Formation of the mouse cochlea: roles of Sonic hedgehog

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The formation of the mammalian cochlea is dependent on signalling from its surrounding tissues during embryogenesis. Using genetically engineered mutant mice and surgically manipulated chicken embryos, we demonstrated that Sonic hedgehog (Shh) secreted from the developing notochord and the floor plate is important for specification of ventral inner-ear structures that include the cochlear duct. Additionally, tissue-specific knockout of Shh in the developing spiral ganglion indicates that this source of Shh is required for mediating growth of the cochlear duct, timing of terminal mitosis of hair cell precursors, and subsequent differentiation of nascent hair cells along the cochlear duct.

INNER-EAR FORMATION

In the mouse, inner-ear formation initiates at embryonic day 8.5 (E8.5) from a region of the ectoderm next to the developing hindbrain. This specialized epithelial region, namely the otic placode, deepens to form a cup that separates from the rest of the ectoderm by pinching off to form a cyst (Fig. 1). Starting at the otic cup stage, some epithelial cells leave the antero-ventral region (defined as the neural-sensory competent domain) of the cup and these neuroblasts coalesce to form the cochleo-vestibular ganglion (CVG, VIII cranial ganglion). This neural delamination process in the mouse continues until at least E11.5, well after the otocyst is formed (Koundakjian et al., 2007; Raft et al., 2007). The neuroblasts within the CVG subsequently develop into bipolar neurons that send processes to innervate the sensory hair cells within the inner ear as well as nuclei in the brainstem and cerebellum.

Concomitant with the neuronal development, the otocyst undergoes a series of complex morphogenetic processes starting at E9.5 and reaching its adult pattern at approximately E16.5 (Fig. 2; Morsli et al., 1998). The first structure that emerges from the rudimentary cyst is the endolymphatic duct, which subsequently develops into the endolymphatic sac and duct. This structure is essential for maintaining fluid homeostasis of the endolymph within the membranous labyrinth. Without a functional endolymphatic system for maintaining a proper balance of high potassium and low sodium ions in the endolymph, mechanotransduction of sensory hair cells will fail.

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The dorsal region of the otocyst proper develops into the vestibule consisting of three orthogonally arranged semicircular canals and the associated ampullae that are responsible for detecting angular acceleration. Additionally, the two macular organs, the utricle and saccule, develop within the mid-region of the otocyst and are important for detecting linear acceleration. The most ventral region of the otocyst develops into the auditory apparatus, the cochlear duct, which starts out in a postero-lateral position of the inner ear and then extends in a ventral-medial and anterior direction before coiling laterally to complete its 1.75 turns. While these series of morphogenetic events sculpture the otocyst into a labyrinth, they also coordinate the gradual separation of the neural-sensory competent domain into various sensory patches: one auditory and five vestibular sensory organs of the mouse.

**MOLECULAR MECHANISMS REGULATING INNER-EAR DEVELOPMENT**

What are the molecular mechanisms that underlie the formation of this intricate sensory organ? Similar to other organs of the body, the ear primordium first acquires its axial information from the surrounding tissues. Studies from chicken and mouse embryos suggest that the signals that confer this axial information may be quite conserved (Riccomagno et al., 2002; Bok et al., 2005; Riccomagno et al., 2005; Bok et al., 2011). For example, retinoic acid secreted by the somites caudal to the developing inner ear is important for patterning the anterior and posterior axes of the
inner ear (Bok et al., 2011). In the chicken, this posterior retinoic acid (RA) signal is opposed by the RA degradation enzyme, Cyp26c1, expressed in the ectoderm anterior to the ear primordium. As a result, the ear primordium receives a gradient of RA signalling: the anterior otic region that receives low levels of RA signalling develops into the neural-sensory competent domain. In contrast, the posterior otic region, closer to the source of RA emanating from the somites, receives higher levels of RA and develops into largely non-sensory structures (Bok et al., 2011).

**Fig. 2:** Development of the inner ear from the otocyst stage to E17. Various stages of the developing mouse inner ear were injected with a paint solution in methyl salicylate. Abbreviations: aa, anterior ampulla; asc, anterior semicircular canal; co, cochlear duct; csd, cochlear-saccular duct; endolymphatic duct; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle; usd, utricular-saccular duct. Adapted from Morsli et al. (1998).

The establishment of dorsal-ventral axis of the inner ear is dependent on secreted molecules from the developing hindbrain. For example, Wnts from the dorsal hindbrain are important for inner-ear development (Riccomagno et al., 2005). On the other hand, Shh, secreted by the floor plate and notochord, is important for patterning ventral structures of the inner ear such as the cochlear duct and saccule (Riccomagno et al., 2002). Thus, the developing hindbrain appears to be a key tissue in providing instructive signals for patterning the dorsal-ventral axis of the inner ear.

Does the inner ear develop autonomously once it acquires its axial identity? Studies on extirpating mouse or chicken inner ears at various developmental stages to a heterologous environment indicate that the three primary cell fates – neural, sensory and non-sensory – are established early during development (Li et al., 1978; Swanson et al., 1990). However, proper morphogenesis requires continuous instructions from the surrounding tissues and extirpated ears do not recapitulate well the anatomy of inner ears at equivalent stages in vivo. Thus, identifying the key molecules alone is insufficient to fully understand the molecular mechanisms underlying inner-ear formation. It is equally important to know when and where
these key signalling molecules are required, especially since many of them are used repeatedly throughout inner ear development.

**ROLES OF SONIC HEDGEHOG IN COCHLEAR FORMATION**

**Patterning of the cochlear duct**

Many tissues such as the hindbrain, neural crest, mesenchyme, and spiral ganglion are important for the proper formation of the cochlear duct (Wu and Kelley, 2012; Bok et al., 2013). Whether all of these tissues participate in conferring axial information to the cochlear anlage will require further investigation. Although it is clear that the cochlear duct is a ventral structure and will require ventral signalling, it is difficult to discern based on the location of the mature cochlea whether it is derived from the anterior or posterior compartment of the ear primordium, or both (Fig. 2). Chicken inner ears that received posteriorizing signals from both anterior and posterior sides of the ear rudiment developed a mirror duplication of the posterior half of the inner ear (Bok et al., 2011). In these ears, a shortened cochlear duct (basilar papilla) without sensory tissue was found. Based on these results, we postulate that the cochlear duct is comprised of both anterior and posterior components: The organ of Corti, being part of the neural-sensory competent domain, belongs to the anterior compartment of the otic cup, whereas the rest of the cochlea, the non-sensory component, is derived from the posterior compartment. Thus, in chicken ears with duplicated posterior halves, the cochlear duct is comprised of non-sensory tissues only.

In chicken embryos, ablation or anterior-posterior inversion of a segment of the hindbrain adjacent to the developing inner ear affects the formation of the basilar papilla more readily than the vestibule, suggesting that cochlear formation is more sensitive to local signalling from the hindbrain (Liang et al., 2010). While a majority of the cochlear phenotypes in the hindbrain manipulated embryos is attributed to the loss of Shh signalling (see below; Bok et al., 2005), additional undetermined signal(s) are thought to be important for mediating the shape of the cochlear duct (Liang et al., 2010). Additionally, mesenchyme surrounding the ear primordium also plays an important role in the proper coiling of the cochlear duct. Several genes expressed in the mesenchyme such as Pou3f4 and Tbx1 have been shown to affect the length and shape of the cochlear duct when deleted from the genome (Phippard et al., 1999; Braunstein et al., 2008).

**Sonic hedgehog**

*Shh*, one of the three vertebrate homologs of the *Drosophila hedgehog* gene, is by far the most important vertebrate Hedgehog gene during embryogenesis. It encodes a secreted molecule that is involved in formation of various organs such as the neural tube, retina, limbs, and gut (Ingham and McMahon, 2001). Shh functions by binding to the cell membrane receptor Patched, which allows the transducer of Shh signalling, Smoothened (also a trans-membrane protein), to be activated. A major role of Shh is to regulate the levels of the transcription factor Gli3 in target cells...
The role of Shh is to regulate the levels of the transcription factor Gli3 in target cells. Signalling, Smoothened (also a trans-membrane protein), is activated by binding to the cell membrane receptor Patched, which allows the transduction of Shh, a secreted molecule that is involved in the formation of various organs such as the far the most important vertebrate, one of the three vertebrate homologs of the Shh, Sonic hedgehog (Liang et al., 1999; Braunstein et al., 2013). Whether all of these tissues participate in conferring axial information to the cochlear anlage will require further investigation. Although it is repeatedly throughout inner ear development, these key signalling molecules are required, especially since many of them are used in paraxial structures such as the somites (Borycki et al., 2000). The first indication that Shh from the midline is also important for patterning the inner ear stemmed from analyses of inner ears in Shh-/- mouse mutants (Riccomagno et al., 2002). No ventral inner-ear structures are evident in these mouse embryos (Fig. 3A,B). To address the specific contribution of Shh secreted from the ventral midline in mediating the inner-ear phenotypes, we surgically ablated a segment of ventral neural tube and notochord near the developing chicken otic cup in ovo. As a result, the inner ear shows a normal vestibule but lacks the basilar papilla and saccule (Fig. 3C,D; Bok et al., 2005). Remarkably similar phenotypes were also obtained when cells secreting antibodies blocking Shh bioactivities were implanted beside the ventral midline, suggesting that Shh is the main effector of ventral inner-ear patterning from the midline (Bok et al., 2005). Taken together these results from chicken and mouse indicate that Shh secreted from the notochord and floor plate has a conserved role in specifying the ventral axis of the inner ear.

**Contribution of Sonic hedgehog from the midline**

Shh secreted by the floor plate and notochord in the midline is important for establishing dorsal-ventral patterning of the neural tube as well as for patterning paraxial structures such as the somites (Borycki et al., 2000). The first indication that Shh from the midline is also important for patterning the inner ear stemmed from analyses of inner ears in Shh-/- mouse mutants (Riccomagno et al., 2002). No ventral inner-ear structures are evident in these mouse embryos (Fig. 3A,B). To address the specific contribution of Shh secreted from the ventral midline in mediating the inner-ear phenotypes, we surgically ablated a segment of ventral neural tube and notochord near the developing chicken otic cup in ovo. As a result, the inner ear shows a normal vestibule but lacks the basilar papilla and saccule (Fig. 3C,D; Bok et al., 2005). Remarkably similar phenotypes were also obtained when cells secreting antibodies blocking Shh bioactivities were implanted beside the ventral midline, suggesting that Shh is the main effector of ventral inner-ear patterning from the midline (Bok et al., 2005). Taken together these results from chicken and mouse indicate that Shh secreted from the notochord and floor plate has a conserved role in specifying the ventral axis of the inner ear.

**Fig. 3:** Paint-filled mouse inner ears of (A) wildtype and (B) Shh null mutants at E15.5 as well as E7 chicken inner ears of (C) controls and (D) those with a segment of the notochord and floor plate beside the developing inner ear removed at E1.5 (midline removal, MR). This surgical operation affects cochlear development and results in an inner ear that resembles the Shh-/- mouse mutants. Abbreviations: bp, basilar papilla. Adapted from Riccomagno et al. (2002); Bok et al. (2005).
Since a major role of Shh is to remove the repressor function of Gli3 in other systems (Litingtung et al., 2002; te Welscher et al., 2002), we investigated the inner ear in Shh and Gli3 double mutants. Gli3 knockout ears are largely normal and only the lateral canal is absent (Bok et al., 2007). If one of Shh’s major roles in the inner ear is to remove the repressor function of Gli3, one would expect the inner ear phenotypes in Shh<sup>-/-</sup>; Gli3<sup>-/-</sup> double mutants to be milder than that of Shh<sup>-/-</sup> alone. This is indeed the case. In Shh<sup>-/-</sup>; Gli3<sup>-/-</sup> double mutant ears, the saccule and a shortened cochlear duct are present (Fig. 4B; Bok et al., 2007) as opposed to the lack of all ventral structures in the Shh<sup>-/-</sup> mutants (Fig. 3B). The shortened cochlear duct in Shh<sup>-/-</sup>; Gli3<sup>-/-</sup> double mutants also lacks Msx1 expression (Fig. 4E), which is a marker for the apical region of the cochlear duct (Fig. 4D). This indicates that the duct is truncated, not simply smaller. While the absence of Gli3 alleviated some of the phenotypes observed in Shh null mutants (presumably due to the loss of Gli3 repressor functions), the persistence of the missing apical region in the Shh<sup>-/-</sup>; Gli3<sup>-/-</sup> double mutants suggests that the apical region of the cochlea requires the activator function of Gli3.

The notion that the apical cochlea requires higher levels of Shh signalling relative to the rest of the cochlea is also supported by the inner-ear phenotypes of A699/A699 mouse mutants, modelled after mutations observed in Pallister-Hall syndrome in humans. Both the mouse and human mutations resulted in a truncated Gli3 protein that has only repressor but no activator activity (Kang et al., 1997; Bose et al., 2002). In A699/A699 mutants, the cochlear duct is shortened and is missing Msx1 expression (Fig. 4C,F). Thus, this mutant cochlea is presumably truncated and missing the apical region. The apical cochlear phenotypes in A699/A699 mouse mutants are consistent with patients with Pallister-Hall syndrome showing a prevalence of low-frequency hearing loss (Driver et al., 2008).

**Contribution of Sonic hedgehog from the spiral ganglion**

*Spiral ganglion Sonic hedgehog mediates growth of the cochlear duct:*

In addition to the source of Shh from the ventral midline, Shh is also expressed in the developing spiral ganglion, first detectable at E11.75 (Bok et al., 2013). What is the role of Shh secreted by the spiral ganglion? This question was addressed by generating tissue-specific knockout of Shh using the cre-lox approach. Three cre strains were used in the study: Neurogenin1<sub>cre</sub> (Ngn1<sup>cre</sup>), Neurogenin1<sub>creER</sub> (Ngn1<sup>creER</sup>), and Foxg1<sub>cre</sub> (Bok et al., 2013). All three promoters driving cre in these strains are active in the developing spiral ganglion and not in the floor plate and notochord (Hebert and McConnell, 2000; Koundakjian et al., 2007; Quinones et al., 2010). In the Ngn1<sub>creER</sub> strain, the cre is fused to a mutated form of the estrogen receptor (ER), which provides a temporal control of cre activation pending tamoxifen administration (Hayashi and McMahon, 2002). The conditional knockout of Shh using each of the three cre strains generated inner ears with a shortened cochlear duct, the shortest being the Foxg1<sub>cre</sub>; Shh<sub>lox/-</sub> mutants (Fig. 5). The length of the cochlear duct in the Ngn1<sub>creER</sub>; Shh<sub>lox/-</sub> ears is also dependent on the timing of tamoxifen administration such that earlier administration leads to a shorter cochlea.
The notion that the apical cochlea requires higher levels of Shh signalling relative to the rest of the cochlea is also supported by the inner-ear phenotypes of double mutants. The persistence of the missing apical region in the inner-ear phenotypes of Shh-/-; Gli3-/- mutant cochlea is presumably truncated and not simply smaller. While the absence of the lateral canal is truncated, not simply smaller. This is indeed the case. In Shh-/-; Gli3-/-; Msx1∆699/∆699, 2010). In the mouse mutants, modelled after mutations observed in Pallister-Hall syndrome showing a prevalence of low-frequency hearing loss (Driver et al., 2002). In contrast to the aforementioned Shh-/-; Gli3-/- and ∆699/∆699 mutant cochleae or a globally shortened duct? It is possible that specification of the apical cochlear duct requires higher or prolonged levels of Shh than other regions of the cochlear duct and that this extra Shh is supplied by the spiral ganglion acting in conjunction with the notochord and floor plate. Under such a scenario, the reduction of Shh signalling from the spiral ganglion in the Shh conditional mutants should affect apical cochlear development and abolish Msx1 expression. In contrast, our analyses indicate that Msx1 is expressed in the apical region of the shortened Foxg1cre; Shhlox/cochlea suggesting that this cochlear duct is only shortened and not truncated, unlike the Shh-/-; Gli3-/- and ∆699/∆699 cochleae. Taken together, these results suggest that Shh secreted by the spiral ganglion mediates only the growth of the cochlear duct, pre-patterned by Shh in the notochord and floor plate.
**Fig. 5:** The cochlear duct in the Shh conditional knockout mutant, Foxg1<sup>Cre</sup>; Shh<sup>lox/+</sup>, is globally shortened. The cochlear duct in Foxg1<sup>Cre</sup>; Shh<sup>lox/-</sup> ears is shortened (C) but Msx1 expression in the apex (D, arrow) is similar to controls (A, B). Adapted from Bok et al. (2013).

Spiral ganglion Sonic hedgehog mediates timing of cell cycle exit and hair-cell differentiation:

An unusual feature of hair-cell development in the organ of Corti is that hair-cell precursors exit from cell cycle in an apex-to-base direction along the cochlear duct, whereas hair-cell differentiation is initiated at the mid-basal region and progresses bi-directionally after terminal mitosis is completed (Ruben, 1967; Lee et al., 2006). Thus, hair cells at the basal region exit from cell cycle promptly after cell cycle exit whereas their counterparts in the apex delay the differentiation process for several days. Previous in vitro studies indicate that Shh inhibits cochlear hair-cell formation (Driver et al., 2008). Using a Shh reporter strain, it was shown that Shh expression in the spiral ganglion gets restricted towards the apical cochlear region over time (Liu et al., 2010). Thus it was postulated that this restriction of Shh expression in the apex might be regulating the basal to apical wave of hair cell differentiation in the organ of Corti (Liu et al., 2010).

We reasoned that if Shh in the spiral ganglion is inhibiting hair-cell differentiation after terminal mitosis, then the lack of Shh in the spiral ganglion should cause hair-cell differentiation to proceed in the same direction as cell cycle exit (progressing from apex to base), provided that Shh has no effect on cell cycle exit of hair-cell precursors. We first determined the timing of cell cycle exit in mutants by injecting a thymidine analog, EdU, at different developmental times and determined the percentages of labelled hair cells present at E18.5, when hair cells can be unequivocally identified (Bok et al., 2013). In principal, cells that undergo terminal mitosis shortly after EdU injection should retain the EdU and thus be heavily labelled, whereas cells that are post-mitotic or have undergone multiple rounds of cell division during this developmental period should not be labelled. The results...
from this cell-cycle-exit analysis indicate that hair-cell precursors exit from cell cycle prematurely in the Foxg1\textsuperscript{cre}; Shh\textsuperscript{lox/loox} cochlea but still in an apical to basal direction, similar to the wildtype (Bok \textit{et al.}, 2013). In contrast, immunostaining of nascent hair cells indicates that hair-cell differentiation proceeds in the reverse apex-to-base direction predicted by our hypothesis (Fig. 6; Bok \textit{et al.}, 2013). Together, these results indicate that Shh generated in the spiral ganglion promotes growth of the cochlear duct and proliferation of hair-cell precursors but inhibits hair-cell differentiation. Then, as Shh expression becomes restricted towards the apex of the cochlear duct, hair-cell differentiation is initiated starting at the basal cochlear region.

\textbf{CONCLUSIONS}

In summary, our results indicate that multiple sources of Shh are required for proper cochlear formation. Shh secreted by the notochord and floor plate is important for patterning the cochlear duct. At a slightly later time in development, Shh generated
in the spiral ganglion mediates the growth of the cochlear duct. The dynamic relationship between the growing cochlear duct and the location of Shh expressing cells in the spiral ganglion dictate the timing of cell cycle exit of hair-cell precursors as well as differentiation of nascent hair cells in the cochlea. Finally, there are good evidence that suggest most of the aforementioned Shh functions are mediated by Shh acting directly on otic epithelial cells (Brown and Epstein, 2011; Tateya et al., 2013).

REFERENCES


Are receptive fields fixed or fluid?

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Neural representations of sensory stimuli are affected by stimulus- and task-context. These effects can be long term, such as observed after intensive training or sensory deprivation, or short term, for instance when stimuli are repeated or attended. Long-term effects are generally associated with changes in neural receptive fields, such as expanded representation of, and increased selectivity for, learned features after training, or cortical remapping after hearing loss. In contrast, short-term context effects are usually explained in terms of either suppressive (e.g., repetition suppression) or facilitatory (e.g., attentional facilitation) gain control, without any change in neural coding parameters. More recent models, however, propose that short-term effects, such as repetition suppression or attention, act not only through gain control of neuron populations, but also change the receptive fields of individual neurons. In this view, receptive fields are considered not as fixed, but rather as fluid and instantly adaptable.

In this paper, new data are presented, based on non-invasive electrophysiological recordings in humans, which support the notion that short-term context effects cause rapid receptive-field plasticity.

INTRODUCTION

Neural receptive fields

The receptive field (RF) of a sensory neuron describes the selectivity with which that neuron responds to a particular stimulus feature. For example, the RF of an auditory neuron is characterised by the sound frequency that it is most responsive to, and by the steepness with which its responsiveness falls off with distance from this characteristic frequency (CF). This variation of responsiveness with frequency is also referred to as the RF ‘tuning’. For primary auditory neurons, the RF is determined by the mechanical frequency tuning of the cochlea, and the neurons particular location along the tonotopic cochlear axis. At more central stages of the auditory pathway, the neural RF is determined by the synaptic input circuitry to the neuron, which receives converging afferent input from multiple units from more peripheral layers. Despite this convergence, the tonotopic arrangement originating from the cochlea is maintained along the ascending auditory pathway all the way to the auditory cortex, where CF varies gradually across the cortical surface, giving rise to a topographic representation of frequency. Such topographic maps of stimulus