but their population was severely reduced in the prospective stapes region (medially) compared to that of the controls (Fig.11O, red asterisk). These results indicate that the absence of stapedial condensation in the *Smad4*^{cko} embryos was due to lack of NCCs and Smad4 mediated Bmp signaling is essential for the NCCs to migrate into prospective stapes region and initiate the stapedial condensation. When EdU staining was performed to analyze the cell proliferation, EdU stained cells were almost similarly stained in the malleus-incus or stapedial condensation regions of both littermates (Fig.12A and B in malleus-incus region; and 12F and G in stapes region). When the total number of EdU positive cells was counted in the middle ear condensation regions, there was no significant difference in the total number of

EdU positive cells in either of the littermates ie., The number of EdU proliferating cells in the boxed region of malleus-incus region are 157±3 cells/20000 um^2 area in controls and 149±5 cells/20000 um^2 in *Smad4*^{cko} embryos (Fig.12K; p>0.05). The number of EdU proliferating cells in the stapes of control is 53±2 cells/7500 um^2 and in *Smad4*^{cko} is 49±2 cells/7500 um^2 (Fig.12L; p>0.05). This result suggests that inactivation of Bmp signaling is not necessary for the NCCs to proliferate in the prospective stapes region. When TUNEL staining was performed to analyze whether NCCs were failed to survive, we observed very few TUNEL positive cells in malleus-incus condensation regions of *Smad4*^{cko} embryos (Fig.12C and D). But, when the cells were counted in the boxed, they were not significantly changed ie., the number of TUNEL positive cells in the boxed region of malleus-incus region are 7±1 cells/20000 um^2 area in controls and 9±2 cells/20000 um^2 in *Smad4*^{cko} embryos (Fig.12M; p>0.05). Very few or no TUNEL positive cells were observed in the stapedial condensation in both control and *Smad4*^{cko} embryos (Fig.12H and I). When counted the total number TUNEL positive cells in the boxed area of stapes region, there was no significant difference in either of the both littermates ie., the number of TUNEL positive cells in the stapes of control is 1±0 cells/7500 um^2 and in *Smad4*^{cko} is 1±0 cells/7500 um^2 (Fig.12N; p>0.05). However, a lot of TUNEL positive cells were observed in the BA1 mesenchyme which might become the Meckel's cartilage. These results suggest that Bmp signaling is not essential for the NC survival in the middle ear condensations.

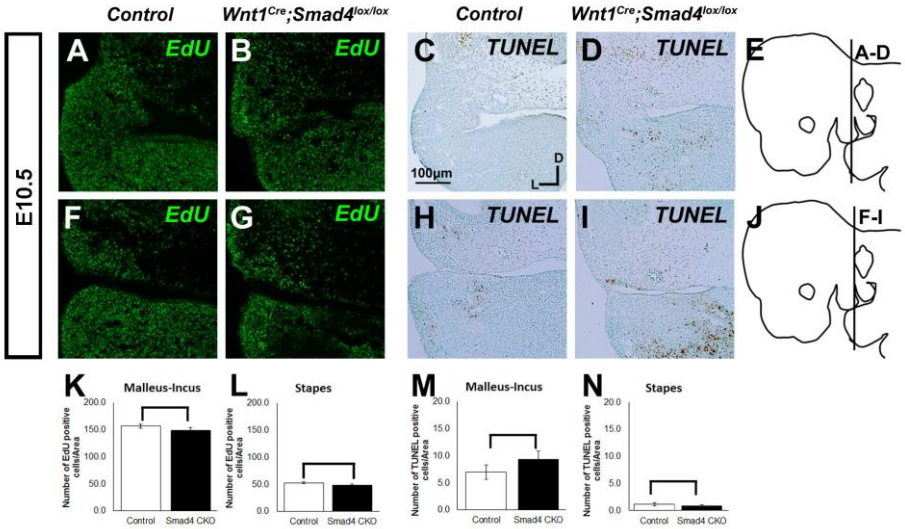

Figure 11. Deletion of *Smad4* in the NCCs did not alter the cell proliferation or cell survival rates. In *Smad4*^{cko} embryos, EdU labeled cells in malleus-incus (B) or stapes (G) regions were appeared to be similar with EdU positive cells in malleus-incus (A) or stapes (F) regions of control embryos at E10.5. Similarly, TUNEL stained cells in malleus-incus (D) or stapes (I) regions of E10.5 *Smad4*^{cko} embryos were similar with TUNEL positive cells in malleus-incus (C) or stapes (H) regions of control embryos at E10.5. There were no significant differences observed either in the EdU or TUNEL stained cells in the malleus-incus and stapes regions of both littermates. Schematic representation of each level of section in E10.5 mouse embryos was shown in E, and J. Scale bar: 100μm.

9. Endodermal *Bmp4* is required for the NCCs to initiate the stapedial condensation

Since, I observed the *Bmp4* expression in the endoderm beneath the stapedial condensation (Fig.10F), and also I demonstrated that Bmp signaling is essential for the NCCs to migrate into the prospective stapes region and initiate the stapedial condensation. I hypothesized that endoderm is the key source of BMP4 signaling required for the NCCs to migrate into stapes region and initiate the stapedial condensation. In order to investigate this, I deleted *Bmp4* in the endoderm using $Foxg1^{Cre}$ mice ($Foxg1^{Cre};Bmp4^{Tm1/lox}$, $Bmp4^{cko}$), and compared the expression patterns of *Sox9*, *Bmp4* and *Transcription factor AP2-alpha* (*AP2α,* a migrating NCC marker) at E10.5[43,53]. *Bmp4* was not expressed in the endoderm of malleus-incus condensation region in either of the littermates (Fig.13A and F, red arrow). *Bmp4*, which was expressed in the endoderm present beneath the stapedial condensation (Fig.13K, black arrow) of control littermate, was lost in the endoderm of $Bmp4^{cko}$ embryos (Fig.13P, red arrow) confirming that *Bmp4* was deleted successfully in the endoderm. *Sox9* was expressed similarly in the malleus-incus condensation region of both control and $Bmp4^{cko}$ embryos, suggesting that endodermal *Bmp4* is not required for NCCs to initiate malleus-incus condensation regions (Fig.13B and G, black arrows). However, *Sox9* was failed to express in the stapedial region of $Bmp4^{cko}$ embryos (Fig.13Q, red asterisk), which was a phenocopy of $Smad4^{cko}$. Then, I examined whether endodermal *Bmp4* is essential for NCCs to migrate

into the prospective stapes region by comparing *Ap2a* expression between the two littermates. In controls, *Ap2a* was expressed in the mesenchyme adjacent, dorsal and complimentary to *Sox9* expression in malleus-incus region (Fig.13C, black arrow) and completely overlaps with *Sox9* expression in the stapedial region (Fig.13L, black arrow). In *Bmp4*^{cko} embryos, *Ap2a* was normally expressed in the malleus-incus region and did not express in the stapes region (Fig.13H and R, black arrow and red asterisk). When I observe the malleus-incus cartilages at E13.5, they are normally formed in *Bmp*^{cko} embryos (Fig.13O and T) and no stapes is present (Fig.13T, red asterisk). This result confirms that endodermal *Bmp4* is crucial and essential for the NCCs to migrate into stapes region and initiate the stapedial condensation.

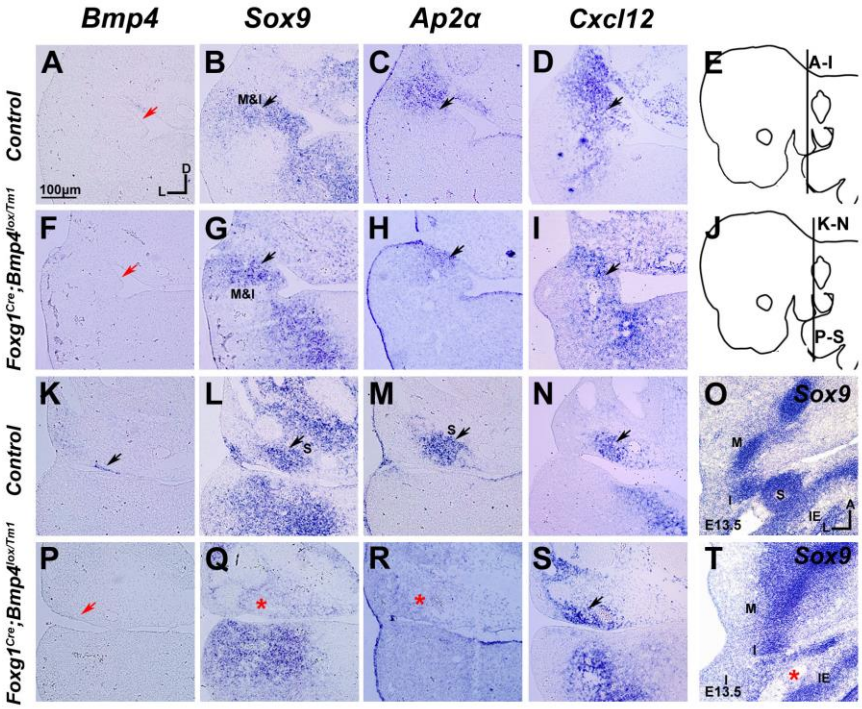

Figure 12. Endodermal *Bmp4* is essential for the NCCs to initiate the stapedial condensation. In *Bmp4*^{cko} embryos, no *Bmp4* was expressed in the pharyngeal endoderm of malleus-incus (K, red arrow) region similar to controls (A, red arrow), and in the pharyngeal endoderm region of stapes region (P, red arrow) which was expressed in the control pharyngeal endoderm (K, black arrow). *Sox9* was similarly expressed in malleus-incus condensation regions of both control and *Bmp4*^{cko} embryos (B and G, black arrow). In controls, *Sox9* was expressed in the stapes region (L, black arrow), but was failed to express in the stapes region of *Bmp4*^{cko} embryos (Q, red asterisk). Similar *Ap2a* expression patterns were observed in the malleus-incus regions of both the littermates (C and H, black arrows). Although *Ap2a* was expressed in the stapes region of control (M, black arrow), its expression in the *Bmp4cko* embryos was not seen (R, red asterisk). *Cxcl12* was expressed in the malleus-incus and stapes regions of controls and *Bmp4*^{cko} embryos (D, I, N, and S). Schematic representation of each level of section in E10.5 mouse embryos was shown in E, and J. *Sox9* was expressed in the malleus, incus and stapes region of control embryos at E13.5 (O); and its expression remains unaltered in the malleus and incus of *Bmp4*^{cko} embryos (T), but was not seen in the stapes (T, asterisk). Normal sections were performed with anterior on the top and lateral on the left side. M – malleus, I – incus, S – stapes and IE – inner ear. Scale bar: 100μm.

10. Endodermal *Bmp4* is not necessary to up-regulate *Cxcl12* expression in the prospective stapes region

In the above, I demonstrated that endodermal *Bmp4* is essential for NCCs to migrate into prospective stapes region. But, it is still puzzling how endodermal *Bmp4* can direct the NCCs into the prospective stapes region. Previous studies on NC-derived sympatho-adrenal tissue development demonstrated that Bmp signaling from dorsal aorta is essential for up-regulating the CXCL12 expression in the aortic mesenchyme. This CXCL12, which acts as a chemoattractant, is essential to attract the sympatho-adrenal progenitors into the aortic mesenchyme[23]. First I examined the expression pattern of the *Cxcl12* in the middle ear regions of E10.5 control embryos. *Cxcl12* was expressed strongly and gradiently in the adjacent mesenchyme to pharyngeal endoderm, where malleus-incus condensation is present (Fig.13D, black arrow). Also, *Cxcl12* expression was partially overlapping with *Sox9* expression in this region (Fig.13B). *Cxcl12* was also expressed in the mesenchyme (Fig.13N, black arrow) and overlapping with *Sox9* and *AP2a* expression patterns (Fig.13L and M, black arrow). Based on these observations and previous reports[23], I hypothesized that endodermal *Bmp4* can up-regulate the *Cxcl12* expression in the prospective stapes region, such that it can act as the chemoattractant for the NCCs to migrate into the prospective stapes region. So, I analyzed the expression pattern of *Cxcl12* in both control and *Bmp4*^{cko} embryos at E10.5. I observed that *Cxcl12* was expressed in the mesenchyme of

both malleus-incus and stapedial condensation (Fig. 13I and S). These results indicate that endodermal *Bmp4* is not necessary to up-regulate *Cxcl12* expression. Also, these results suggest that *Cxcl12* alone may not be sufficient to attract the NCCs into the prospective stapes region.

11. *Cxcl12* is necessary for stapes development

Previous studies using zebrafish, it was demonstrated that CXCL12-CXCR4 signaling is essential for NCC migration and patterning during the craniofacial development[22]. So, I hypothesized that whether deletion of *Cxcl12* can result in middle ear defects. To examine this, exon2 of *Cxcl12* gene was deleted[44] and examined the expression patterns *Sox9* and *Col2a1* (chondrogenesis marker) of E14.5 middle ears. *Sox9* and *Col2a1* were expressed in all the middle ear ossicles of *wildtype* mouse embryo (Fig.14A and B). In *Cxcl12*$^{-/-}$, *Sox9* and *Col2a1* were expressed in the middle ear ossicles (Fig.14C and D), but the morphology of the stapes is greatly reduced and malformed. Stapedial artery was absent (Fig.14C and D). Malleus and incus were normally present in *Cxcl12*$^{-/-}$ embryos. These results suggest us that *Cxcl12* is essential to form a normal stapes, but not required for malleus and incus.

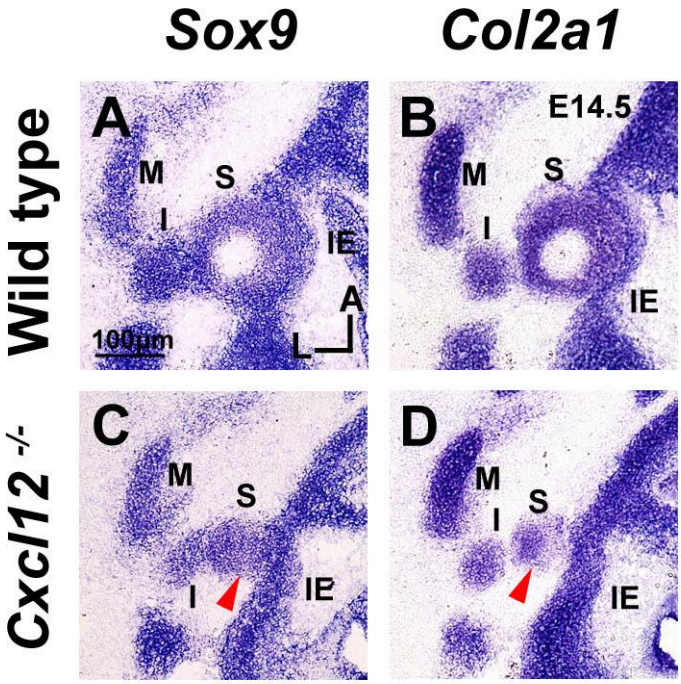

Figure 13. *Cxcl12* is necessary for stapes development. *Sox9* and its downstream gene *Col2a1* were expressed in the malleus, incus and stapes of control embryos at E14.5 (A and B). In *Cxcl12*$^{-/-}$, *Sox9* and *Col2a1* were normally expressed in the malleus and incus (C and D), but their expressions in stapes were severely altered (C and D, arrowheads). Normal sections were performed with anterior on the top and lateral on the left side. M – malleus, I – incus, S – stapes and IE – inner ear. Scale bar: 100μm.

IV. DISCUSSION

In this work, I demonstrated that the signals such as SHH and BMP4 emanating from endoderm are essential for NCCs to initiate the malleus-incus and stapedial condensations, respectively. I also showed that HH signaling is required for the NC survival during the middle ear development. Additionally, I report that CXCL12 is required for normal development of stapes including the development of stapedial artery.

1. Endodermal HH signaling is essential for NC-derived middle ear condensation

It is well known that endodermal *Shh* is essential to maintain the BA patterning and can also act as the survival factor for adjacent and corresponding BA mesenchymal cells[9]. In the $Shh^{-/-}$, the initial formation of BAs and their patterning were normal[9]. Different studies using Smo^{cko} or $Shh^{-/-}$ mice embryos or by inhibiting the SHH signaling by introducing hybridoma cells (line5E1) shown that reduction in the size of the head, with NCCs in the BAs were failed to survive[9,12,13,15]. As a result most of the craniofacial structures including the middle ear cartilages were absent in Smo^{cko} or $Shh^{-/-}$ or Shh^{cko} mice. Also, Smo^{cko} studies revealed that the cell proliferation rate was not altered at the early embryonic ages (E9.5 or E10.5), but the number of proliferating cells were reduced from E11.5[15]. From the previous studies, it was also confirmed that the NCC migration into the branchial arches were populated normally suggesting that HH signaling is not essential for the NCC

migration[12,15]. These results show that the absence of malleus-incus condensation in both the *Smo*^{cko} and *Shh*^{cko} embryos with no change in the proliferating NCCs and increase in the number of apoptotic NCCs. These studies were consistent with the previous observations that NCCs in BAs were failed to survive and able to proliferate at early E10.5 stage. I also found that the NCCs were able to migrate into the prospective stapes region at the early stages and were able to initiate the stapedial condensation. But, the stapedial condensation in the *Shh*^{cko} was greatly reduced when compared with *Smo*^{cko} embryos at E10.5. This might be because arrest of BA development in the absence of the *Shh* or due to the failure of NCCs survival within the BA2 mesenchyme[9,12]. Hence results from *Smo* and *Shh* mutant studies demonstrate that endodermal *Shh* is essential for the NCCs survival during the initial middle ear condensations, but is not essential for NCC migration or NCC proliferation.

2. HH signal can control the *Sox9* expression in the NCCs

Constitutive activation of HH signaling in the NCCs resulted in craniofacial malformation with lack of head skeletal structures[15]. However, it is not clear whether these defects are due to either SHH or IHH (another HH family member). Forced expression of *SHH* in the cartilage using *Col2a1* transgenic mice had lead severe craniorachischisis, and other skeletal defects in ribs, sternum and long bones[54]. Along with these, continuous expression of *Shh* in the also induce the high-level of *Sox9* expression in the cartilaginous bones[54]. The another HH family IHH is essential in regulating the chondrocyte

proliferation, and chondrocyte maturation at the specific target sites during the bone formation[26]. The results from *SmoM2* mutants suggest that there was an ectopic increase in the *Sox9* expression of both malleus-incus and stapedial condensations (Fig.8F and N) and also no change in the cell proliferation rate. The constitutive activation of SHH signaling in the NCCs could induce the high-level of *Sox9* expression in the malleus-incus and stapedial condensations because of the presence of the *cis*-elements in the 6.8kb of *Sox9* is sufficient to initiate the effect of *Shh*[54]. It was also reported that forced expression of *Shh* in the cartilage had led to other defects include the fusion of joints, as they lack the joint markers such as *Growth differentiation factor 5* (*Gdf5*)[55]. I also observed that middle ear cartilages were fused with each at later stages (E15.5) in Smo^{M2} mutant embryos. The fusion of middle ear ossicles could be from failure of joint formations. Usually, the joints are formed either switching of cartilage markers in the presumptive regions and start to express the joint markers in the presumptive joint regions or the mesenchymal cells present with in the joint regions have to undergo apoptosis. The malleus and incus were initially developed from a single condensation, later the malleus-incus joint was formed by the down-regulation of cartilage markers and the up-regulation of joint markers[56]. There was no evidence how the incus-stapes joint was formed. In Smo^{M2} mutant embryos, the fusion of middle ear cartilages might result either due to the continuous expression of cartilage markers or due to the increase in the NCC survival rates[55].

3. Endodermal *Bmp4* directs the NCCs into the prospective stapes region

BMP signaling had a lot of roles in various developmental processes. BMP signaling is required for NCC formation and migration either in the cranial or caudal regions[17]. Among the BMP family members, Bmp2 or Bmp4 play crucial roles in providing the NCC formation, positional information or NCC migration. Inactivation of Bmp2/4 signaling in the NCCs by *Xnoggin* under the enhancer of *Hoxa2* gene resulted in a wide variety of craniofacial skeletal abnormalities. In the same transgene activation in the NCCs resulted in the absence of the NCCs migration into the respective BAs suggesting that BMP signaling is required for the NCC migration[18]. Chicken embryo mandibular explant culture studies had demonstrated that positional information is provided exogenous BMP4 bead and was able to induce the *Sox9* expression in the proximal position where BMP4 bead was implanted[35]. Using the chicken explant studies, it is also shown that implanting BMP4 bead alone in the mesenchyme was able to induce the *Sox9* expression[34]. In this work, I showed that endodermal BMP4 signaling is essential for the NCCs to initiate the stapedial condensation due to the absence of both *Sox9* and *Ap2a* expression patterns and similar phenotypes were observed in the *Smad4*^{cko} embryos. No apoptotic NC cells were observed in the middle ear condensation regions of *Smad4*^{cko} embryos suggesting that Bmp signaling is not essential for NCCs survival during the middle ear condensation. But, this result is contradictory to that of the previous observations that NCC migration remains unaffected or NCC migration was delayed[17,41]. So, it is still puzzling whether the endodermal

Bmp4 directs the NCCs into the prospective stapes region is direct or indirect effect. During the development of sympatho-adrenal system development (a NC derivative), the Bmp signaling was inactivated by electroporation of *Noggin* cDNA into the dorsal aorta that has led to the failure of NCCs migration into the aortic arch mesenchyme due to lack of Cxcl12 expression in the aortic mesenchyme[23]. But when dominant-negative type I Bmp receptor was electroporated into the sympatho-adrenal progenitors, they were normally migrated into the aortic mesenchyme. Hence the effect of aortic Bmp signaling was indirect on NCC migration. I also hypothesized that similar mechanism can be implied by endodermal BMP4 signaling to direct the NCC migration into the prospective stapes region. Unfortunately, even in the absence of endodermal *Bmp4*, *Cxcl12* was able to express in the prospective stapes region. This observation was not consistent with our assumption. It is essential to find how the endodermal *Bmp4* can direct the NCCs into the prospective stapes region.

V. CONCLUSION

In this work, I summarize that SHH and BMP4 signals from the endoderm are required to initiate the malleus-incus and stapedial condensations, respectively. Also, HH signaling is essential for the NCC survival during the initial middle ear condensation stages. The above results report that constitutive expression of HH signaling in the NCCs had increased the middle ear condensation regions and which might also result in the fusion of middle ear cartilages at later stage. Inhibition of endodermal BMP4 signaling resulted in the absence of the NCCs in the prospective stapes region and hence the NCCs were failed to initiate the stapedial condensation. Based on these observations, I conclude that the signals those are emanating from the pharyngeal endoderm are essential for the NCCs to initiate and differentiate into the middle ear condensations in their respective BAs. Additionally, it is also demonstrated that *Cxcl12* is essential for the normal development of the stapes with proper stapedial artery.

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ABSTRACT IN KOREAN

중이 발생 과정에서 hedgehog (HH), bone morphogenetic protein (BMP)4, CXCL12 신호 역할 규명

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포유류의 청각 시스템은 귀의 세가지 형태인 외이, 중이, 내이가 포함 되어있으며 특히, 중이는 세 가지 뼈인 추골(malleus), 침골(incus), 등골(stapes)로 구성되어있다. 중이의 세 가지 이소골은 외이에서 들어온 소리신호를 내이로 전달하는 역할을 하고, 이소골의 발달 장애는 전도성 난청을 유도한다. 중이 이소골은 신경능선세포(neural crest cells)에서 유래된다. 마름뇌분절(rhombomere, R) 1과 R2, R3에서 유래한 신경능선세포가 새궁(branchial arch, BA) 1로 이동하여 매켈연골(Meckel's cartilage), 추골, 침골로 발생된다. R4의 신경능선세포 및 일부 R3, R5의 세포는 BA2로 이동하여 등골을 형성한다. 발달 과정은 알려져 있으나 신경능선세포가 어떻게 그들의 최종 목적지를 찾고, 목표 조직으로 분화할 수 있는 지에 대해서는 여전히 잘 알려져 있지 않다.

따라서, 우리는 cre-loxp 기술을 이용하여 신경능선세포 또는 인두

내배엽에서 관심 유전자를 결손 시키고, 대조군과 조건부 녹아웃(cKO) 마우스의 유전자 발현 양상을 비교분석 하여 연구를 진행하였다. *Wnt1*^{Cre} 또는 SHH 를 이용하여 신경능선세포에서 *Smoothened* (*Smo*) 유전자를 결손 시키면, 배아시기(E) 10.5일의 BA1에서 추골-침골의 초기 응축(condensation)이 크게 감소하고, BA2에서 등골의 응축이 약간 감소한다. E11.5에서는 *Smo*^{cko} 배아에서 등골의 응축이 결국 사라져있는 것을 관찰하였다. 이러한 추골-침골 응축 또는 등골 응축의 감소는 응축 단계 동안 신경능선세포의 생존을 위해 HH 신호가 필요하다는 것을 확인할 수 있었다. 반대로 HH 신호가 신경능선세포에서 지속적으로 활성화(*Smo*^{M2}) 되었을 때는 중이 이소골 모두 응축이 증가되었다. 이후 시기에서 *Smo*^{M2} 마우스에서 융합되고, 전위되어있는 중이 이소골이 관찰되었다. 이와 유사하게 신경능선세포에서 *Smad4* 또는 인두 내배엽에서 *Bmp4* 를 결손 시켰을 때는 E10.5 배아에서 추골-침골의 응축에는 변화가 없었지만 등골의 응축은 사라져있었다. 그러나 등골 응축의 결손은 등골이 될 예정인 지역(등골예정부위)에서의 신경능선세포의 결손으로 인한 것이므로, *Bmp4* 신호는 신경능선세포의 증식과 생존에는 필수적이지 않음을 알 수 있었다.

이전 연구를 바탕으로 등골이 만들어지기 위해서는 케모카인 신호 물질인 *Cxcl12*가 신경능선세포의 화학유인물질로써 역할을 하며, 내배엽성 *Bmp4* 가 *Cxcl12*의 발현을 유도하는데 필요하다는 가설을 세워 실험을 수행했다. 그러나 인두 내배엽에서 *Bmp4*가 결손 되었지만 등골예정부위에서 여전히 *Cxcl12*가 발현하는 것을 관찰하였다. 이를 통해 내배엽성 *Bmp4*가 등골예정부위에서는 *Cxcl12* 발현을 유도하는 데 필요하지 않다는 것을 알 수 있다. 마우스 몸 전체에서 *Cxcl12* 유전자를 결손 시켰을 때, E14.5 배아에서 추골-침골이 정상적인 것을 관찰했으며 이를 통해 *Cxcl12*

가 정상적인 등골 발생에는 중요하지만 초기 등골 응축에는 중요하지 않다는 것을 다시 한 번 확인할 수 있었다.

이 연구를 통해 신경능선세포의 HH, 내배엽성 Bmp4 신호는 중이의 세 뼈, 추골, 침골, 등골의 초기 응축을 위해서 매우 중요한 역할을 한다는 것을 제안한다.

핵심되는 말: 추골, 침골, 등골, 응축, 신경능선세포, 내배엽, Hedgehog, BMP4, CXCL12

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