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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

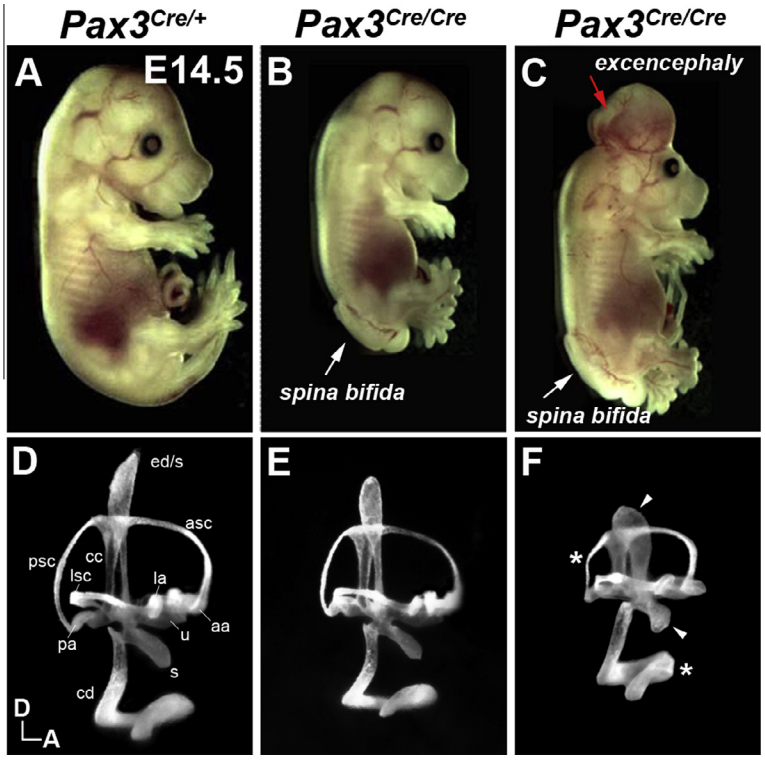

Fig. 1. Inner ear morphological defects were observed only in *Pax3*-null embryos with neural tube defects in the cranial region. Gross inner ear morphologies of $Pax3^{Cre}$ heterozygous and $Pax3^{Cre/Cre}$ homozygous embryos were analyzed at E14.5 by the paint-fill injection method. (A–C) $Pax3^{Cre/Cre}$ 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.)