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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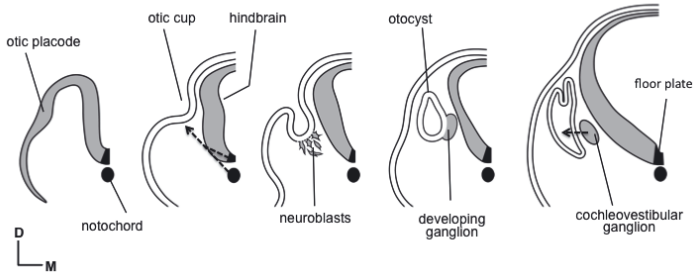

Fig. 1: Development of the inner ear from the otic placode to otocyst stage. Starting at the otic cup stage, neuroblasts delaminate from the antero-ventral region of the otic cup to form the cochleo-vestibular ganglion. Dotted arrows indicate the two sources of Shh that are important for cochlear development: floor plate and notochord required for specification of the cochlea at an early stage and the spiral ganglion required for growth of cochlear duct and timing of cell cycle exit and hair-cell differentiation at later stages. Orientations: D, dorsal; M, medial. Adapted from Chang *et al.* (2003).

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).

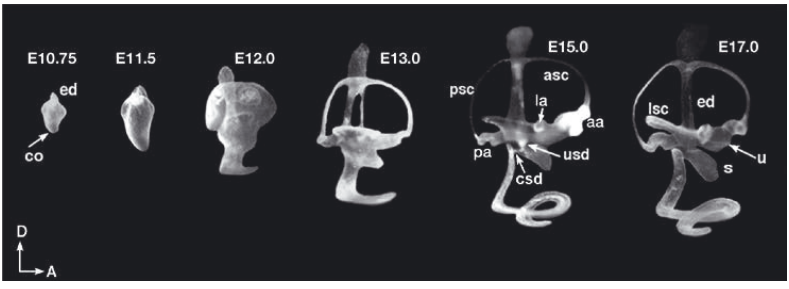

Fig. 2: Development of the inner ear from the otocyst stage to E17. Various stages of the developing mouse inner ear were injected with a paint solution in methyl salicylate. Abbreviations: aa, anterior ampulla; asc, anterior semicircular canal; co, cochlear duct; csd, cochlear-saccular duct; endolymphatic 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

(Litingtung *et al.*, 2002; te Welscher *et al.*, 2002). In the absence of Shh signalling, the Gli3 protein is cleaved and the N-terminus of the protein functions as a transcription repressor. In the presence of activated Smoothened, this enzymatic cleavage of Gli3 is inhibited and the full length of Gli3 protein functions as a transcription activator, which activates Shh target genes in the nucleus.

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.

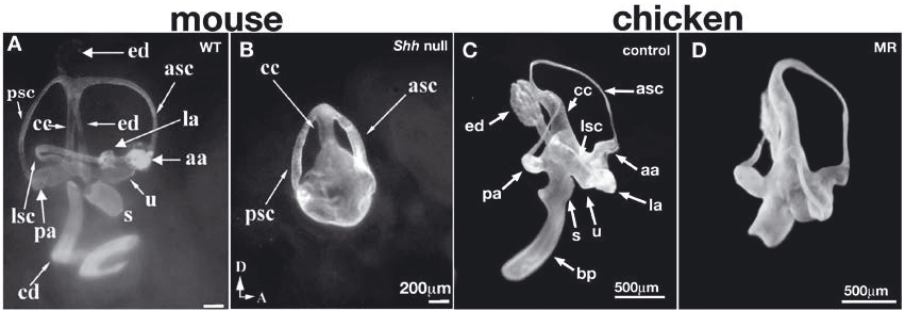

Fig. 3: Paint-filled mouse inner ears of (A) wildtype and (B) *Shh* null mutants at E15.5 as well as E7 chicken inner ears of (C) controls and (D) those with a segment of the notochord and floor plate beside the developing inner ear removed at E1.5 (midline removal, MR). This surgical operation affects 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 $Shh^{-/-}$; $Gli3^{-/-}$ double mutants to be milder than that of $Shh^{-/-}$ alone. This is indeed the case. In $Shh^{-/-}$; $Gli3^{-/-}$ 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 $Shh^{-/-}$ mutants (Fig. 3B). The shortened cochlear duct in $Shh^{-/-}$; $Gli3^{-/-}$ 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 $Shh^{-/-}$; $Gli3^{-/-}$ 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 Δ699/Δ699 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 Δ699/Δ699 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 Δ699/Δ699 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: $Neurogenin1^{cre}$ ($Ngn1^{cre}$), $Neurogenin1^{creER}$ ($Ngn1^{creER}$), and $Foxg1^{cre}$ (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 $Ngn1^{creER}$ 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 $Foxg1^{cre}$; $Shh^{lox/-}$ mutants (Fig. 5). The length of the cochlear duct in the $Ngn1^{creER}$; $Shh^{lox/-}$ ears is also dependent on the timing of tamoxifen administration such that earlier administration leads to a shorter cochlea.

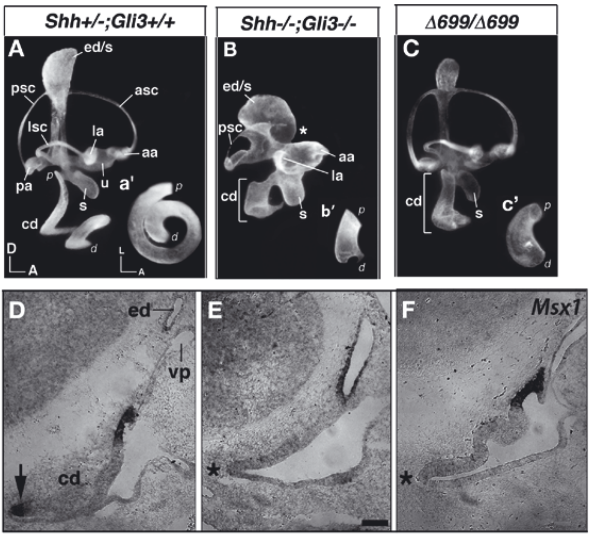

Fig. 4: Truncated cochlear ducts in *Shh*$^{-/-}$; *Gli3*$^{-/-}$ and *Δ*699/*Δ*699 mutants. (A-C) Paint-filled inner ears at E13.5 and (D-F) *Msx1* expression at E12.5 of *Shh*$^{+/-}$, *Gli3*$^{+/-}$ (A,D), *Shh*$^{-/-}$; *Gli3*$^{-/-}$ (B, E) and *Δ*699/*Δ*699 (C,F) mouse embryos. Cochlear ducts are shorter in *Shh*$^{-/-}$; *Gli3*$^{-/-}$ (B) and *Δ*699/*Δ*699 (C) ears than controls (A), and they are missing *Msx1* expression (E-F, asterisk), which marks the apical region of the cochlear duct (D, arrow). (a'-c') Ventral views of the cochlear duct shown in (A-C), respectively. Asterisk in (B) indicates the truncated anterior canal. Abbreviation: vp, vertical canal pouch. Adapted from Bok *et al.* (2007).

Does the shortened cochlear duct in these conditional mutants represent a truncation similar to the aforementioned *Shh*$^{-/-}$; *Gli3*$^{-/-}$ and *Δ*699/*Δ*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 *Foxg1*^{cre}; *Shh*$^{lox/-}$ cochlea suggesting that this cochlear duct is only shortened and not truncated, unlike the *Shh*$^{-/-}$; *Gli3*$^{-/-}$ and *Δ*699/*Δ*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.

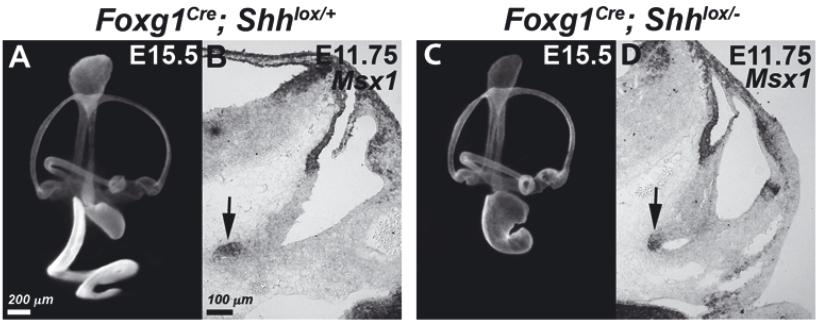

Fig. 5: The cochlear duct in the *Shh* conditional knockout mutant, *Foxg1*^{cre}*; Shh* $^{lox/-}$, is globally shortened. The cochlear duct in *Foxg1*^{cre}*; Shh* $^{lox/-}$ ears is shortened (C) but *Msx1* expression in the apex (D, arrow) is similar to controls (A, B). Adapted from Bok *et al.* (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*^{cre}*; Shh* $^{lox/-}$ cochlea but still in an apical to basal direction, similar to the wildtype (Bok *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 *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.

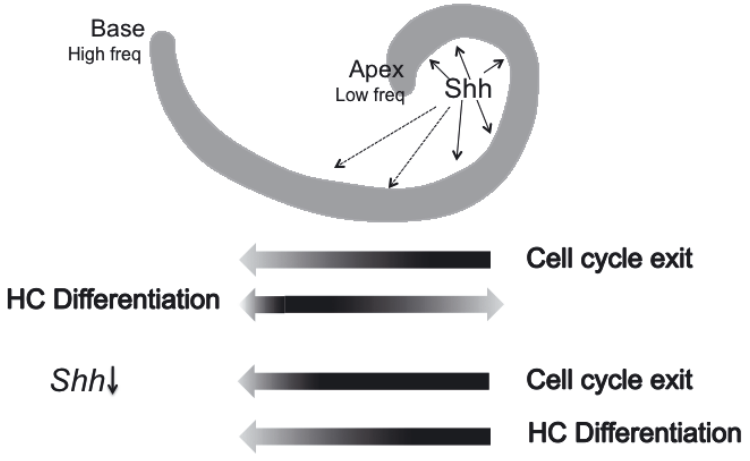

Fig. 6: Schematic summary of the role of Shh in regulating the timing of cell cycle exit and hair-cell differentiation in the mammalian cochlea. The mammalian cochlear duct is tonotopically organized such that hair cells at the base of the cochlea are tuned to high-frequency sound and hair cells at the apex to low-frequency sound. In the developing wildtype cochlea, hair-cell precursors exit from cell cycle in an apical to basal direction along the cochlear duct. Nevertheless, hair-cell differentiation is inhibited pending the restriction of Shh expression in the spiral ganglion towards the apical cochlear region (dotted arrows). Knocking out *Shh* expression in the spiral ganglion increases the timing of cell cycle exit and causes hair cells to differentiate promptly after cell cycle exit.

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).

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