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Lineage tracing of Sox2-expressing progenitor cells in the mouse inner ear reveals a broad contribution to non-sensory tissues and insights into the origin of the organ of Corti

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ABSTRACT

The transcription factor Sox2 is both necessary and sufficient for the generation of sensory regions of the inner ear. It regulates expression of the Notch ligand Jag1 in prosensory progenitors, which signal to neighboring cells to up-regulate Sox2 and sustain prosensory identity. However, the expression pattern of Sox2 in the early inner ear is very broad, suggesting that Sox2-expressing progenitors form a wide variety of cell types in addition to generating the sensory regions of the ear. We used *Sox2-CreER* mice to follow the fates of Sox2-expressing cells at different stages in ear development. We find that Sox2-expressing cells in the early otocyst give rise to large numbers of non-sensory structures throughout the inner ear, and that Sox2 only becomes a truly prosensory marker at embryonic day (E)11.5. Our fate map reveals the organ of Corti derives from a central domain on the medial side of the otocyst and shows that a significant amount of the organ of Corti derives from a Sox2-negative population in this region.

1. Introduction

The perception of sound and linear and angular acceleration are mediated by dedicated sensory organs in the inner ear. The specialized array of hair cells and supporting cells in each sensory organ derives from prosensory patches that are induced shortly after formation of the otocyst, and which express the transcription factor Sox2 and the Notch ligand Jag1 (Adam et al., 1998; Eddison et al., 2000; Kiernan, 2013, 2005; Lewis, 1998; Morrison et al., 1999; Morsli et al., 1998; Neves et al., 2007; Ohyama et al., 2010). Gain- and loss-of-function studies support a model in which Jag1 signals through Notch receptors to activate Sox2 expression in adjacent prosensory cells. Consequently, mutation of *Sox2* or *Jag1* either abolishes or greatly reduces the size of inner ear sensory patches and their derived sensory organs (Brooker et al., 2006; Kiernan et al., 2001, 2005, 2006). Ectopic expression of *Sox2*, *Jag1*, or activation of the canonical Notch signaling pathway is sufficient to induce sensory patches in non-sensory parts of the inner ear (Hartman et al., 2010; Neves et al., 2011, 2012; Pan et al., 2013, 2010).

Several lines of evidence suggest Jag1-Notch signaling serves to *maintain* prosensory patch identity, but may not be necessary to *initiate* it. *Jag1* expression in the otocyst is initially regulated by canonical Wnt signaling (Jayasena et al., 2008), and the presence of Wnt-responsive elements in the *Jag1* promoter suggests this regulation is direct (Estrach et al., 2006). Moreover, both Jag1 and Sox2 are initially expressed in broad domains in the developing otocyst, extending well beyond the regions that will ultimately form sensory patches (Jayasena et al., 2008; Mak et al., 2009; Morrison et al., 1999; Neves et al., 2007). These domains later refine to the prosensory patches, and it is likely that Notch signaling acts at these times to stabilize sensory patch identity (Neves et al., 2013a; Raft and Groves, 2015).

To understand the decisions that govern the production of sensory versus non-sensory epithelium in the inner ear, we used *Sox2-CreER* mice (Arnold et al., 2011) to follow the fate of Sox2-expressing cells between embryonic days 8–12. We find that most non-sensory regions of the inner ear derive from Sox2-expressing progenitors at early stages. Surprisingly, significant regions of the organ of Corti are not generated from Sox2-expressing regions of the inner ear at these stages. Rather, we show that the apical-basal axis of the organ of Corti can be mapped onto the interface of a $Sox2^{+}$ and $Sox2^{-}$ region in the ventromedial face of the otocyst. Our data suggest that distinct sets of

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signals are necessary to restrict the initially broad patterns of Sox2 and Jag1 to prosensory patches, and then to maintain the identity of these patches as the sensory organs of the ear differentiate.

2. Materials and methods

2.1. Experimental animals

Sox2-CreER^{T2} knock-in mice (MGI: *Sox2*$^{tm1(Cre/ERT2)Hoch}$; Arnold et al., 2011) were provided by Dr. Konrad Hochedlinger. These mice are now available from the Jackson Laboratory (stock number 017593). *Ai3* Cre reporter mice (MGI: Gt(ROSA)26Sor$^{tm3(CAG-EYFP)Hze}$; Madisen et al., 2010) were purchased from the Jackson Laboratory, (stock number 007903). Genotyping was performed by PCR using the following primers: *Sox2-CreER*^{T2}: Cre1F (GCC TGC ATT ACC GGT CGA TGC AAC GA) and Cre1R (GTG GCA GAT GGC GCG GCA ACA CCA TT) yield a 700 bp band. *Ai3*: P020 (AAG GGA GCT GCA GTG GAG TA), P021 (CCG AAA ATC TGT GGG AAG TC), P102 (ACA TGG TCC TGC TGG AGT TC) P103 (GGC ATT AAA GCA GCG TAT CC), yielding a 297 bp wild type band and a 212 bp transgene band.

2.2. Fate mapping of Sox2 progenitors

For fate mapping experiments, heterozygous male *Sox2-CreER*^{T2} mice were mated with homozygous *Ai3* Cre reporter female mice and the presence of a vaginal plug the following morning was used to designate the day of pregnancy. A single dose of 2.5 mg tamoxifen and 2.5 mg progesterone (0.1 ml; dissolved in peanut oil at a concentration of 25 mg/ml each) was administered to pregnant females by oral gavage at embryonic day (E)8.5, E9.5, E10.5, E11.5 or E12.5. The genotypes of embryos or newborn pups from offspring of *Sox2-Cre; Ai3* crosses were determined by the presence of EYFP fluorescence and confirmed by PCR as described above. In all cases, 4 samples were analyzed with each marker for each age of tamoxifen administration. All animal experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use committee.

2.3. Tissue preparation

Inner ears were collected for analysis at either E18.5 or the day of birth. After fixing with 4% paraformaldehyde for 2 h at room temperature, the inner ears were kept in 30% sucrose in PBS for cryoprotection overnight at 4 °C, then embedded in OCT and frozen. Tissue was sectioned at 8 μm and sections collected as 3 serial sets. For vestibular organs, the utricle, saccule and the three ampullae were isolated separately and embedded for serial cryosectioning at 8 μm. For whole mount preparations of the cochlea, the membranous cochlea duct was dissected and the lateral wall tissue, including the stria vascularis and spiral ligament, was trimmed away after immunostaining prior to mounting and photographing.

2.4. Immunohistochemistry

The primary antibodies used in this study were: anti-GFP (1:1000, chicken, Abcam), anti-Myosin 7a (1:200, rabbit, Proteus), anti-Sox10 (1:250, goat, Santa Cruz Biotechnology), anti-Jag1 (1:75, rabbit, Santa Cruz Biotechnology), anti-NeuN (1:500, mouse, Millipore), anti-GFAP (1:500, rabbit, Dako Z0334) and anti-Cre (1:150, mouse, Millipore).

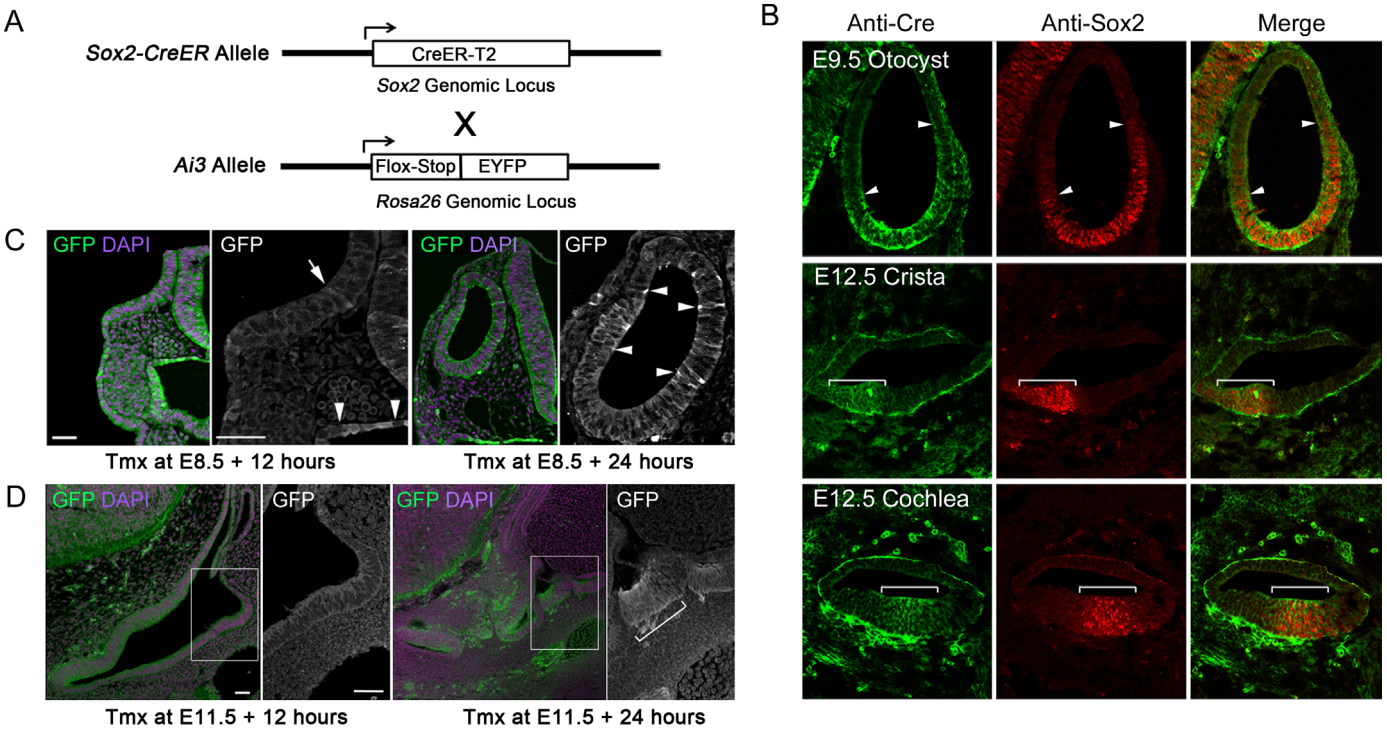

Fig. 1. (A) Diagram of the *Sox2-CreER* knock-in allele (Arnold et al., 2011). Heterozygous *Sox2-CreER* male mice were mated with homozygous Ai3 reporter female mice (Madisen et al., 2010). (B) Sections of E9.5 or E12.5 inner ears from *Sox2-CreER* mice, stained with antibodies to Cre and Sox2 protein. The co-localization of both proteins is shown with arrowheads and brackets in the otocyst (E9.5) and anterior crista and cochlea (E12.5). Note that the Cre protein is localized in the cytoplasm, as animals were not given tamoxifen. (C) Monitoring the speed of recombination in *Sox2-CreER; Ai3 ROSA* reporter mice. After a single dose of tamoxifen at E8.5, embryos were harvested at either 12 or 24 h and processed for immunostaining with EYFP antibodies (green) and DAPI to label nuclei (magenta). After 12 h, strong EYFP labeling can be seen in the neural tube, pharyngeal endoderm and epidermis ventral to the otic placode. A higher power image with brightly labeled EYFP cells in pharyngeal endoderm (arrowheads) shows a single bright EYFP cell in the invaginating otic placode (arrow). After 24 h, more brightly labeled EYFP cells can be seen in the otocyst (arrowheads in higher power image) as well as pharyngeal endoderm and the neural tube. (D) We observed a similar delay in EYFP expression when tamoxifen was applied at E11.5 and embryos examined 12 or 24 h later. After 12 h, labeling in ear tissue is very weak (box shows a higher power view of the developing utricular macula), but after 12 h, strong EYFP fluorescence is seen in both the cochlear primordium (c) and the developing utricle (box and higher power view; brackets). Scale bars = 50 μm.