Pax3 lineage contribution to the mammalian inner and middle ear

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Pax3 lineage contribution to the mammalian inner and middle ear

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감사의 글

제가 대학원에 들어온 지도 어느덧 2 년여의 시간이 흘렀습니다. 이 곳에서의 지난 시간을 돌이켜보면, 이 기간 동안 학문적으로 많이 배울 수 있었고, 그래서 제가 더욱 성장할 수 있었습니다. 여전히 부족한 저 이지만, 이 곳에서의 배움을 바탕으로 앞으로 제가 더 발전될 것을 기대합니다. 그리고 학위 기간 동안 저를 위해 많은 도움을 주신 분들께 감사의 말씀을 전하고 싶습니다.

먼저는 아무것도 모르는 저를 제자 삼아주시고, 공부하는 기간 동안 물심양면으로 지원해주시고, 학문의 가르침뿐만 아니라 인생의 가르침을 주신 저의 스승님, 복진웅 교수님께 감사의 말씀을 드리고 싶습니다. 학자의 모습을 몸소 보여주시는 김명희 교수님, 실험실의 정신적 지주이신 박형우 교수님, 바쁘신 와중에도 제 논문을 위해 시간을 내어주시고, 관심을 가지고 심사해주신 최재영 교수님께 감사 드립니다. 그리고 실험실의 아버지이신 유병기 선생님, 진정한 프로가 무엇인지를 몸소 보여주시는 아이 셋의 어머니이신 이지연 박사님, 언제나 밝은 웃음과 미소로 인사해주시는 이유라 박사님, 실험의 문제가 있을 때에 정성껏 Discussion 해주신 김홍경 박사님, 언제나 제게 칭찬을 아끼지 않고 격려해주신 윤희제 박사님, 인자한 표정의 소유자 Dr. Mahesh, 실험실의 살림꾼이신 친절한 공경아 선생님, 도움이 필요할 때 언제나 아낌없이 도와주신 똑순이 민혜현 선생님, 실험에 있어서는 누구보다도 열정적이셔서 그 모습을 보며 도전이 된 오령 선생님, 멀리 인도에서 왔고, 열심히 공부하며 연구하는 두 딸의 어머니 Kalyani, 실험실의 어머니 권현정 선생님, 지금은

안 계시지만, 예전에 조교로 계셨던 두 분, 김현주 선생님과 김선희 선생님, 성실히 맡은 바 최선을 다하는 한두열 선생님, Chicken 실험의 달인이 될 마지현 선생님, 호기심 많은 Hari, 동기이자 공부하는 동안 힘이 되었던 상훈이, 하나밖에 없는 귀한 실험실 후배 성주, 작년 한 해 우리 실험실에 활력소를 불어 넣어 주었던 박해동 선생님, 그리고 조직학, 해부학 실험실 선생님들 모든 분께 감사의 말씀을 전합니다. 여러분들의 배려와 도움으로 제가 무사히 학위 과정을 마칠 수 있었습니다.

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2011년 6월 이 동 진 올림

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ABSTRACT

Pax3 lineage contribution to the mammalian inner and middle ear

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The vertebrate inner ear develops from a specialized region of the ectoderm located on either side of the caudal hindbrain known as the otic placode. During development, the otic placode invaginates to form the otic cup, from which some cells delaminate and migrate into neighboring mesenchyme to form neurons of the cochlear-vestibular ganglion. The otic cup deepens further and pinches off from the ectoderm to form the otocyst that will develop into the membranous labyrinth of the inner ear. While a majority of the cells in the membranous labyrinth are derived from the otic placode, some of the cells in the labyrinth are derived from the neural crest. Similarly, the middle ear ossicles, whose role is to relay and to amplify the environmental sound to the inner ear, are also known to be derived from the neural crest. Neural crest cell are originated from the junction between the

epidermis and dorsal region of the neural tube and give rise to a variety of cell types such as neurons, glia, melanocytes, bones and cartilages.

To better understand the role of neural crest in inner and middle ear development, I genetically fate-mapped the progenies of neural crest using *Cre/loxP* system. *Pax3* is a member of the *Pax* family of transcription factors and is known to be important for various aspects of embryogenesis including neural crest differentiation. In human, mutations in *PAX3* cause Waardenburg's syndrome Type I, characterized by neurosensory hearing loss. *Pax3* is expressed in the dorsal neural tube, from which the neural crest cells are migrated, but not in the developing inner and middle ears. By crossing *Pax3-Cre* mice with *R26R* reporter mice, I genetically labeled the cells expressing *Pax3* in the dorsal neural tube and identified their descendants during inner and middle ear development using β-galactosidase (β-gal) staining. At E15.5, β-gal positive cells are detected in various substructures of the inner ear including endolymphatic duct, common cruz, semicircular canal, utricle, saccule, vestibular dark cells, stria vascularis, and ganglia. In addition, β-gal positive cells are present in some parts of the otic capsule and in all three middle ear ossicles. Furthermore, analyses of *Pax3-Cre* homozygous mutant embryos showed that *Pax3* is required for melanocytes differentiation of the stria vascularis in the cochlear lateral wall but not for other neural crest-derived cell types in the inner and middle ears.

Key words : Waardenburg' syndrome, inner ear, middle ear, neural crest, *Pax3*, fate map

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I. INTRODUCTION

The mammalian ear consists of three parts, the outer, middle and inner ear. The outer ear collects sound which vibrates the tympanic membrane. The vibration is transferred to the middle ear ossicles composed of malleus, incus and stapes. The middle ear is a composite organ formed from endoderm, mesoderm, ectoderm and neural crest [1 2 3 4 5]. Its function is to transmit and amplify sound wave from the outer ear to inner ear where sensory receptors are located [6 7]. The inner ear is an unusually complex organ. The inner ear originates from a transient embryonic structure, the otic vesicle. The otic vesicle is derived from the otic placode, a specialized ectoderm located adjacent to rhombomeres 5 and 6 of the hindbrain [8, 9]. The inner ear is a sensory organ responsible for hearing, balance and detection of gravity. The vestibular sensory organs including the Utriculi, Sacculi, and cristae detect

gravity and linear and angular movement. The cochlear duct contains the auditory machinery for hearing.

The neural crest (NC) is an unique embryonic structure. It contains a remarkable multipotent stem cell population during embryogenesis. NC has been referred to as the "fourth germ layer" [10]. NC migrates from the embryonic neural epithelium into various regions of the body and differentiates into many kinds of cell types such as neurons and glial cells, melanocytes of pigment cells, bones and connective tissues [4, 11].

Pax3 is a member of a gene family characterized by the presence of a conserved 120 amino acid pared-type DNA binding domain [12]. It encodes a transcription factor and expresses in the dorsal neural tube from which neural crest cells emerge during embryogenesis. *Pax3* is expressed in pre-migratory neural crest cells and in presomitic mesoderm [13]. Mutations in *Pax3* has been shown to be responsible for the mouse splotch phenotype and the human Waardenburg syndrome (WS) type I. In Splotch mice in the heterozygous state, white spotting are visible because of pigmentation defect. Homozygous splotch mutations are embryonic lethal, and show exencephaly and/or spina bifida as well as various defects associated with neural crest cell deficiency [14].

In this report, I described in detail where the neural crest derivatives migrate in the inner and middle ear and the role of *Pax3* in inner and middle ear development.

II. MATERIALS AND METHODS

1. Mouse

Pax3-Cre mice [15] and *Rosa26* reporter mice [16] were obtained from the Jackson Labs. Embryos and adult mice were genotyped by PCR analysis of DNA derived from tail and toes biopsy, respectively. *Pax3-Cre* mice are Knock-in mice replaced the first exon of *Pax3* gene with *Cre* recombinase gene, expressing *Cre* recombinase in dorsal neural tube where endogenous *Pax3* is expressed. *Cre* recombinase can remove DNA fragment located between *loxP* sites through DNA recombination. In *Rosa26* reporter mice, transcription termination sequence was inserted between consititutive promoter and *LacZ* gene. Thus, when *Rosa26* reporter mice are bred to *Pax3-Cre* mice, the termination sequence of the *Rosa26* reporter mice is deleted by recombination, and the expression of *LacZ* gene can be activated. As a result, the cells expressing the Cre recombinase constitutively express *LacZ* gene, which can be observed by β-galactosidase staining (fate-mapping).

2. ß-galactosidase staining and RNA in situ hybridization

To detect β-galactosidase activity in mouse embryos, the embryos were stained in the following the manner: Fixed embryos were incubated 1-3

hr(s) in a staining solution consisting of 5mM K3Fe(CN)6, 5mMk4Fe(CN)6, 2mM MgCl2, 0.01% Deoxycholic Acid, 0.02% NP-40, and 1mg/ml X-gal at 37℃. The embryos were then washed twice in PBS containing 2mM MgCl2, rinsed in H2O, and stained in the Orange G solution for 30 seconds.

Frozen sections of mouse embryos were processed for in situ hybridization. Embryos were fixed overnight in 4% paraformaldehyde in PBS, dehydrated in 30% sucrose, and embedded in OCT (Tissue-Tek). Embryos were then sectioned at 12 um thickness onto superfrost slides (VWR Scientific) and stored at -70℃. Before in situ hybridization, slides were dried at room temperature, post-fixed, and permeabilized using 10 ug/ml proteinase K for depending 1-5min. Hybridization was performed in pre-hybridization solution. Each bag contained four slides and 10ml of hybridization solution with a probe.

3. Immunohistoflorescence(IHF)

Embryos were harvested at E10.5 and fixed in 4% paraformaldehyde (PFA) in PBS for 2-3hrs. After then, the embryos were embedded and frozen in OCT compound (TissueTek) for cryosection. The cryosections were cut at 12-um thickness. In this study, the dilution and sources of antibodies we used chicken anti-lacZ (1:1500, ABcam #ab9361-250), goat anti-Sox10 (1:1500, Santa CruZ sc-17342), and mouse anti-Islet1 (1:1500, Devel opmental Studies

Hybridoma Bank 40.2D6). Alexa-conjugated (invitrogen) and Texas Red-conjugated (Abcam) secondary antibodies were used a concentration of 1:200. Immunofluorescence images were obtained on a Olympus IX70 fluorescence microscope.

4. Paint fill injection

Mouse at E15.5 was harvested and fixed overnight in Bodian's fixative. Specimens were then dehydrated in ethanol and cleared in methyl salicylate. The inner ears were visualized by injecting commercial correction liquid in methyl salicylate into the lumen of the inner ear. The micropipette was inserted in the lateral surface of otocyst. For more mature ears, the superior ampulla, the utricle, or the common cruz were targeted depending on the ease of visualization of the lumen.

III. RESULTS

1. *Pax3* lineage overlaps with endogenous *Pax3* gene expression in rhombomere 4 (r4) and 5 region (Otic region)

To analyze the *Pax3* lineage in the inner ear, I bred *Pax3-Cre* mice with *Rosa26* reporter (R26R) line, which can genetically label *Pax3*-expressing cells through *Cre-loxP* system. The *Pax3-Cre* reporter effectiveness and specificity were observed by comparing between ß-gal staining in *Pax3-Cre;R26R-lacZ* embryos and in situ hybridization with *Pax3* RNA probe in wild type embryos. *Pax3* expression was observed in the dorsal part of the neural tube at embryonic day 9.5 (E9.5) (Fig. 1A,B; arrows). However, *Pax3* transcripts were not detected in the otic vesicle (Fig. 1B) as well as in the mature inner ear epithelium at E15.5 (Fig. 2A-F). Compared to the *Pax3* expression, *Pax3* lineage, which can be traced by ß-gal staining, was detected in dorsal part of the neural tube similar to *Pax3* expression pattern (Fig. 1C,D; arrows) and also in the migrating NCCs from r4 to the adjacent mesenchyme (Fig. 1C; arrowheads). *Pax3* lineage was also observed in the dorsolaterally migrating NCCs (Fig. 1C,D; red arrows) and in the otic vesticle (Fig. 1D; open arrowhead). These results show that *Pax3-Cre* line can be used for the *Pax3* lineage analysis, which includes the migrating NCCs.

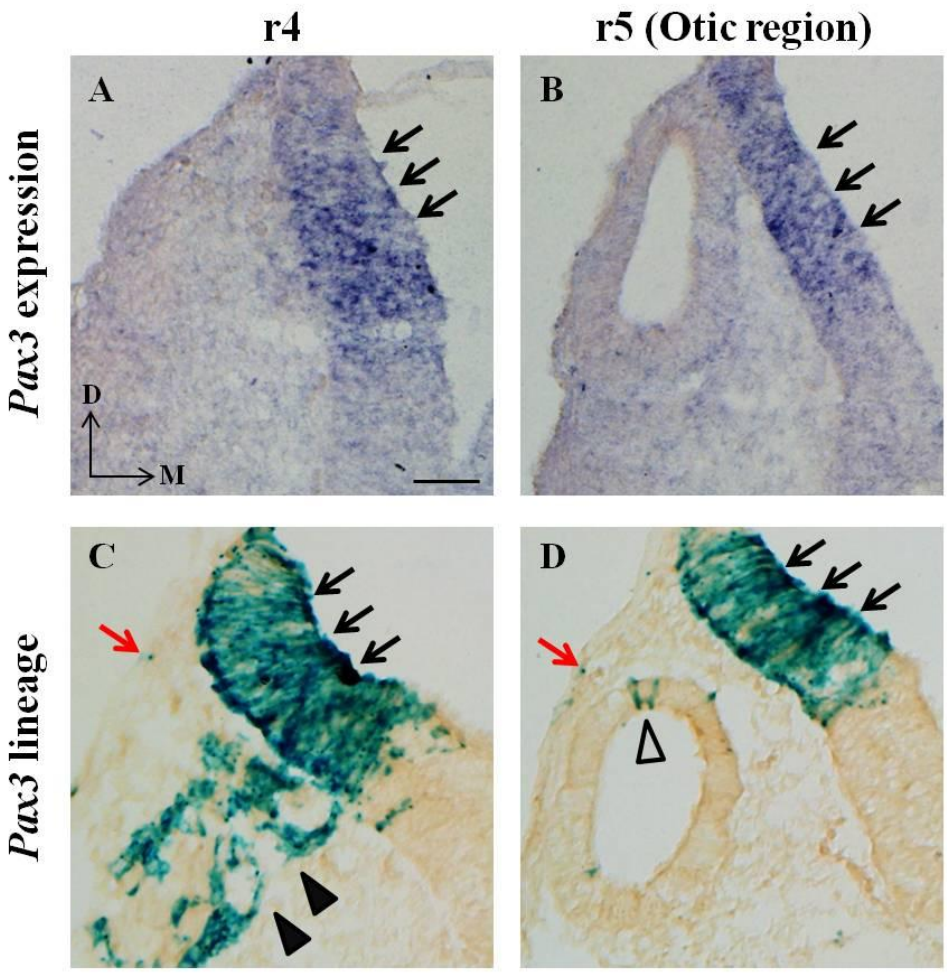

Figure 1. *Pax3* mRNA expression pattern and *Pax3* lineage analysis At E9.5, *Pax3* transcripts were detected in dorsal part of the neural tube (A,B; arrows), where neural crest cells migrate. At E9.5, ß-gal positive cells were detected in dorsal part of the neural tube (C,D; arrows), in the migrating neural crest cells (C,D; arrowheads and red arrows), and in otic epithelium (C,D; open arrowhead). R, rhombomere; D, dorsal; M, medial. Scale bar: 50um.

2. *Pax3* lineage is found in various regions of the inner ear

Although *Pax3* is not expressed in the inner ear, *Pax3* lineage was found in various regions of the inner ear at E15.5. *Pax3* lineage was found in endolymphatic duct (Ed) (Fig. 2G; arrows), common cruz (CC) (Fig. 2H; arrows), and semi-circular canals (SCC) (Fig. 2I; arrow). Especially, the descendants of *Pax3*-expressing cells were overlaped with subpopulation of vestibular dark cells around the component. Furthermore, the lineage was examined in. There was also vestibular dark cell whose subpopulation is overlapped with *Pax3* lineage. In addition, *Pax3* lineage was observed in utricle (UC) and saccule (SC) (Fig. 2J,L). In most of the vestibular structures, the descendants of *Pax3*-expressing cells were observed in the vestibular dark cells (Fig. 2H,I,J; arrows). Glial cells (Schwann cells) of peripheral nervous system (PNS) are known to be originated from NC [17] [18]. Consistently, I observed *Pax3* lineage under the UC, SC, and cristae in vestibule as well as modiolus region in the cochlea where vestibular or cochlear ganglion is present (Fig. 2J,K,L; arrows). In the cochlea, *Pax3* lineage was observed in in stria vascularis (SV) (Fig. 2K; red arrow). SV consists of three kinds of cells such as marginal, intermediate, and basal cells. These cells are derived from different origins. Marginal cells are generated from otic epithelium, intermediate cells are melanocyte differentiated from NCCs, and basal cells are originated from mesenchymal cells [19]. Thus, *Pax3* lineage appeared to

migrate into the intermediate cell region of SV in the cochlea. Finally, *Pax3* lineage was observed in the epithelium of cochlea duct (Fig. 2K).

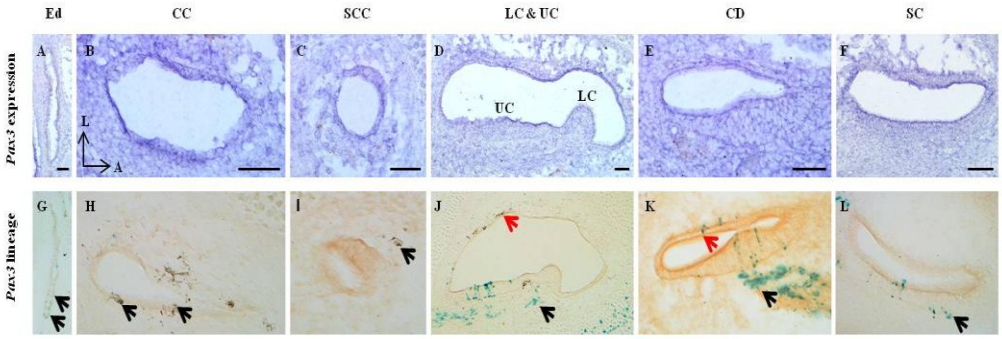

Figure 2. *Pax3* lineage migrates a variety of regions in the inner ear but *Pax3* is not expressed in the regions Descendants of *Pax3*-expressing cells were found in endolymphatic duct (Ed) (G; arrows), common cruz (CC) (H; arrows), semi-circular canal (SCC) (I; arrow), utricle (U) & around the region (J; arrow), saccule (SC) (L; arrow), epithelium of cochlea duct (K; arrow), stria vascularis (SV) (K; red arrow), and cochleo-vestibular ganglion (CVG) (J,K; arrows). Note is that vestibular dark cells are overlapped with descendants of *Pax3*-expression cells (H,I; arrows, J; red arrow). However, *Pax3* was not expressed in the inner ear. Scale bar: 50um.

3. *Pax3* lineage contributes extensively to the bony otic capsule and middle ear ossicles

Cranial neural crest cells is important for bone formation in the vertebrate head [20]. In addition, NCCs has been shown to contribute to bony otic capsule and middle ear ossicles in chicken [21]. Thus I examine whether NCCs also contribute to bony capsule and middle ear ossicles in mammals. I observed *Pax3* lineage in the bony capsule in the cochlear duct, but not in the cartilages of the vestibular structures at Postnatal6 (P6) (Fig. 3A). Pax3 lineage was also foudnin all three middle ear ossicles including malleus, incus, and stapes (Fig. 3B-D). Interestingly, the footplate of stapes was negative for β-gal staining (Fig. 3C,D; arrowheads, arrows). To confirm whether *Pax3* lineage does not contribute to the stapedal footplate, I did ß-gal staining in stapes region by section at P1. ß-gal staining was negative in the footplate of stapes, which was marked by expression of *Aggrecan*, a cartilage marker [22] (Fig. 3E,F; arrows). These results show that *Pax3* lineage contributes to the bony otic capsule and all three middle ear ossicles in mammals.

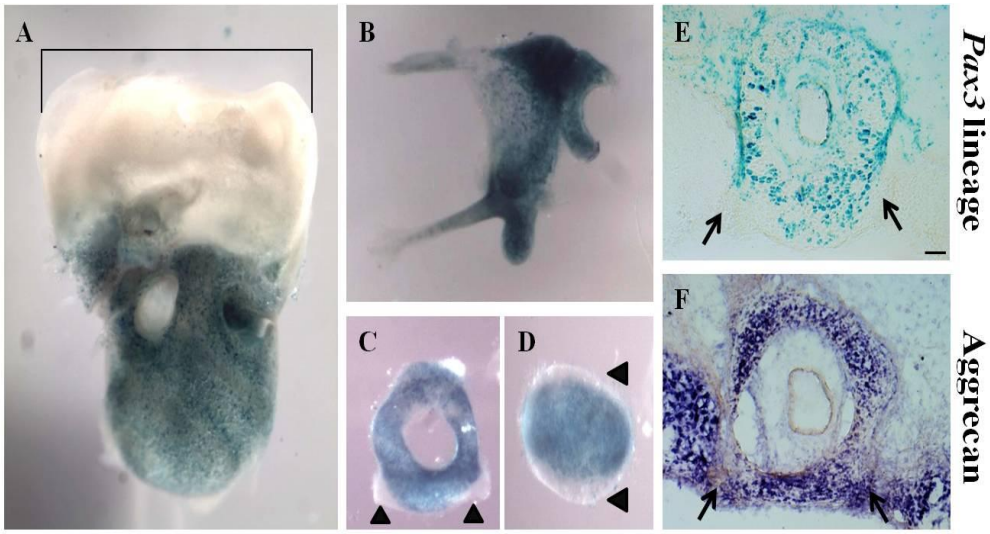

Figure 3. *Pax3* lineage migrates in the bony otic capsule and middle ear ossicles

Descendants of *Pax3*-expressing cells were found in the otic capsule (A) and the middle ear ossicles at Postnatal 6 (B,C,D). The cartilages in the vestibule (A; braket) and the stapedal footplate (C,D; arrowheads, E; arrows) were negative for β-gal staining. Scale bar: 50um.

4. Morphogenesis of the inner ears in the absence of *Pax3* function

Abnormal function of *Pax3* has been shown to cause a failure to close the neural tube during embryogenesis [23]. Consistently, *Pax3-Cre* homozygotes which have no *Pax3* function [15] showed defects in the neural tube such as spina bifida (Fig. 4B,C; arrow) which is spinal cord openand excencephaly which is brain open (Fig. 4C; arrows). The size of homozygote embryos was smaller than *Pax3-Cre* heterozygotes (Fig. 4A-C). I examined the gross morphology of the inner ears of the *Pax3-Cre* homozygotes embryos by paint-fill injection. The inner ears of *Pax3-Cre* heterozygotes was used as controls (Fig. 4D). The inner ears of *Pax3-Cre* homozygotes with spina bifida were slightly smaller than *Pax3-Cre* heterozygotes, but the morphology was completely normal (Fig. 4E). The inner ears of *Pax3-Cre* homozygotes with with excencephaly was even smaller and showed mild morphological defects such as shorter and fatter Ed irregular patterning of SCC and cochlear duct (Fig. 4F). These results show that the inner ear morphology of *Pax3-Cre* homozygotes appears to depend on the location of neural tube defects. Because the inner ear in excenphaly was affected more than it in spina bifida in *Pax3* knockout embryos. Likewise this, previous paper shows that inner ear of exencephaly is severely affected more than it of the others in *Gli3* knockout embryos [24].

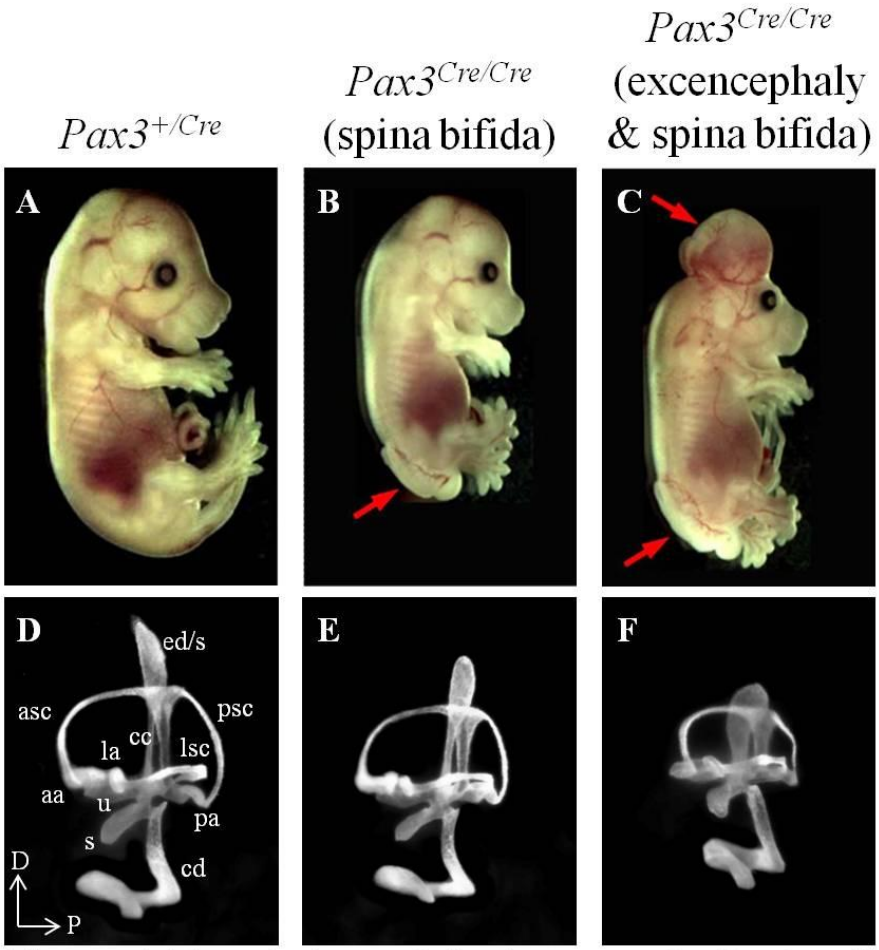

Figure 4. Morphology of the inner ear in *Pax3* null embryos *Pax3*^{Cre} homozygotes displayed spina bifida (B; red arrow), excencephaly, or both (C; red arrows). The size of homozygote embryos was smaller compared to heterozygote litter mates. Inner ears of *Pax3*^{Cre} homozygotes with spina bifida looked grossly normal although slightly smaller in size (E). Inner ears of *Pax3*^{Cre} homozygotes with excencephaly were even smaller in size and the contour of the membranous labyrinth was not smooth (F). aa, anterior ampulla; asc, anterior semicircular canal; cc, common crus; cd, cochlear duct; ed/s, endolymphatic duct and sac; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle; D, dorsal; P, posterior.

5. Melanocytes but not glial cells are affected in the absence of *Pax3* function

Next, I examined in detail the *Pax3* lineage in *Pax3-Cre* homozygote embryos.*Pax3* lineage appeared to be normal in Ed and glial cells of UC, SC, and cochlea duct in the *Pax3-Cre* homozygotes (Fig. 5A,D,E,F; arrows). . However, *Pax3* lineage was not observed in CC SCC, UC as well as in intermediate cells of SV (Fig. 5B-E; red asterisks). Consistent with the lack of *Pax3* lineage, I could not detect any vestibular dark cell in *Pax3* null embryos. The abnormal melanocyte differentiation in *Pax3-Cre* homozygotes was also confirmed by *Trp2* expression patterns. In *Pax3-Cre* heterozygotes, *Trp2*-positive melanocytes were observed in vestibular dark cells of CC, SCC and LC & UC (Fig. 5G,H,I) as well as the intermediate cells of SV (Fig. 5J). These *Trp2* expressions were completely disappeared in *Pax3-Cre* homozygotes (Fig. 5K-N; red asterisks). In contrast, expression of *Sox10*, a marker for glial cell [25], which is overlapped with a subpopulation of ß-gal positive cells (Fig. 6A,B; white rectangles) appeared to be normal in *Pax3* null embryos at E10.5 (Fig. 6E,F) and E15.5 (Fig. 6C,D,G,H).. Finally, the middle ear ossicles were not affected in the *Pax3-Cre* homozygotes embryos (Data not shown).

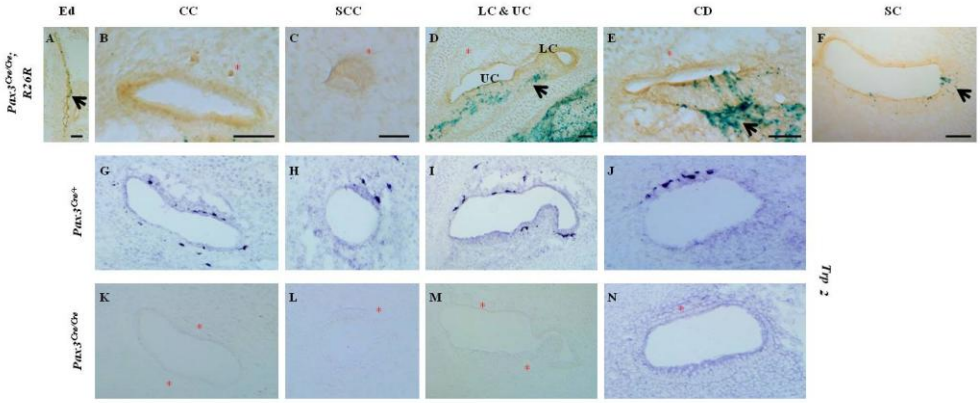

Figure 5. *Pax3* lineage in the stria vascularis of the cochlea and the dark cells of the vestibules are affected in *Pax3* null embryos Descendants of *Pax3*-expressing cells were compared between $Pax3^{Cre/+}$;*R26R* (Fig. 2) and $Pax3^{Cre/Cre}$;*R26R* embryos at E15.5. β-gal positive cells in the presumptive stria vascularis and dark cells in the vestibular region were disappeared in $Pax3^{Cre/Cre}$ homozygotes (B,C,D,E; red asterisks). In other inner ear components such as spiral (E; arrow) and vestibular ganglia (D; arrow), macular (D,F; arrow) and endolymphatic duct (A; arrow), obvious differences in β-gal staining were not observed between $Pax3^{+/Cre}$;*R26R* and $Pax3^{Cre/Cre}$;*R26R* embryos.*Trp2*, melanoblast marker, was diappeared in the region where the expression was in $Pax3^{Cre/Cre}$ embryo compared to in $Pax3^{Cre/+}$ (K,L,M,N ; red asterisks). Scale bar: 50um.

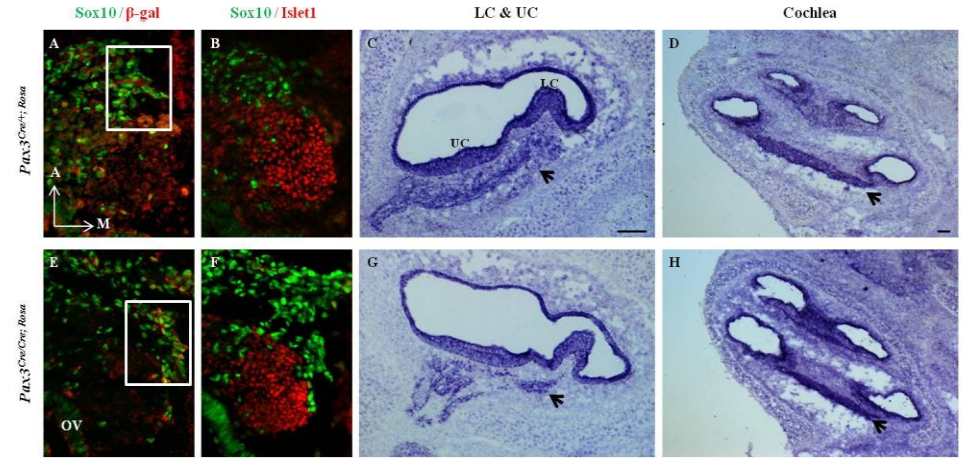

Figure 6. *Sox10*, glial cell marker, expression is appeared to be unaffected in *Pax3* null embryos *Sox10* expression was still appeared both $Pax3^{Cre/+}$ and $Pax3^{Cre/Cre}$ embryos in the cochlea-vestibule ganglia region during inner ear development (A-H; arrows). *Islet1* is a marker for neurobalst. Green color indicates *Sox10* and red color indicates *Islet1*. OV, otic vesicle; A, anterior; M, medial. Scale bar: 50um.

IV. DISCUSSION

In this study, I have shown that *Pax3* lineage, which is originated from neural crest, was found in a various substructures of the inner ear as well as in the middle ear ossicles. I have also shown that vestibular dark cells and melanocytes, but not glial cells, were disappeared in *Pax3* knockout embryos.

1. Neural crest migrate into various inner ear components and middle ear ossicles

Pax3 is expressed in dorsal part of neural tube containing migratory neural crest (NC) (Fig. 1C,D). Consistently, *Pax3* lineage was found not only in the dorsal neural tube but also in migratory neural crest. In our study, I found that neural crest cells (NCCs) migrate into various substructures of the inner and middle ears.

First of all, neural crest cells were observed in Ed (Fig. 2G). In a previous report, it has been shown that *Trp2*, a melanocyte marker, was expressed in Ed of E14 mouse embryos [26]. However we could not observe *Trp2* expression in Ed by in situ hybridization and the *Pax3* lineage in Ed was not affected in *Pax3* null embryos.

In avian, neural crest cells do not migrate into the cochlear duct epithelium [21]. In contrast, I observed that neural crest cells migrate into the

epithelium of cochlear duct, especially into SV, in mammals (Fig. 2K). The melanocyte in stria vascularis of cochlear duct was completely disappeared in *Pax3* knockout embryos (Fig. 5E; red asterisk), consistent with the known role of *Pax3* in melanocyte differentiation [27].

Neural crest cells were also found to migrate into UC and SC which are responsible for linear movement (Fig. 2J,L; arrows). In addition, the neural crest cells migrated to the bony otic capsule of cochlear part (Fig. 3A). This observation was indicate that consistent with a previous report showing the importance of neural crest cells in craniofacial bone formation [20], neural crest cells are essential for formation of bony otic capsule in cochlear region.

I also found that neural crest cells migrate to all three middle ear ossicles including malleus, incus, and stapes (Fig. 3B-D), consistent with the case of avian [21]. Interestingly, the neural crest cells did not appear to contribute to the footplate of stapes (Fig. 3C-F; arrowheads and arrows). This results confirmed previous reports proposing two different sources of the stapes human [28].

2. Vestibular dark cell is disappeared in *Pax3* null embryos

Neural crest cells migrate to the vestibular dark cells associated with commom cruz (cc), semi-circular canal (scc), and the periphery of utricle and cristae (Fig. 2H,I; arrows, J; red arrow). When *Pax3* is knocked out, *Pax3*

lineage as well as vestibular dark cells were disappeared in these regions, suggesting that *Pax3* is essential for differentiation of vestibular dark cells and survival of neural crest cells. There are many ion channels in the vestibular dark cells like NKCC1, KCNE1, and KCNQ1 important for normal vestibular function such as balance [29], [30], [31], [32]. Consistently, , mutations in *PAX3* in human cause Waardenburg syndrome (WS) type I, characterized by dizziness and vertigo [33].

3. Melanocytes are disappeared in *Pax3* knockout embryos

Intermediate cell of SV in the cochlear duct is originated from NC [19]. In *Pax3* knockout embryos, melanocytes, assessed by *Trp2* expression, are disappeared in the SV (Fig. 5E; red asterisk), consistent with the important role of *Pax3* in melanocyte differentiation [34], [27]. *Kir4.1* (potassium channel) in the intermediate cell is shown to be critical for the generation of endocochlear potential (EP), which is essential for sound transduction, [35]. It has been reported that 30% to 70% of WS type I patients, suffer from sensorineural hearing loss (SNHL)[36] and a recent report showed that there is defects in melanocytes in the inner ears of WS type I patients [37]. These results suggest that the SNHL in WS type I patients could be due to defects in melanocyte differentiation and resulting abnormal *Kir4.1* function.

4. Schwann cell is appeared in *Pax3* knockout embryos

NCCs migrate to cochleo-vestibular ganglion (CVG) region (Fig. 2J-L; arrows), consistent with the report from avian, in which NC has been shown to contributes to CVG of the avian inner ears [38]. Although it has been suggested that *Pax3* function is important for the establishment and maintenance of SC precursors [39], SCs in the inner ear appeared normal in *Pax3* null embryos, suggesting that *Pax3* function is not required for SC differentiation or compensated by other factors.

V. CONCLUSION

By using *Cre/loxP* system, we genetically labeled *Pax3*-expressing cells and analyzed the whereabouts of the *Pax3* lineage, which are mostly neural crest cells, and the role *Pax3* during inner and middle ear development. .

1. *Pax3* lineage was observed in various substructures of inner ear, including
    1) Epithelium of endolymphatic duct, utricle, saccule, cochlea
    2) Vestibular dark cells of common cruz, semi-circular canal, utricle
    3) Glial cells of vestibular and cochlear ganglion
    4) Melanocytes of stria vascularis
    5) Middle ear ossicles including malleus, incus, stapes

2. *Pax3* knockout embryos, the vestibular dark cells and melanocytes were abolished but glial cells appear to be unaffected.

REFERENCES

1. Couly GF, Coltey PM, Le Douarin NM. The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. Development 1993;117(2):409-29.

2. Hanken J, Gross JB. Evolution of cranial development and the role of neural crest: insights from amphibians. J Anat 2005;207(5):437-46.

3. Le Lievre CS. Participation of neural crest-derived cells in the genesis of the skull in birds. J Embryol Exp Morphol 1978;47:17-37.

4. Noden DM. The control of avian cephalic neural crest cytodifferentiation. I. Skeletal and connective tissues. Dev Biol 1978;67(2):296-312.

5. O'Gorman S. Second branchial arch lineages of the middle ear of wild-type and Hoxa2 mutant mice. Dev Dyn 2005;234(1):124-31.

6. Mallo M. Formation of the middle ear: recent progress on the developmental and molecular mechanisms. Dev Biol 2001;231(2):410-9.

7. Wood JL, Hughes AJ, Mercer KJ, Chapman SC. Analysis of chick (Gallus gallus) middle ear columella formation. BMC Dev Biol 2010;10:16.

8. Noramly S, Grainger RM. Determination of the embryonic inner ear. J Neurobiol 2002;53(2):100-28.

9. Solomon KS, Fritz A. Concerted action of two dlx paralogs in sensory placode formation. Development 2002;129(13):3127-36.

10. Hall BK. The neural crest as a fourth germ layer and vertebrates as quadroblastic not triploblastic. Evol Dev 2000;2(1):3-5.

11. Noden DM. Interactions and fates of avian craniofacial mesenchyme. Development 1988;103 Suppl:121-40.

12. Chi N, Epstein JA. Getting your Pax straight: Pax proteins in development and disease. Trends Genet 2002;18(1):41-7.

13. Goulding MD, Chalepakis G, Deutsch U, Erselius JR, Gruss P. Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J 1991;10(5):1135-47.

14. Auerbach R. Analysis of the developmental effects of a lethal mutation in the house mouse. J Exp Zool 1954(127):305-29.

15. Engleka KA, Gitler AD, Zhang M, Zhou DD, High FA, Epstein JA. Insertion of Cre into the Pax3 locus creates a new allele of Splotch and identifies unexpected Pax3 derivatives. Dev Biol 2005;280(2):396-406.

16. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 1999;21(1):70-1.

17. Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M. Sox10, a novel transcriptional modulator in glial cells. J Neurosci 1998;18(1):237-50.

18. Peirano RI, Goerich DE, Riethmacher D, Wegner M. Protein zero gene expression is regulated by the glial transcription factor Sox10. Mol Cell Biol 2000;20(9):3198-209.

19. Trowe MO, Maier H, Schweizer M, Kispert A. Deafness in mice lacking the T-box transcription factor Tbx18 in otic fibrocytes. Development 2008;135(9):1725-34.

20. Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. Genes Dev 2004;18(8):937-51.

21. Noden DM. The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. Dev Biol 1983;96(1):144-65.

22. Glumoff V, Savontaus M, Vehanen J, Vuorio E. Analysis of aggrecan and tenascin gene expression in mouse skeletal tissues by northern and in situ hybridization using species specific cDNA probes. Biochim Biophys Acta 1994;1219(3):613-22.

23. Deol MS. Influence of the neural tube on the differentiation of the inner ear in the mammalian embryo. Nature 1966;209(5019):219-20.

24. Bok J, Dolson DK, Hill P, Ruther U, Epstein DJ, Wu DK. Opposing gradients of Gli repressor and activators mediate Shh signaling along the dorsoventral axis of the inner ear. Development 2007;134(9):1713-22.

25. Breuskin I, Bodson M, Thelen N, Thiry M, Borgs L, Nguyen L, et al. Glial but not neuronal development in the cochleo-vestibular ganglion requires Sox10. J Neurochem 2010;114(6):1827-39.

26. Steel KP, Davidson DR, Jackson IJ. TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. Development 1992;115(4):1111-9.

27. Tachibana M, Kobayashi Y, Matsushima Y. Mouse models for four types of Waardenburg syndrome. Pigment Cell Res 2003;16(5):448-54.

28. Anson BJ, Cauldwell EW. Stapes, fistula ante fenestram and associated structures in man; from the fetus of 160 mm. (5 months) to newborn infant. Arch Otolaryngol 1948;48(3):263-300.

29. Flagella M, Clarke LL, Miller ML, Erway LC, Giannella RA, Andringa A, et al. Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. J Biol Chem 1999;274(38):26946-55.

30. Dixon MJ, Gazzard J, Chaudhry SS, Sampson N, Schulte BA, Steel KP. Mutation of the Na-K-Cl co-transporter gene Slc12a2 results in deafness in mice. Hum Mol Genet 1999;8(8):1579-84.

31. Delpire E, Lu J, England R, Dull C, Thorne T. Deafness and imbalance associated with inactivation of the secretory Na-K-2Cl co-transporter. Nat Genet 1999;22(2):192-5.
32. Vetter DE, Mann JR, Wangemann P, Liu J, McLaughlin KJ, Lesage F, et al. Inner ear defects induced by null mutation of the isk gene. Neuron 1996;17(6):1251-64.

33. Black FO, Pesznecker SC, Allen K, Gianna C. A vestibular phenotype for Waardenburg syndrome? Otol Neurotol 2001;22(2):188-94.

34. Moustakas A. TGF-beta targets PAX3 to control melanocyte differentiation. Dev Cell 2008;15(6):797-9.

35. Marcus DC, Wu T, Wangemann P, Kofuji P. KCNJ10 (Kir4.1) potassium channel knockout abolishes endocochlear potential. Am J Physiol Cell Physiol 2002;282(2):C403-7.
36. Newton V. Hearing loss and Waardenburg's syndrome: implications for genetic counselling. J Laryngol Otol 1990;104(2):97-103.

37. Merchant SN, McKenna MJ, Baldwin CT, Milunsky A, Nadol JB, Jr.

Otopathology in a case of type I Waardenburg's syndrome. Ann Otol Rhinol Laryngol 2001;110(9):875-82.

38. Le Douarin N, Dulac C, Dupin E, Cameron-Curry P. Glial cell lineages in the neural crest. Glia 1991;4(2):175-84.

39. Kioussi C, Gruss P. Making of a Schwann. Trends Genet 1996;12(3):84-6.

ABSTRACT (IN KOREAN)

포유동물의 내이와 중이 발생에 기여하는 *Pax3*의 lineage분석

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이 동 진

척추 동물의 내이는 외배엽의 otic placode라고 알려진 곳으로부터 발생을 한다. 발생이 진행되는 동안, otic placode는 otic cup으로 되고, 그 후에 otic cup은 외배엽으로부터 떨어져 나와 otocyst가 된다. 결국에 이 otocyst는 내이로 분화한다. 그리고 발생 초기에 이 otocyst의 일부 세포들은 otocyst의 갈라짐과 갈라진 이 세포들의 이동을 통해서 내이의 신경절을 형성하게 된다. 이렇게 otic placode로부터 기인한 세포들이 내이를 구성하는 동안, 신경능선으로부터 기인한 세포들 또한 내이 발생에 기여한다. 내이에서와 마찬가지로, 내배엽,중배엽,외배엽 뿐만 아니라 신경능선세포들이 중이 발생에 관여를 한다.

이 신경능선세포들은 외배엽과 신경관의 등축 사이에서 기원하여서 뉴런, 아교세포, 멜라닌 세포, 뼈 등의 여러 종류의 세포들로 분화한다.

신경능선세포들이 마우스 내이와 중이 발생에 어떤 기여를 하는지 알아 보기 위해서, 본 실험에서 *Pax3-Cre* 마우스를 사용하였다. *Pax3*는 전사인자이며, *Pax* family의 멤버이다. 그리고 신경능선세포의 분화를 포함하여 배아 발생에 중요한 것으로 알려져 있다. *PAX3*의 돌연변이에 의해서 사람에서는 난청의 소견을 보이는 Waardenburg's syndrome type I을 일으킨다. *Pax3*는 신경능선을 포함한 신경절의 배측에서 발현을 하지만, 내이에서는 발현하지 않는다. *Pax3-Cre* 마우스와 Rosa26 reporter 마우스의 교배로 인해서, 본 실험에서는 *Pax3* lineage가 내이와 중이의 여러 부위로 이동했음을 알 수 있었다. 더 나아가 *Pax3* knockout 마우스의 분석을 통해서 *Pax3*가 멜라닌 세포의 형성에 필요함을 관찰할 수 있었다.

핵심되는 말 : Waardenburg's syndrome, 내이, 중이, 신경능선, *Pax3*