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# Pax3 function is required specifically for inner ear structures with melanogenic fates

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ABSTRACT

*Pax3* mutations result in malformed inner ears in *Splotch* mutant mice and hearing loss in humans with Waardenburg's syndrome type I. In the inner ear, Pax3 is thought to be involved mainly in the development of neural crest. However, recent studies have shown that *Pax3*-expressing cells contribute extensively to multiple inner ear structures, some of which were considered to be derived from the otic epithelium. To examine the specific functions of Pax3 during inner ear development, fate mapping of *Pax3* lineage was performed in the presence or absence of functional Pax3 proteins using $Pax3^{Cre}$ knock-in mice bred to *Rosa26* reporter (R26R) line. β-gal-positive cells were widely distributed in $Pax3^{Cre/+}$; *R26R* inner ears at embryonic day (E) 15.5, including the endolymphatic duct, common crus, cristae, maculae, cochleovestibular ganglion, and stria vascularis. In the absence of Pax3 in $Pax3^{Cre/Cre}$; *R26R* inner ears, β-gal-positive cells disappeared from regions with melanocytes such as the stria vascularis of the cochlea and dark cells in the vestibule. Consistently, the expression of *Dct*, a melanoblast marker, was also absent in the mutant inner ears. However, when examined at E11.5, β-gal positive cells were present in $Pax3^{Cre/Cre}$ mutant otocysts, whereas *Dct* expression was absent, suggesting that *Pax3* lineage with a melanogenic fate migrated to the inner ear, yet failed to differentiate and survive without Pax3 function. Gross inner ear morphology was generally normal in $Pax3^{Cre/Cre}$ mutants, unless neural tube defects extended to the cranial region. Taken together, these results suggest that despite the extensive contribution of *Pax3*-expressing cells to multiple inner ear tissues, Pax3 function is required specifically for inner ear components with melanogenic fates.



## 1. Introduction

The mammalian inner ear is responsible for sensing and relaying sound and balance information. It is a complicated organ composed of a variety of specialized cell types derived from the ectoderm, mesoderm, and neural crest [1,2]. Most of the epithelial components of the inner ear are derived from the otic placode, a thickened ectoderm located on either side of the hindbrain. The otic placode gives rise to most of the cells enclosing the endolymphatic fluid including hair cells and neurons. In contrast, cells enclosing the perilymphatic space and otic fibrocytes are derived from mesenchymal cells surrounding the otic epithelium. Bony inner ear structures also have a mesenchymal origin [3]. The neural crest migrates from the neural tube and differentiates into melanocytes and glial cells in the inner ear [4,5]. Middle ear ossicles also originate from the neural crest [6].

Pax3, a member of the Pax family of transcription factors, is expressed in the dorsal neural tube that contains premigratory neural crest cells. Pax3 is involved in multiple steps of neural crest development such as proliferation, migration, and differentiation processes including myogenesis, melanogenesis, and neurogenesis [7,8]. Homozygous *Splotch* mice carrying mutations in *Pax3* are embryonic lethal and display multiple defects associated with abnormal neural crest development including failure of neural tube closure [9,10].

*Pax3* function in inner ear development is suggested by the malformed inner ear morphology in *Splotch* mutants [11]. In addition, the inner ears of *Splotch* mutants show decreased sialylation of neural cell adhesion molecule (NCAM) and reduced expression of

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S100 proteins in the cochleovestibular ganglia [12]. In humans, the ortholog of the *PAX3* gene is associated with Waardenburg's syndrome type I (WS-I), which is characterized by pigment abnormalities and sensorineural hearing loss [13,14]. Although Pax3 function in the inner ear has been suggested in melanocyte development [15], recent lineage analyses show that *Pax3*-expressing cells in the neural tube migrate and populate multiple inner ear components [16,17]. The *Pax3* lineage is found not only in well-known neural crest derivatives such as glial cells and melanocytes, but also in inner ear components that are considered to be derived exclusively from the otic placode [16,17]. These observations suggest that Pax3 could have multiple roles in inner ear development.

To examine specific roles of Pax3 during inner ear development, we traced the *Pax3* lineage in the inner ear in the presence or absence of functional Pax3 protein. *Pax3*-expressing cells in the neural tube were genetically labeled using $Pax3^{Cre}$ mice bred to *R26R* mice, and the *Pax3*-lineage was traced by β-galactosidase (β-gal) analysis [18,19]. In $Pax3^{Cre}$ knock-in mice, the first exon of the *Pax3* gene is replaced with Cre recombinase so $Pax3^{Cre/Cre}$ homozygote embryos are *Pax3* null [18]. Our results demonstrated that despite the extensive contribution of *Pax3* lineage in the inner ear, Pax3 function was specifically required for inner ear components with melanogenic fates.

## 2. Materials and methods

### 2.1. Mouse

$Pax3^{Cre}$ mice [18] and *Rosa26* reporter (*R26R*) mice [19] were from Jackson Laboratory (ME, USA). $Pax3^{Cre/+}$ heterozygotes were bred to *R26R* to obtain $Pax3^{Cre/+}$; *R26R* double heterozygote mice, which were bred to obtain $Pax3^{Cre/+}$; *R26R* control or $Pax3^{Cre/Cre}$; *R26R* mutant embryos at embryonic day (E) 15.5 or E11.5. The day of vaginal plug detection was defined as E0.5. All animal procedures were approved by and conducted according to the guidelines of the Animal Care and Use Committee of Yonsei University College of Medicine.

### 2.2. β-galactosidase staining

Embryos were fixed in 2% paraformaldehyde, 0.1M PIPES, 2 mM $MgCl_2$, 5 mM EGTA for 2 hours, dehydrated with 30% sucrose with 2 mM $MgCl_2$ in 1× PBS overnight at 4 °C, embedded in Tissue-Tek OCT compound, and stored at −80 °C until use. Frozen samples were sectioned to 12 μm samples using a cryotome (Microme HM525, Thermo Scientific) and collected on superfrost slides (VMR Scientific, West Chester, PA, USA). Slides were postfixed in fixative solution, washed with 1× PBS with 2 mM $MgCl_2$, and permeabilized in 0.02% NP-40 in 0.1M phosphate buffer ($Na_2HPO_4$ and $NaH_2PO_4$) at 4 °C. β-gal activity was visualized using a 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 and 1X PBS at 37 °C for overnight. The slides were counterstained with Orange G solution, cleared with xylene, and mounted with synthetic mount solution (Thermo Scientific).

### 2.3. In situ hybridization, TUNEL assay, and paint-fill analysis

*In situ* hybridization was performed as previously described [20]. Riboprobes for *dopachrome tautomerase* (*Dct*; also known as

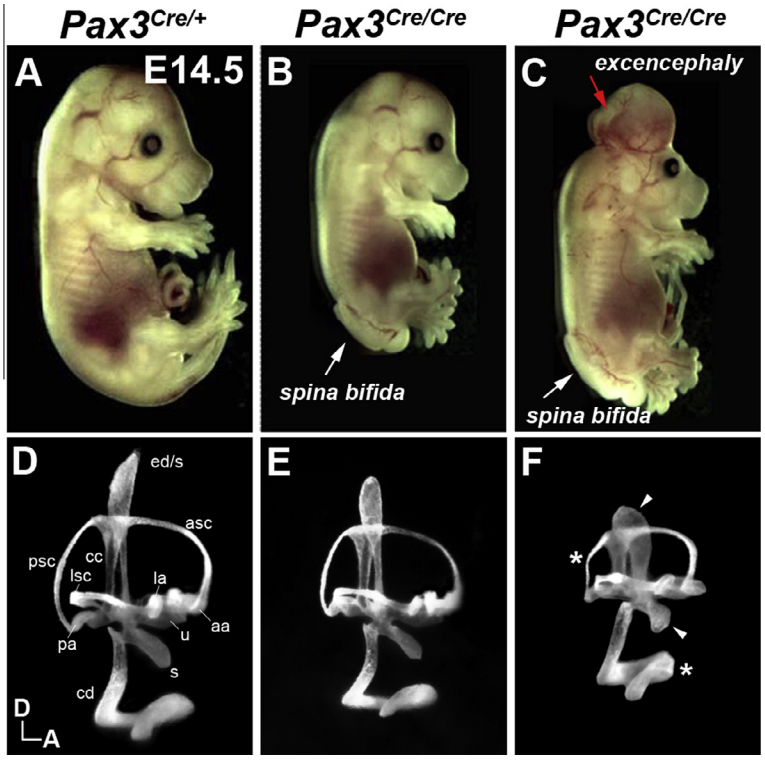


**Fig. 1.** Inner ear morphological defects were observed only in *Pax3*-null embryos with neural tube defects in the cranial region. Gross inner ear morphologies of $Pax3^{Cre}$ heterozygous and $Pax3^{Cre/Cre}$ homozygous embryos were analyzed at E14.5 by the paint-fill injection method. (A–C) $Pax3^{Cre/Cre}$ homozygotes usually displayed neural tube defects in the spinal cord (spina bifida) (B, white arrow), in the cranial region (excencephaly), or both (C, red and white arrows). $Pax3^{Cre/Cre}$ homozygous embryos were generally smaller than heterozygous litter mates. (E) The inner ears of homozygotes with spina bifida were smaller than those of heterozygous, but morphology was generally normal. (F) The inner ears of homozygotes with exencephaly were also smaller than heterozygous inner ears, and contours of the semicircular canals and cochlear duct were irregular (asterisks), and the endolymphatic duct and saccule were stunted and thick (arrowheads). 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

*tyrosinase related protein 2, Trp2*) [21], *Atoh1* [22], *Sox10* [23], and *Sox2* [24] were prepared as described. Paint-fill analysis [20] and TUNEL assay [25] were performed as previously described.

## 3. Results and discussion

### 3.1. Pax3-null inner ear morphological defects indirectly result from abnormal neural tube formation

Inner ear morphology is reported to be defective in *Splotch* homozygous mutants [11,16]. However, previous studies examined the inner ear morphology of sectioned specimens or before inner ear morphogenesis was complete. This prevented appropriate evaluation of the effect of Pax3 on inner ear morphogenesis. We paint-filled the inner ears of $Pax3^{Cre/+}$ or $Pax3^{Cre/Cre}$ embryos at E14.5, when inner ear morphogenesis was almost complete and most inner ear structures could be identified (Fig. 1D). The inner ears of $Pax3^{Cre/Cre}$ mutants with neural tube defects in the spinal cord (i.e., spina bifida) were slightly smaller than the inner ears of heterozygotes, although morphology was generally normal (Fig. 1A, B, D and E). In contrast, the inner ears of $Pax3^{Cre/Cre}$ mutants with cranial neural tube defects (i.e., excencephaly) displayed stunted, thick endolymphatic ducts and saccules and irregular contours of the semicircular canals and cochlear duct (Fig. 1C and F). These results indicated that Pax3 function was not directly required for inner ear morphogenesis and the morphological defects observed in *Pax3*-null mutants were an indirect consequence of abnormal neural tube development in the cranial region.

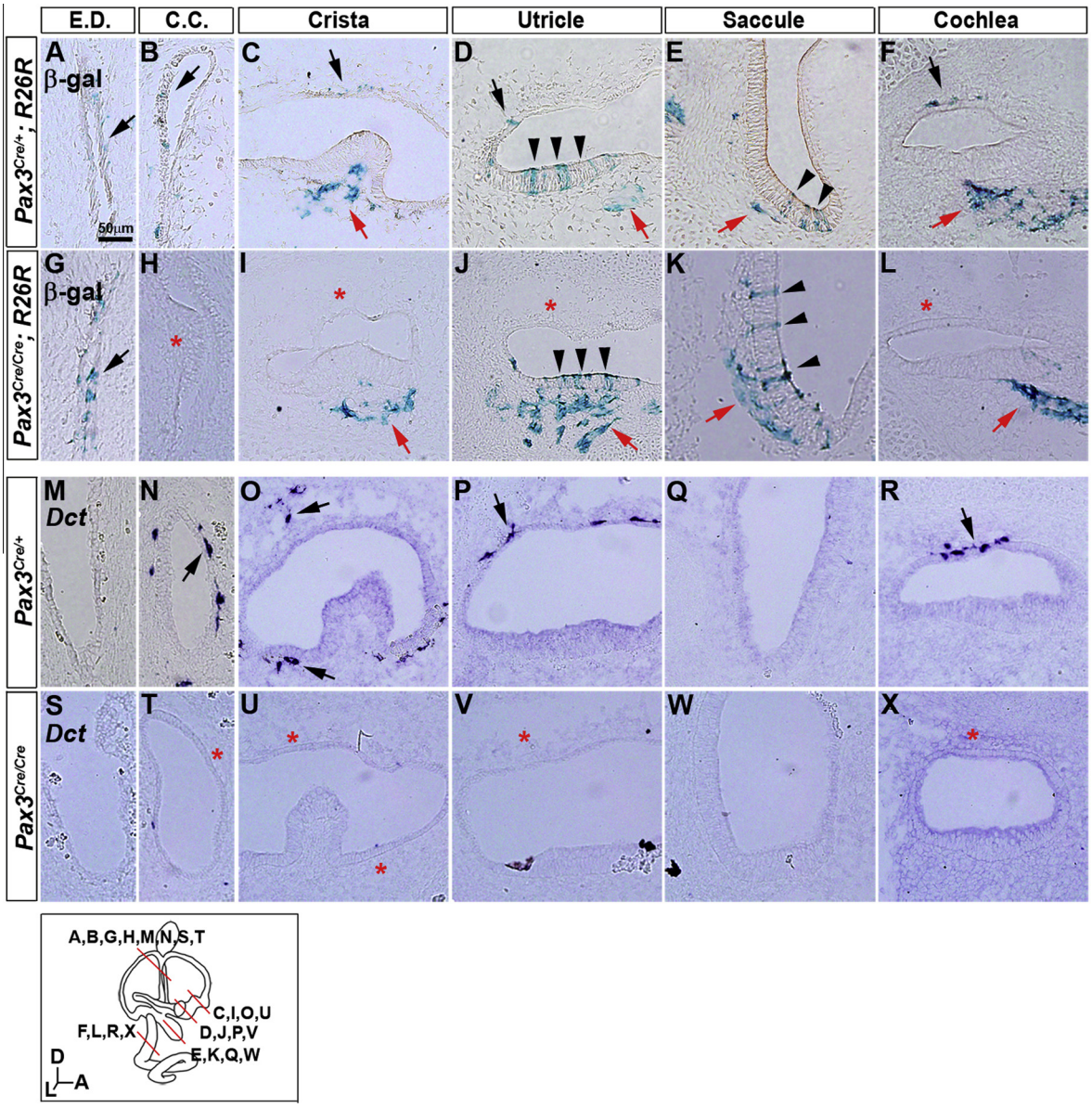


**Fig. 2.** Progeny of *Pax3*-expressing cells with melanogenic fates were specifically absent from *Pax3*-null inner ears. (A–L) *Pax3* lineage in the inner ear was traced in $Pax3^{Cre/+}$; *R26R* (A–F) and $Pax3^{Cre/Cre}$; *R26R* (G–L) embryos at E15.5. (A–F) In $Pax3^{Cre/+}$; *R26R* inner ears, β-gal-positive cells were found in the endolymphatic duct (ED) and common crus (CC) (A, B, arrowheads), glial cells in the vestibular and cochlear ganglia (C–F, red arrows), epithelial region of maculae (D, E, arrowheads), roof of the cristae and utricle and lateral sides of the cristae where dark cells were located (C, D, black arrows), and stria vascularis of the cochlea (F, black arrow). (G–L) In $Pax3^{Cre/Cre}$; *R26R* inner ears, β-gal-positive cells were absent from structures with melanogenic fates, including CC, dark cell regions, and stria vascularis (H–L, red asterisks). (M–X) *Dct* transcripts were examined in $Pax3^{Cre/+}$; *R26R* (M–R) and $Pax3^{Cre/Cre}$; *R26R* (S–X) inner ears at E15.5. (M–R) *Dct* was normally observed in CC (N), dark cell regions of the cristae and utricle (O, P), and stria vascularis of the cochlea (R). (S–X) In the $Pax3^{Cre/+}$; *R26R* inner ears, *Dct* expression was absent (asterisks). Diagram represents section planes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Pax3 lineages with melanogenic fates were absent in Pax3-null cochleae

A recent study showed that the progeny of *Pax3*-expressing cells in the neural tube migrate and populate multiple inner ear regions [16]. However, specific roles of *Pax3* in inner ear development have not been investigated in detail. Thus, we first determined if Pax3 function regulated the inner ear distribution of the *Pax3* lineage. To genetically label *Pax3*-expressing cells, $Pax3^{Cre}$ knock-in mice were bred to *R26R* mice. *Pax3* transcripts were not detected in the otic epithelium (data not shown) [16]; therefore, β-gal-positive cells in the inner ears of $Pax3^{Cre/+}$; *R26R* embryos were considered the progeny of *Pax3*-expressing cells that migrated from the neural tube. β-gal-positive cells were found in nonsensory structures such as the endolymphatic duct, common crus, dark cell regions of cristae and maculae, and the stria vascularis of the cochlea (Fig. 2A–F, black arrows). In addition, β-gal-positive cells were observed in neurosensory inner ear structures such as the maculae of the utricle and saccule and the cochlear and vestibular ganglion regions (Fig. 2C–F, arrowheads and red arrows). These results confirmed that the *Pax3* lineage contributed to multiple inner ear structures [16].

We next determined if the inner ear distribution of the *Pax3* lineage was altered in the absence of functional Pax3 proteins in $Pax3^{Cre/Cre}$; *R26R* homozygous embryos [18]. β-gal-positive cells were no longer seen in some inner ear structures. β-gal-positive cells were observed in the endolymphatic duct (Fig. 2G, arrow), but not the common crus (Fig. 2H, asterisk). In the cristae and maculae, β-gal-positive cells were not observed in the roof regions (Fig. 2I and J, red asterisks), but were maintained in the epithelium and ganglion (Fig. 2I–K, arrowheads and red arrows). In the cochlea, β-gal-positive cells were not observed in the stria vascularis (Fig. 2L, red asterisk) but were seen in the spiral ganglion region (Fig. 2L, red arrow).

These results suggested that although the *Pax3* lineage contributed to multiple inner ear structures, Pax3 function was required specifically for migration or survival of a subpopulation of the lineage. Since Pax3 is important for melanogenesis [26], we investigated whether the lineage that was not observed in the absence of Pax3 function was melanogenic. We performed *in situ* hybridization for *Dct*, a melanoblast marker, to localize melanogenic cells in the inner ear [21]. In $Pax3^{Cre/+}$ heterozygotes, *Dct* transcripts were observed in the dark cell regions of the cristae and macula of the utricle, the common crus, and the stria vascularis of the cochlea

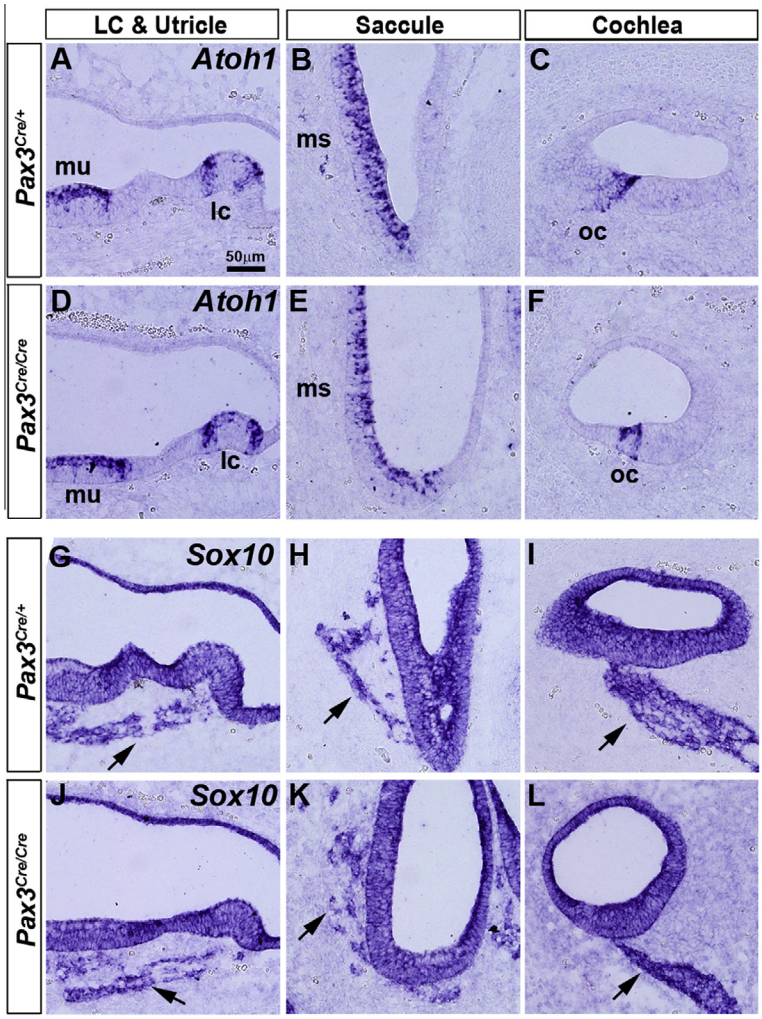


**Fig. 3.** Markers for hair cell and glial differentiation with normal expression in *Pax3*-null inner ears. (A–F) Expression of *Atoh1* in the cristae (A, D), maculae of utricle and saccule (A, B, D, E), and the organ of Corti in the cochlea (C, F) were similar between $Pax3^{Cre/+}$; *R26R* (A–C) and $Pax3^{Cre/Cre}$; *R26R* (D–F) embryos. (G–L) Expression of *Sox10* in the glial cells of vestibular ganglion (G, H, J, K, arrows) and spiral ganglion (I, L, arrows) were also similar between $Pax3^{Cre/+}$; *R26R* (G–I) and $Pax3^{Cre/Cre}$; *R26R* (J–L) embryos. mu, maculae of utricle; ms, maculae of saccule; lc, lateral cristae; oc, organ of Corti.

(Fig. 2N–R, black arrows). Interestingly, regions with *Dct* expression overlapped regions where β-gal-positive cells appeared in *Pax3*$^{Cre/+}$; *R26R* embryos but not in *Pax3*$^{Cre/Cre}$; *R26R* embryos (Fig. 2A–R). *Dct* expression was completely downregulated in the absence of Pax3 function (Fig. 2T–X, red asterisks). These results demonstrated that Pax3 function was required specifically for migration or survival of the subpopulation of the *Pax3* lineage with melanogenic fates in the inner ear.

### 3.3. Non-melanogenic Pax3 lineage was largely unaffected in Pax3-null inner ears

Unlike the melanogenic lineage, β-gal-positive cells were observed in the sensory and ganglion regions of *Pax3*-null inner ears (Fig. 2I–L). We thus investigated whether differentiation of hair cells and glial cells proceeded normally without Pax3 function. We compared the expression of *Atoh1*, a key regulator of hair cell differentiation, in E15.5 *Pax3*$^{Cre/+}$ and *Pax3*$^{Cre/Cre}$ inner ears (Fig. 3A–F). *Atoh1* expression in the cristae, maculae, and organ of Corti appeared to be normal in *Pax3*$^{Cre/Cre}$ inner ears (Fig. 3D–F). Expression of *Sox10*, which is important for glial cell differentiation [23], was also similar between *Pax3*$^{Cre/+}$ and *Pax3*$^{Cre/Cre}$ inner ears (Fig. 3G–L, arrows). The embryonic lethality of *Pax3*$^{Cre/Cre}$ embryos around E15.5 precluded the analyses of mature hair cells and glial cells; nonetheless, these results suggested that *Pax3* did not play a major role in normal development of hair cells and glial cells, at least until mid-gestation. The normal development of glial cells in the cochleovestibular ganglia of *Pax3*$^{Cre/Cre}$ embryos was consistent with a previous report showing a varying degree of defects in motoric nerve ensheathment by Schwann cells along the rostrocaudal axis in *Splotch* mutants, with cervical levels largely unaffected and thoracic and lumbar levels gradually more affected [27]. Thus, Pax3 did not appear to be a major factor in glial development in the cranial regions including in inner ear.

### 3.4. Pax3 is required for melanocyte differentiation and survival but not migration in the inner ear

The absence of the melanogenic lineage in *Pax3*$^{Cre/Cre}$ inner ears could be due to migration or survival failure. Pax3 has been impli-

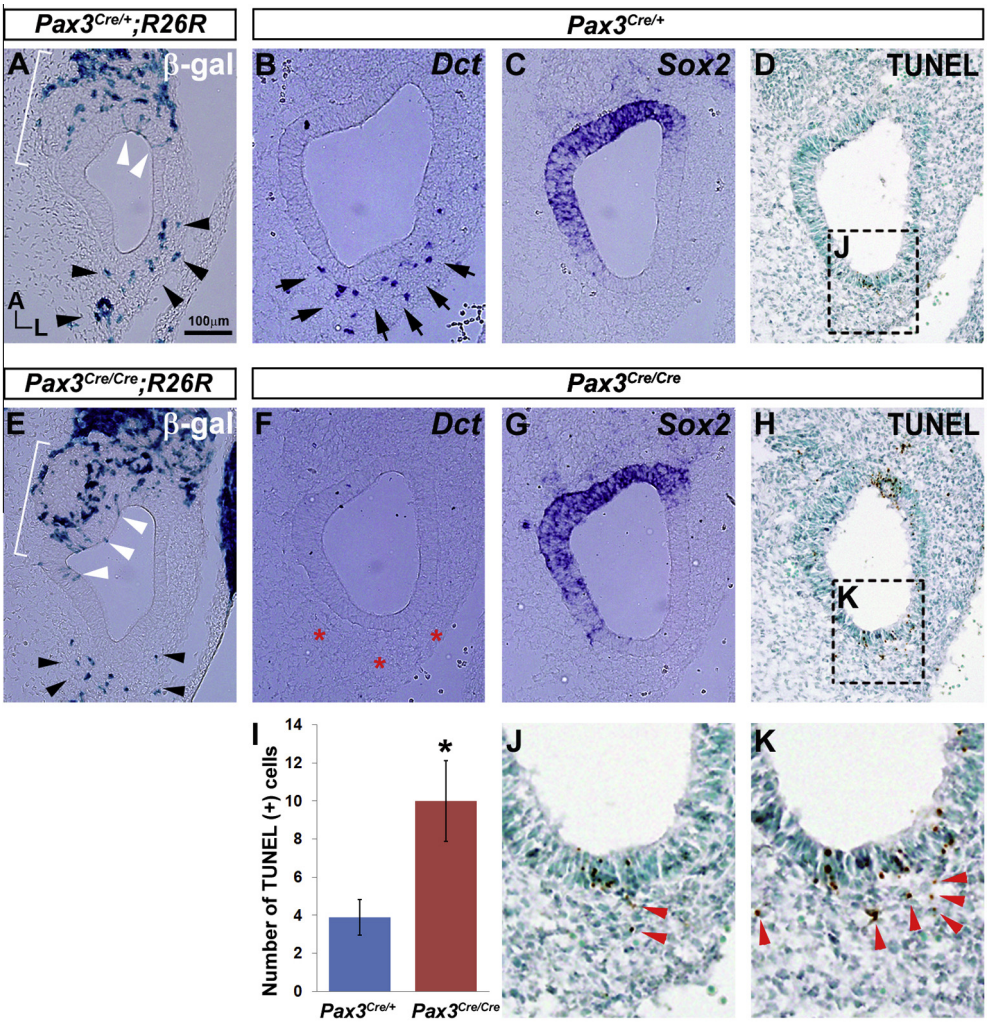


**Fig. 4.** Pax3 function was required for melanogenic differentiation and survival but not for migration of the *Pax3* lineage. (A, E) *Pax3* lineage was examined in E11.5 otocysts. β-gal-positive cells were observed in the mesenchyme anterior (brackets) and posterior (black arrowheads) to the otocyst and in the otic epithelium (white arrowheads) in both *Pax3*$^{Cre/+}$; *R26R* (A) and *Pax3*$^{Cre/Cre}$; *R26R* (E) otocysts. (B, C, F, G) *Dct* and *Sox2* transcripts were examined in *Pax3*$^{Cre/+}$ (B, C) and *Pax3*$^{Cre/Cre}$ (F, G) embryos. *Dct* expression was observed in the posterior mesenchyme of *Pax3*$^{Cre/+}$ embryos (B, arrows) but not in *Pax3*$^{Cre/Cre}$ mutants (F, red asterisks), while *Sox2* expression patterns were similar between the two otocysts (C, G). (D, H–K) Cell death was analyzed using the TUNEL assay. TUNEL(+) cells were increased in the posterior mesenchyme of *Pax3*$^{Cre/Cre}$ mutants (H, K, red arrows) compared to *Pax3*$^{Cre/+}$ controls (D, J, red arrows). Average number of TUNEL(+) cells in the posterior mesenchyme per section was significantly higher in the mutants than controls (I, asterisk, $P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cated in both events, yet the requirements for Pax3 in the neural crest migration appeared to be context dependent. Neural crest migration was delayed in explants of the caudal neural tube in *Splotch* mutants, but the neural crest populating the heart migrated normally without Pax3 function [28–30]. In addition, disruption of *Pax3* expression is reported to increase cell death in the presomitic mesoderm, whereas elevated *Pax3* expression in rhabdomyosarcoma tumor cells results in resistance against cell death [9,31].

We thus examined β-gal positive cells at otocyst stages when the *Pax3* lineage was actively migrating. In E11.5 $Pax3^{Cre/+}$; *R26R* embryos, β-gal-positive cells were observed in the mesenchyme both anterior and posterior to the otocyst as well as in the otic epithelium (Fig. 4A). The *Pax3* lineage in the otic epithelium was within the prosensory domain marked by *Sox2* expression (Fig. 4A and C, white arrowheads). The *Pax3* lineage in the anterior mesenchyme was observed in the cochleovestibular ganglion region and appeared to be glial cell precursors (Fig. 4A, bracket). β-gal-positive cells in the posterior mesenchyme co-localized with *Dct* expression, indicating migrating melanoblasts (Fig. 4A and B, arrows and arrowheads). In $Pax3^{Cre/Cre}$; *R26R* otocysts, *Dct* expression was completely absent (Fig. 4F, red asterisks), whereas β-gal-positive cells were observed in the posterior mesenchyme (Fig. 4E, arrowheads). These results suggested that the melanogenic *Pax3* lineage migrated to the posterior mesenchyme of the inner ear, yet failed to differentiate into melanoblasts.

β-gal-positive cells observed in the posterior mesenchyme of E11.5 otocysts disappeared in E15.5 *Pax3*-null inner ears (Fig. 2). Since Pax3 has been implicated in cell survival in other systems [9,31], we examined if the disappearance of β-gal-positive cells was due to a failure of cell survival by performing the cell death (TUNEL) assay. We observed that there was an increased number of TUNEL(+) cells in the posterior mesenchyme of $Pax3^{Cre/Cre}$ mutants compared to control embryos (Fig. 4D and H–J, red arrows), suggesting that the disappearance of β-gal-positive cells later in development is, at least in part, due to a failure of cell survival. Together, these results suggest that while Pax3 function is not required for migration of the melanogenic *Pax3* lineage, it is essential for differentiation and survival.

### 3.5. Pax3 in hearing loss in human WS-I

Our data demonstrated that despite the extensive contribution of *Pax3* lineage to multiple inner ear structures, Pax3 function is specifically required for differentiation and survival of the melanogenic lineage. In the cochlea, the melanogenic lineage mainly gives rise to the intermediate cells of stria vascularis, which play a crucial role in hearing function especially by generating endocochlear potential essential for transducing sound information in the hair cells [32,33]. Thus, melanogenic defects could be a major cause of hearing loss observed in human WS-I patients carrying mutations in the *PAX3* gene. Consistently, a histopathologic analysis of temporal bone of a WS-I patient who had suffered from unilateral hearing loss revealed melanogenic defects on the affected side of the stria vascularis [15]. The temporal bone analysis also showed abnormalities in non-melanogenic structures such as hair cells, spiral ganglion, and tectorial membrane [15]. Whether Pax3 function was required for long-term survival or maintenance of these structures is unknown. However, these defects appeared to be secondary effects of abnormal strial function in the cochlea, because *Pax3* was no longer expressed in the *Pax3* lineage once cells were migrated and incorporated into developing inner ear tissues [16]. Taken together, our observations suggest that Pax3 function is specifically required for normal development of melanogenic lineage in the cochlea, and melanogenic defects caused by abnormal PAX3 function appears to be a major cause of hearing loss in human WS-I.

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