Dual embryonic origin of the mammalian otic vesicle forming the inner ear

Laina Freyer¹*, Vimla Aggarwal² and Bernice E. Morrow¹,³,*

SUMMARY
The inner ear and cochleovestibular ganglion (CVG) derive from a specialized region of head ectoderm termed the otic placode. During embryogenesis, the otic placode invaginates into the head to form the otic vesicle (OV), the primordium of the inner ear and CVG. Non-autonomous cell signaling from the hindbrain to the OV is required for inner ear morphogenesis and neurogenesis. In this study, we show that neuroepithelial cells (NECs), including neural crest cells (NCCs), can contribute directly to the OV from the neural tube. Using Wnt1-Cre, Pax3Cre¹ and Hoxb1Cre¹ mice to label and fate map cranial NEC lineages, we have demonstrated that cells from the neural tube incorporate into the otic epithelium after otic placode induction has occurred. Pax3Cre¹ labeled a more extensive population of NEC derivatives in the OV than did Wnt1-Cre. NEC derivatives constitute a significant population of the OV and, moreover, are regionalized specifically to proneurosensory domains. Descendants of Pax3Cre¹ and Wnt1-Cre labeled cells are localized within sensory epithelia of the sacculus, utricle and cochlea throughout development and into adulthood, where they differentiate into hair cells and supporting cells. Some NEC derivatives give rise to neuroblasts in the OV and CVG, in addition to their known contribution to glial cells. This study defines a dual cellular origin of the inner ear from sensory placode ectoderm and NECs, and changes the current paradigm of inner ear neurosensory development.

KEY WORDS: Inner ear, Neural crest cells, Fate mapping, Sensory placode, Mouse

INTRODUCTION
Cranial sensory placodes are thickenings of ectoderm that are the source of complex sensory organs and ganglia that innervate the head and neck (D’Amico-Martel and Noden, 1983; Le Douarin, 1986). The otic placode is induced next to the hindbrain and invaginates into the head to form the otic cup. The otic cup then closes off from the surface ectoderm of the head, thus creating the OV (Anniko and Wikstrom, 1984). Neuroblasts are specified within the otic epithelium and delaminate into the mesenchyme where they condense to form the CVG (Wikstrom and Anniko, 1987; Ma et al., 1998). The OV undergoes morphogenesis to give rise to the inner ear labyrinth, a continuous epithelium that makes up the vestibular [endolymphatic duct (ED), semicircular canals (SCC), utricle, saccule] and auditory (cochlea) components of the inner ear (Morsli et al., 1998). This is accompanied by development of six sensory patches: three cristae (at the base of each SCC), two maculae (utricle, saccular) and the organ of Corti (within the cochlea). Sensory epithelia are defined by the presence of mechanosensory hair cells that are associated with supporting cells and innervated by CVG neurons.

To date, it is widely accepted that the otic placode ectoderm is the only source for the inner ear labyrinth and neurons of the CVG (for reviews, see Fekete and Wu, 2002; Barald and Kelley, 2004). Contributions of other tissues to inner ear development include melanocytes, which are derived from NCCs. NCCs are specified in the dorsal neural tube and migrate throughout the embryo (Bronner-Fraser, 1995; Graham et al., 2004). Cranial NCC migratory streams are organized by rhombomeric segments of the hindbrain and respond to cues from the pharyngeal endoderm (Graham et al., 2004; Birgabauer et al., 1995; Sauka-Spengler and Bronner-Fraser, 2008). In mice, melanocyte progenitor cells originate from the midbrain-hindbrain junction and cervical trunk regions of the neural tube, and then migrate around the inner ear later in development to give rise to the intermediate cells of the stria vascularis (SV) that is located along the lateral wall of the cochlea (Wilson et al., 2004; Steel and Barkway, 1989; Cable et al., 1992; Cable et al., 1995). We decided to examine other potential functions of NCCs in inner ear development.

In this study, we used Wnt1-Cre, Pax3Cre¹ and Hoxb1Cre¹ mice to genetically fate map GFP-expressing reporter cells from the neural tube. Although Wnt1-Cre has been widely used to fate map NCCs, we found that Pax3Cre¹ labeled a broader population of NECs in the neural tube, including NCCs. Our fate-mapping results demonstrate that NEC/NCC derivatives contribute a significant population of cells to the inner ear. Using time-lapse microscopy, we documented Pax3Cre¹ labeled cells invading the otic epithelium in vivo. NEC descendants remain in the inner ear throughout development and localize to the CVG and sensory epithelia of the utricle, saccule and cochlea where they are distinct from pigment-producing melanocytes. Wnt1-Cre and Pax3Cre¹-labeled reporter cells in the CVG express the early neuroblast markers NeuroD and Islet-1. Pax3Cre¹ derivatives differentiate into hair cells that express myosin VIIA (MyoVIIA) and supporting cells that express the Ca²⁺-binding protein S100 or the neurotrophin receptor P75. In adult mice, Pax3Cre¹-labeled derivatives persist in the CVG, maculae and cochlea. Our fate-mapping results confirm an NEC origin of glial cells in the CVG that express Sox10, along with some Pax2-Cre-labeled derivatives that also express Sox10 in the CVG.
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MATERIALS AND METHODS

Mouse models
Mice were used in this study according to the regulatory standards of the Institutional Animal Care and Use Committee (IACUC) of Albert Einstein College of Medicine. All mouse lines used in this study have been previously described. Pax3<sup>Cre</sup>/+ (stock number 005549), Wnt1-lacZ (stock number 002865) and Hoxb1<sup>Cre</sup>/+ (stock number 01273) mice were purchased from Jackson Laboratories. RCE:LoxP mice were obtained from Dr Gordon Fishell (NYU Langone Medical Center, NY, USA). Sequences of primers used for PCR genotyping are in supplementary material Table S1. Embryos were dissected according to date of vaginal plug (considered to be E0.5). Embryonic stage was confirmed by counting somite pairs.

Direct fluorescence
GFP was detected by direct fluorescence. Whole-mount images were taken immediately following dissection. For tissue sections, embryos were fixed accordingly: 2 hours for ≤10.5, 4 hours for E11.5, and overnight for ≥12.5. E17.5 embryos and adults required removal of the temporal bone for overnight fixation. Fixation was carried out in 4% paraformaldehyde (PFA) in PBS at 4°C. Adult inner ears required perfusion of the cochlea. After fixation, tissue was washed in phosphate-buffered saline (PBS) then cryoprotected in 30% sucrose in PBS overnight at 4°C. Adult inner ears were decalcified in 0.5 M EDTA in PBS (pH 8.0) at 4°C for 48 hours prior to sucrose. Tissue was embedded in OCT and cryosectioned at 12-14 μm.

X-gal staining
Whole embryos were fixed in 1% formaldehyde, 0.2% gluteraldehyde, 2 mM MgCl<sub>2</sub>, 5 mM EGTA and 0.02% NP-40 in PBS. Fixation time varied with embryonic stage (15 minutes for E8.5, 20 minutes for E9.5, 30 minutes for E10.5). Embryos were stained in 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 2 mM MgCl<sub>2</sub>, 0.01% deoxycholic acid, 0.02% NP-40 and 1 mg/ml X-gal in PBS at 4°C overnight. Embryos were post-fixed in 4% PFA and cryosectioned at 10 μm. Tissue sections were dehydrated in an ethanol series into xylene and mounted in Permount.

RNA in situ hybridization
For whole-mount RNA in situ hybridization, embryos were fixed in 4% PFA at 4°C overnight and dehydrated in a methanol series with PBS/0.1% Tween-20 and stored at -20°C. The detailed protocol for RNA in situ hybridization has been previously described (Franco et al., 2001). An RNA probe template for Cre was synthesized with T7 polymerase from a Hi (Dr Moises Mallo, Spain). For RNA in situ hybridization on sections, embryos were cryoembedded, sectioned and labeled by microdissection of the surrounding tissue. Immunofluorescence was performed as described for tissue sections except that primary antibody (MyoVIla) was incubated 1:10 overnight at 4°C. Tissue was embedded in 0.6% agarose/PBS in a MatTek glass-bottom dish for imaging with a Leica SP2 AOBS confocal microscope.

Immunofluorescence on whole-mount tissue
Sensory epithelia from the utricle, saccule and organ of Corti were exposed by microdissection of the surrounding tissue. Immunofluorescence was performed as described for tissue sections except that primary antibody (MyoVIla) was incubated 1:10 overnight at 4°C. Tissue was embedded in 0.6% agarose/PBS in a MatTek glass-bottom dish for imaging with a Leica SP2 AOBS confocal microscope.

Paintfilling of the inner ear labyrinth
Embryos were cut below the forelimbs and fixed in 5% glacial acetic acid, 2% formaldehyde and 75% ethanol overnight. This was followed by dehydration to ethanol and clearing in methyl salicylate. Embryos were bisected dorsally and the brain was removed. A micropipette was used to microinject 0.2% correction fluid diluted in methyl salicylate into the utricle. Paintfilled inner ears were imaged and stored in methyl salicylate.

RESULTS

Fate mapping of NEC lineages to the mouse OV and CVG
Cranial NCCs emigrate from the neural tube during a developmental stage at which the otic cup is in direct contact with the hindbrain (Serbedzija et al., 1992; Schneider-Maunoury and Pujades, 2007). Close proximity of the OV to the neural tube allows for non-autonomous signaling from the hindbrain that is required for neurogenesis and morphogenesis (Liang et al., 2010). Direct cellular contribution of NECs from the hindbrain to the OV has not been investigated.

We genetically fate mapped NEC populations using Wnt1-Cre transgenic mice (Danielian et al., 1998), Pax3<sup>Cre</sup>/- knock-in mice (Engleka et al., 2005) and Hoxb1<sup>Cre</sup>/+ knock-in mice (O’Gorman, 2005) together with a conditionally activated GFP reporter line (RCE:loxP, referred to as RCE<sup>GFP<sub>Cre</sub></sup>) (Sousa et al., 2009) in order to further investigate the contribution of NECs to inner ear development. Wnts are secreted proteins that are necessary for NCC induction (Sauka-Spengler and Bronner-Fraser, 2008), and Wnt expression is normally limited to the developing central nervous system (McMahon and Bradley, 1990). Wnt1-Cre is widely used to label NCC derivatives in mice and is active in neuroectoderm that forms the most dorsal part of the neural tube where NCC specification occurs (Fig. 1A). Pax3 is a paired box homeodomain transcription factor that is a downstream target of Wnt signaling in premigratory NCCs (Sauka-Spengler and Bronner-Fraser, 2008). Pax3<sup>Cre</sup>/+ is also expressed in the neural tube (Fig. 1B), with additional expression in the somites (Engleka et al., 2005; Goulding et al., 1991). In early to mid-gestation, Hoxb1<sup>Cre</sup>/+ expression is specific to rhombomere 4 in the anterior embryo (Fig. 1C). For comparison, we performed a fate map of the otic placode ectoderm lineage (Fig. 1D) as labeled by Pax2-Cre (Ohyama and Groves, 2007).
Fig. 1. Fate mapping of NEC/NCC and otic placode ectoderm populations. (A-D) Lateral views of whole-mount embryos from E8.5 to E9.5. GFP reporter expression is activated by Wnt1-Cre, Pax3<sup>Cre<sup>+/+</sup></sup>, Hoxb1<sup> Cre+/+</sup> or Pax2-Cre. Otic tissue is outlined in yellow; boxes indicate regions that are magnified in adjacent images. (E, F) NEC derivatives (green) express Ap2<sub>x</sub> (red). (E) Transverse sections through the trunk neural tube (NT) and dorsal root ganglion (DRG). Images on the right are higher magnification demonstrating expression of GFP with Ap2<sub>x</sub> in the DRG. (F) Lateral whole-mount views (left column) of the first and second branchial arches (BA1, BA2). Sagittal sections through the branchial arches (middle column) with magnified views of the BA2 mesoderm (right column). Nuclei are stained with DAPI (blue). ss, somite stage.
Pax2 is one of the earliest markers of the presumptive otic and epibranchial placode domains (Ohyama and Groves, 2004; Ohyama et al., 2007) and Pax2-Cre expression leads to extensive labeling of otic cells (Fig. 1D). Wnt1-Cre, Pax3Cre/+ and Hoxb1Cre/+labeled lineages contribute to known NCC derivatives such as the dorsal root ganglion (DRG, Fig. 1E) and branchial arch mesoderm (Fig. 1F). Derivatives of reporter cells labeled by Hoxb1Cre/+ in the anterior region of the embryo are limited to the second branchial arch, consistent with their migration from rhombomere 4 (O’Gorman, 2005).

The Wnt1-Cre labeled population comprises too few cells to visualize in the inner ear by whole mount (Fig. 1A). Cells labeled by Pax3Cre/+ can be seen but it is not clear whether GFP is in the OV or surrounding cells (Fig. 1B). Upon sectioning of embryos, NEC lineages in Wnt1-Cre, Pax3Cre/+ and Hoxb1Cre/+ mice were observed in the otic epithelium (Fig. 2A). Specifically, GFP reporter cells were localized to the prosensory epithelium of the OV as well as within the CVG. Fate mapping of the otic placode ectoderm by Pax2-Cre showed that the vast majority of cells in the otic placode appear to express GFP at E8.5; however, otic cells that were negative for GFP became evident at later otic cup and OV stages (Fig. 2A). Wnt1-Cre and Pax3Cre/+ fate maps were confirmed with a lacZ reporter line, Rosa26R (Soriano, 1999), which shows a comparable distribution of reporter cells in the neurosensory region of the OV epithelium adjacent to the site of CVG formation, as well as within the CVG at E10.5 (Fig. 2B). Based on rough quantification of the total number of cells within the OV and CVG at E10.5 in Pax3Cre/+;RCEGFP/+ embryos, we estimate that NEC derivatives constitute ~20% of the total number of cells in the OV and ~25% of the total number of cells in the CVG (data not shown, illustrated in Fig. 2C).

We noted that Pax3Cre/+ labels a more extensive population of NECs than Wnt1-Cre in the neural tube, OV and CVG (Figs 1A,B, 2A,B). This discrepancy is not obvious in other NCC derivatives such as the DRG and branchial arches (Fig. 1E,F). These results suggest that while both Pax3Cre/+ and Wnt1-Cre can be used to fate map derivatives of NCCs that have been specified in the most dorsal part of the neural tube, Pax3Cre/+ is additionally active in more ventral neural tube cells that may not necessarily have undergone NCC differentiation. This introduces the possibility that NECs, besides NCCs, may directly enter the otic epithelium from the neural tube.

It was important to rule out ectopic or leaky activity of Cre in otic tissue where Wnt1 and Pax3 are not normally expressed (Kwang et al., 2002; Tajbakhsh et al., 1998). Expression of Cre mRNA was assessed in Wnt1-Cre and Pax3Cre/+ mice by in situ hybridization (Fig. 3A). Cre is strongly expressed in the neural tube.
in both mouse lines and in the somites of Pax3Cre/+ mice. No Cre mRNA expression was detected in the otic epithelium from E8.5 to E10.5, thus implying that activation of the GFP reporter by Cre recombinase occurs within the neural tube prior to incorporation of NEC derivatives into the otic epithelium. We did see expression of Cre in the CVG of Wnt1-Cre mice at E10.5; however, this was not seen in Pax3Cre/ mice. Furthermore, we examined the normal expression of Pax3 protein at the otic cup stage in Wnt1-Cre;RCEGFP/+ , Pax3Cre/+;RCEGFP/+ and Pax2-Cre;RCEGFP/+ mice (Fig. 3B). Pax3 was expressed in the neural tube, consistent with the expression of Cre mRNA, as driven by the endogenous Pax3 regulatory elements (Fig. 3A). We did not observe expression of Pax3 in otic epithelial cells. Additionally, we performed X-gal staining of Wnt1-lacZ embryos (Fig. 3C) in which a lacZ reporter recapitulates endogenous Wnt1 expression (Echelard et al., 1994). Because it takes time for β-galactosidase to decay after Wnt1-lacZ is turned off, some NCC derivatives exhibit residual staining (Echelard et al., 1994). Even after overstaining, minimal X-gal staining was seen (note significantly darker staining in the neural tube where Wnt1-lacZ is still actively expressed, Fig. 3C).

**Invasion of the otic epithelium by NECs during otic cup formation**

Cranial NCCs migrate primarily from even-numbered rhombomeric segments of the hindbrain in both mouse and chick (Sechrist et al., 1993; Serbedzija et al., 1992). The OV is situated directly adjacent to rhombomere 5, which does not contribute significantly to migratory NCCs even though it is capable of generating them (Trainor et al., 2002). Fate mapping of Hoxb1Cre/+ derivatives from rhombomere 4 to the OV and CVG was very similar to that of Wnt1-Cre derivatives at E10.5 (Fig. 2A). However, when we examined the anterior-posterior (AP) orientation of neural tube derivatives in the OV at E9.5 (Fig. 4A), we found that Wnt1-Cre-labeled NCCs and Pax3Cre/+ -labeled NECs were localized anteriorly, whereas Hoxb1Cre/+ derivatives were located posteriorly.

In an attempt to capture the incorporation of neural tube cells into the otic epithelium in vivo, we used multiphoton microscopy for time-lapse imaging of live embryos (Wyckoff et al., 2007). Pax3Cre/+;RCEGFP/+ embryos were counterstained with red-fluorescent wheat germ agglutinin (WGA), a lectin that binds to sugar residues and is used to stain the plasma membrane of live cells. This allowed us to visualize the borders between neural tube epithelia, otic epithelia and mesenchyme. Time-lapse photography was performed for up to 3 hours with 5 μm optical sectioning (supplementary material Movies 1-4; Fig. 4B). Direct invasion of the developing otic cup by reporter cells from the neural tube was documented between the 12- to 14-somite stage (supplementary material Movies 1, 3-4; Fig. 4B). This time point coincides with the emigration of vital dye-labeled NCCs from the neural tube at the 11- to 14-somite stage in mice (Serbedzija et al., 1992), as well as at the stage when the otic epithelium is in direct contact with rhombomere 5 (Mayordomo et al., 1998) (Fig. 4B). Consistent with this is broken
expression of laminin where it appears that the basal lamina of the neuroepithelium is fused to that of the otic epithelium (Fig. 4C). There was also invasion of the otic epithelium by reporter cells in the mesenchyme, a process that would require mesenchymal-to-epithelial transition (supplementary material Movie 2). We cannot rule out the possibility that some neuroectodermal cells adjacent to the otic placode ectoderm are incorporated as the placode invaginates into the head during otic cup formation.

**Localization of NEC/NCC derivatives in the developing mouse inner ear**

Fate mapping through later stages of development (E11.5 to E17.5) revealed the distribution of *Wnt1-Cre* and *Pax3Cre/+* lineages during and after morphogenesis of the inner ear. *Wnt1-Cre* and *Pax3Cre/+* derivatives were localized to the CVG, the maculae of the saccule and utricle, and the cochlea (Fig. 5A,B). GFP reporter cells were also present in the ED at earlier stages (E11.5 to E14.5), but diminished by E17.5 (Fig. 5C). *Pax3Cre/+* derivatives were fate mapped around and within the cochlea, with many GFP reporter cells in the greater epithelial ridge (GER) as well as in the SV (Fig. 5C). We did not observe any *Wnt1-Cre* or *Pax3Cre/+*-labeled derivatives in the SCCs or cristae at any stage.

We observed *Wnt1-Cre* and *Pax3Cre/+* GFP+ derivatives in the periotic mesenchyme from E11.5 to E14.5 (Fig. 5A,B) in a manner that was similar to the known distribution of melanocytes around the inner ear (Steel et al., 1992; Stanchina et al., 2006). By E17.5, melanocytes are easily identified by their production of melanin. Pigmented reporter cells with distinctive dendritic-like morphology...
Fig. 5. NEC derivatives in the developing and mature inner ear. (A) Mapping of Pax3Cre\(^{+}\) derivatives (GFP, green). Sensory epithelia are indicated by brackets. Reporter cells are in the mesenchyme opposite the GER where the SV will form (arrows). (B) Mapping of Wnt1-Cre derivatives (GFP, green). Note GFP\(^{+}\) cells in the periotic mesenchyme (arrows) surrounding the developing SCCs. (C) NEC derivatives (GFP, green) in Pax3Cre\(^{+}\);RCEGFP\(^{+}\) mice remain in sensory epithelia (brackets). Bright-field (bottom) reveals pigment-producing melanocytes in the mesenchyme (arrows) surrounding the non-sensory epithelium and in the SV. (D) Illustration of the distribution of NEC derivatives (green) and NCC-derived melanocytes (brown) in the mature inner ear relative to sensory epithelia (red) and the CVG (blue). (E) Persistence of NEC derivatives (green) in the CVG, maculae and cochlea of adult mice (GFP overlaid with bright field). Nuclei are stained with DAPI (blue).
were identified in the mesenchyme surrounding the non-sensory epithelia of vestibular organs, as well as in the SV, but nowhere else within the inner ear epithelium or CVG (Fig. 5C). Melanocytes also express the GFP reporter as Pax3Cre/ labels NCCs all along the cranial and trunk neural tube (Fig. 1B,E,F). These results indicate that the NECs, and NCCs therein, that we are tracing in the inner ear sensory epithelium and CVG are not melanocytes (illustrated in Fig. 5D). We also fate mapped Pax3Cre/+ labeled cells in the adult inner ear. Between 3 and 4 months of age (P96), Pax3Cre/+ derivatives persisted in the utricular and saccular maculae, as well as in the CVG and organ of Corti (Fig. 5E).

**Differentiation of NEC derivatives into specialized neurosensory cell types**

The localization pattern of NEC derivatives in the developing inner ear suggested that they might function in neurosensory development. As specification of neuroblasts is one of the earliest cell fate determination events in the otic epithelium (Fekete and Wu, 2002), we first analyzed the expression of neural markers. Neurogenic differentiation factor 1 (NeuroD; Neurod1 – Mouse Genome Informatics) is a basic helix-loop-helix (bHLH) transcription factor expressed in neuroblasts both before and after delamination from the otic epithelium (Ma et al., 1998). Islet 1 (Isl1) is a LIM/homeodomain transcription factor expressed in CVG neuroblasts only after they have delaminated. A subset of the Pax3Cre/+ and Wnt1-Cre lineage cells express NeuroD in the OV epithelium and CVG (Fig. 6A) suggesting that at least some GFP-expressing neuroblasts are specified from within the otic epithelium. Based on expression analysis of Isl1, we approximate that 80% of neuroblasts in the CVG derive from the otic placode ectoderm, as fate mapped by Pax2-Cre, whereas 20% derive from NECs, as fate mapped by Pax3Cre/+ (Fig. 6B). Pax3Cre/+ and Wnt1-Cre-labeled reporter cells appear to colocalize with neurofilament (NF), an intermediate filament that is strongly expressed in the axons of differentiating neurons (Fig. 6C). P75 is a neurotrophin receptor that is often used as a NCC marker and is also expressed in CVG neurons (Whitlon et al., 2010; Whitlon et al., 2009). P75 appears to colocalize with reporter cells in the CVG of Wnt1-Cre:RCEGFP/+ mice (Fig. 6D), although some of this lineage may migrate from the mesoderm.

Next, we examined the expression of markers for other specialized neurosensory cell types. Myosin VIIA (MyoVIIA; Myo7a – Mouse Genome Informatics) is an unconventional myosin that is strongly expressed in all hair cells within the inner ear (Bermingham et al., 1999). Colocalization of MyoVIIA with GFP in Pax3Cre/+ -labeled reporter cells can be seen by confocal microscopy of whole-mount preparations (Fig. 7A) as well as tissue sections (Fig. 7B). The organ of Corti includes a single row of inner hair cells (IHC) and three rows of outer hair cells (OHCs) that run the length of the cochlea. Based on whole-mount immunofluorescence for MyoVIIA (Fig. 7A), it appears that NEC derivatives preferentially differentiate into IHCs versus OHCs. However, the general pattern of NEC derivatives differentiating into hair cells seems to be random within the organ of Corti and the maculae. Supporting cells that express P75 and the Ca2+-binding protein S100 (White et al., 2006) and were found to be colocalized with Pax3Cre/+ -labeled reporter cells (Fig. 7C). Similar

![Fig. 6. NECs contribute neurons to the CVG.](image-url)
to what was observed for hair cells, it appears that the distribution of supporting cells derived from the \( \text{Pax3Cre/}^+ \) lineage is randomized within the maculae and organ of Corti. We estimate that \( \text{Pax3Cre/}^+ \) derivatives account for \( \sim 25\% \) of supporting cells and \( 20\% \) of hair cells within the macula (Fig. 7D).

It is currently thought that glial cells within the CVG are NCC derived (Britsch et al., 2001; Breuskin et al., 2010; D’Amico-Martel and Noden, 1983). We sought to confirm this using our fate-mapping approach. Sox10 is a high-mobility group-domain transcription factor that is expressed in NCCs and is necessary for the survival of CVG glia (Breuskin et al., 2010). However, Sox10 is also expressed throughout the OV (Breuskin et al., 2010). When we analyzed the expression of Sox10 protein in CVG nuclei, it was clear that all Sox10+ cells within the CVG were \( \text{Pax3Cre/}^+ \) derivatives (Fig. 8A). When comparing this with the otic placode ectoderm lineage labeled by \( \text{Pax2-Cre} \), we did not observe any GFP reporter cells that were also Sox10+ in the cochlear component of the CVG, but there were some cells with colocalization of Sox10 and GFP in the vestibular component (Fig. 8B).

**DISCUSSION**

In this study, we demonstrate for the first time that the mammalian inner ear originates from otic placode ectoderm and NECs, as determined by genetic labeling of neural tube populations by \( \text{Wnt1-Cre}, \text{Pax3Cre}^+ \) and \( \text{Hoxb1Cre}^+ \). This changes the current dogma – that only the otic placode ectoderm gives rise to the inner ear labyrinth and neurons of the CVG. Our results show that NEC lineages, which probably include NCCs, can invade the otic epithelium where they subsequently localize to the CVG, maculae and cochlea of the inner ear. Together with otic epithelium that originates from non-neural sensory placode ectoderm, cells that derive from the neural tube differentiate into neurons, hair cells and supporting cells. This is in addition to the known contributions of NCC lineages to: (1) the SV that surrounds the cochlea; and (2) glial cells of the CVG.

**Dual origin of the OV and CVG**

In vertebrate development, cranial sensory placodes are embryonic precursors of complex sensory organs (for a review, see Schlosser, 2010). NCCs are another embryonic population of cells that most probably co-evolved with sensory placodes in response to increased predatory behavior in vertebrates (Gans and Northcutt, 1983). It has been shown that NCCs cooperate with neurogenic (epibranchial and trigeminal) placodes for normal ganglia formation in mouse and chick (Begbie and Graham, 2001; Gammill et al., 2007; Shiau et al., 2008). In addition, neuromast formation in fish also requires cooperation between cells derived from the lateral line placode (present in fish only) and NCCs (Collazo et al., 1994; Grant et al., 2005).

Contribution of NCCs to other sensory organs is still being investigated. Only recently has it been shown by lineage tracing that glial ensheathing cells and subpopulations of neurons in the
The olfactory system originate from NCCs that were fate mapped using Wnt1-Cre (Barraud et al., 2010; Forni et al., 2011). We also observed Wnt1-Cre and Pax2CRE/loxp derivatives in the olfactory epithelium in the course of our studies (supplementary material Fig. S1). In the lateral line, it is speculated that NCCs give rise to glia associated with sensory neuromasts; however, a direct lineage has not been shown.

Mixed origins of the CVG have been demonstrated by quail-chick grafting experiments (D’Amico-Martel and Noden, 1983). Presumptive placode ectoderm or neural crest was grafted from quail into chick to determine the cellular origin of sensory ganglia. It was noted that many of these experiments demonstrated chimeric formation of the CVG; however, this was attributed to incomplete transplant of the otic placode as there was also a mixture of donor and host cells in other parts of the inner ear. In light of our findings, these early grafting results in chick may have represented a true mixture of placode and NECs in the CVG.

It has been debated whether or not a population of ventrally emigrating neural tube (VENT) cells exists that do not express the typical NCC marker HNK-1 (Dickinson et al., 2004). VENT cells have been proposed to migrate after NCCs and contribute to the OV and CVG, as well as to the cardiovascular system and enteric nervous system (Sohal et al., 2002; Ali et al., 2003a; Ali et al., 2003b; Sohal et al., 2002). Evidence for the existence of VENT cells has been challenged by some who question the accuracy of tracing techniques used to identify these cells in chick (Yaneza et al., 2002; Boot et al., 2003). The findings have not been further pursued until now.

Perhaps the most compelling evidence in the literature in support of our results is a study that was performed in zebrafish to dissect the functions of Sox10 in the inner ear (Dutton et al., 2009). Individual NCCs were labeled with rhodamine-dextran and a small percentage were traced to the otic epithelium. One cell was later found in a macula and another in the statoacoustic ganglion. These cells were deemed transient with no additional discussion. It is possible that only a small percentage of the NCCs were labeled, explaining a lower frequency than we have found using the Cre/loxP system in mice. When taken together, the data suggest that dual origin of the inner ear may extend beyond mammals to all vertebrates.

Fate-mapping techniques provide extensive information about cell lineages that cannot always be determined by gene expression analysis. Identification of NCCs by marker analysis is complicated by the changing expression profiles of migratory cells and by NCC-independent expression patterns during development. We were unable to identify NCCs in the OV based solely on expression analysis (supplementary material Fig. S2). Antibodies to Ap2α and the zinc-finger protein Snail1 exhibited staining in migratory NCC derivatives in the head mesenchyme but were not expressed by Pax3CRE/loxp-labeled derivatives in the otic cup (supplementary material Fig. S2A,B). Similarly, mRNA expression of cellular retinoic acid-binding protein 1 (Crabp1) was not detected in the otic cup by in situ hybridization (supplementary material Fig. S2C). The AP orientation of NECs in the OV at E9.5 suggests a multifaceted contribution of NECs to the OV and CVG that: (1) probably includes cellular contributions from more than one rhombomere; and (2) may or may not include cells that have been specified as NCCs.

One way to demonstrate the presence of a second cell type in the OV is to use a Pax2-specific antibody. The Pax2-Cre transgene is expressed early in the otic placode ectoderm, whereas endogenous Pax2 protein is expressed in the otic placode and otic cup (Ohyama and Groves, 2004). At slightly later stages of OV development, endogenous Pax2 is localized ventrally where it is required for cochlear outgrowth (Burton et al., 2004). Unfortunately, Pax2 protein shows a dynamic expression pattern in the OV and, because of this, expression of Pax2 protein only partially overlaps with the Pax2-Cre fate map (supplementary material Fig. S2D). Vital dye-tracing experiments in chick have demonstrated significant mixing of cells fated to different tissues prior to specification and formation of the otic placode (Streit, 2002). Our results suggest that recruitment of cells from the neural tube occurs after otic placode formation. It is unclear whether or not NEC derivatives are also subject to otic specification signals.

**Neurosensor development of the inner ear**

Neurons and hair cells of the maculae derive from a common pool of neurosensory progenitors that express neurogenin 1 (Ngn1) (Raft et al., 2007). Ngn1-Cre derivatives are also localized to the CVG, maculae and GER of the cochlea, and are capable of giving rise to neurons, hair cells and supporting cells within the maculae (Raft et al., 2007). Results from our study show that localization and differentiation of NEC derivatives in the inner ear and CVG coincide with the known distribution of Ngn1-Cre fate-mapped cells (Raft et al., 2007). The cristae are sensory epithelia that are defined by expression of the morphogen Bmp4, which is essential for SCC formation (Matei et al., 2005; Chang et al., 2008). Neither the NEC lineages (this study) nor the Ngn1-Cre lineage (Raft et al., 2007) were found in the cristae or SCCs, indicating that this domain may be subject to regulatory mechanisms that are independent of those in the maculae or organ of Corti.

In the mature inner ear, NEC derivatives differentiate into neurons, hair cells and supporting cells; however, there was no single neurosensor cell type that derived exclusively from the neural tube lineages. Therefore, cellular fate within the neurosensor region of the inner ear appears to be independent of cellular origin. This indicates that NEC derivatives in the inner ear...
may not function in cell fate specification, but instead may play a role in spatial organization of proneurosensoryst regions early during development. Alternatively, the contribution of neural tube cells to the proneurosensoryst region of the otic epithelium may be important for establishing proper communication and connectivity between the inner ear and hindbrain.

**Possible functional requirements for neuroepithelial cells in the inner ear**

The best way to test the functional requirements of NCCs in inner ear development would be to inhibit the contribution of NECs to the OV. Pax3\textsuperscript{Cre/Cre} mice are functionally null but do not have defects in NCC migration (Serbedzija and McMahon, 1997). Nonetheless, Splotch (Sp\textsuperscript{3H}/Sp\textsuperscript{3H}) mice that are homozygous null for Pax3 have been reported to have inner ear morphological defects and neuronal degeneration with phenotypic variability that corresponded with the severity of neural tube defects (Deol, 1966; Buckiova and Syka, 2004). We confirmed these findings by piecing of the inner ear labyrinth in Pax3\textsuperscript{Cre/Cre}-null mice (supplementary material Fig. S3). The hindbrain is essential for patterning the OV cell non-autonomously via secretion of morphogens such as Sonic hedgehog and Wnt (Vazquez-Echeverria et al., 2008; Riccomagno et al., 2002; Riccomagno et al., 2005; Liang et al., 2010). Therefore, analysis and phenotypic interpretation of Pax3-null mutants is complicated by the inability to isolate the requirements for direct NEC contribution versus cell non-autonomous signaling to the OV.

We did not attempt to ablate NCCs by Wnt1 loss-of-function because Wnt1-null mice do not have defects in neural crest-derived tissues, probably owing to compensation by other Wnt genes in the neuroectoderm (McMahon and Bradley, 1990). Direct requirements of NCCs in inner ear development could not be tested within the scope of this study and will require alternative approaches, such as conditional cell ablation, to bypass defects in signaling from the hindbrain.

**Origin of CVG glia**

Glia of the CVG are known to be of NCC origin based on early chick-quaill chimera studies (Britsch et al., 2001; Breuskin et al., 2010; D’Amico-Martel and Noden, 1983). Degeneration of glial cells in the CVG occurs in the absence of Sox10 (Breuskin et al., 2010). However, fate-mapping techniques have not been used in mice order to confirm the embryonic origin of these glia. Our results confirm that Sox10-expressing cells in the CVG are NCC derived, but that some cells from the otic placode ectoderm lineage also express Sox10 in the CVG. Our findings emphasize the need for further investigation of glial cell specification and development with respect to the inner ear.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**References**


