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REVIEW

Development of the cochlea

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ABSTRACT

The cochlea, a coiled structure located in the ventral region of the inner ear, acts as the primary structure for the perception of sound. Along the length of the cochlear spiral is the organ of Corti, a highly derived and rigorously patterned sensory epithelium that acts to convert auditory stimuli into neural impulses. The development of the organ of Corti requires a series of inductive events that specify unique cellular characteristics and axial identities along its three major axes. Here, we review recent studies of the cellular and molecular processes regulating several aspects of cochlear development, such as axial patterning, cochlear outgrowth and cellular differentiation. We highlight how the precise coordination of multiple signaling pathways is required for the successful formation of a complete organ of Corti.

KEY WORDS: Atoh1, Deafness, Hearing, Notch, Organ of Corti, Radial intercalation

Introduction

The mammalian inner ear is a remarkable structure, made up of a labyrinth of elaborate ducts and canals (Fig. 1). Together, these facilitate the perception of mechanical stimuli arising from airborne (auditory) pressure waves, physical movement or gravitational forces. Transduction of auditory signals occurs in the cochlea, a coiled structure comprising the ventral half of the inner ear. The cochlea contains three ducts: the scala vestibuli, scala media and scala tympani. The scala vestibuli and scala tympani communicate through the helicotrema, an opening located at the extreme apex of the cochlea, whereas the scala media is a blind duct located between the other two scalae (Fig. 1). Incoming sounds induce fluid-based traveling waves that originate in the cochlear base and propagate towards the apex within the scala vestibuli. These traveling waves induce vibrations in the scala media at frequency-dependent positions along the spiral before generating descending waves that travel back towards the cochlear base in the scala tympani. The central scala media is a triangular structure with three unique walls. The first, Reissner's membrane, creates a barrier between the scala media and the adjacent scala vestibuli. The second wall, the stria vascularis, plays a key role in the generation and maintenance of the unique electrochemical environment within the scala media, which is required for auditory function. The final wall, typically termed the floor, contains the sensory epithelium – the organ of Corti – flanked by two non-sensory regions termed the inner and outer sulci.

The organ of Corti comprises two types of mechanosensory hair cells (inner and outer hair cells; IHCs and OHCs) and at least six types of associated non-sensory supporting cells (SCs), arranged in rows to form a highly ordered asymmetric mosaic extending along the basal-to-apical (tonotopic) axis of the cochlea (Fig. 1). Although the overall cellular pattern of the organ of Corti is invariant along the cochlea, graded differences in morphology and physiology reflect and mediate tonotopic changes in frequency sensitivity. Finally, the epithelium of the organ of Corti is pseudostratified, with hair cells (HCs) located in the lumenal half and SCs spanning from the basement membrane to the lumenal surface.

Cochlear HCs are the primary transducers of auditory stimuli. Each HC includes a mechanosensitive stereociliary bundle located on its lumenal surface. Although all stereociliary bundles include a single true cilium (termed a kinocilium), at least during development, stereocilia are actually modified actin-based microvilli. Each bundle contains 50-200 individual stereocilia arranged in rows to form a chevron shape, with the single kinocilium asymmetrically located at one edge of the bundle. The lengths of individual stereocilia vary by row; those in the row closest to the kinocilium are the tallest, whereas stereocilia in adjacent rows are progressively shorter, leading to the formation of a staircase pattern. All cochlear HC stereociliary bundles are uniformly oriented such that the tallest row of stereocilia is located on the lateral side of each cell. Sound-induced pressure waves deflect the bundles creating a shearing motion at the tips where filamentous links (tip links) connect individual stereocilia. Shearing leads to increased tension on tip links, which causes ion channel opening, HC depolarization and increased neurotransmitter release between HCs and auditory neurons.

Developmental biologists have long been aware of the basic processes that occur as the ventral region of the developing inner ear becomes the cochlear duct and the organ of Corti. In the last 20-25 years, however, molecular biological techniques and targeted mouse genetics have dramatically increased our understanding of the cellular and genetic processes that mediate the formation of this incredible structure. These studies have highlighted, for example, how the major axes of the cochlea are set up, and how cell fates and polarity are established along these axes. In this Review, we focus on recent discoveries examining different aspects of cochlear development, including patterning and outgrowth of the cochlear duct, cellular differentiation within the sensory epithelium, and HC formation and polarization. A central theme will be cellular and subcellular patterning, and how this patterning controls the precise assembly of the different structures that play crucial roles in auditory function.

Initial patterning of the inner ear and cochlear duct

In the mouse, and all other vertebrates, the majority of epithelial cells within the inner ear are derived from the otocyst, a fluid-filled cyst that develops through an invagination of surface ectoderm located adjacent to the developing hindbrain, beginning between embryonic day (E)8 and E9 in the mouse. Factors from surrounding tissues and structures rapidly define the dorsoventral (D-V), anterioposterior (A-P) and mediolateral (M-L) axes of this developing structure. D-V identity is specified through opposing gradients of Wnt and sonic hedgehog (Shh) originating from the dorsal hindbrain and floorplate/notochord, respectively (Bok et al., 2007; Brown and Epstein, 2011; Brown et al., 2015; Freter et al., 2008; Ohta and Schoenwolf, 2018; Riccomagno

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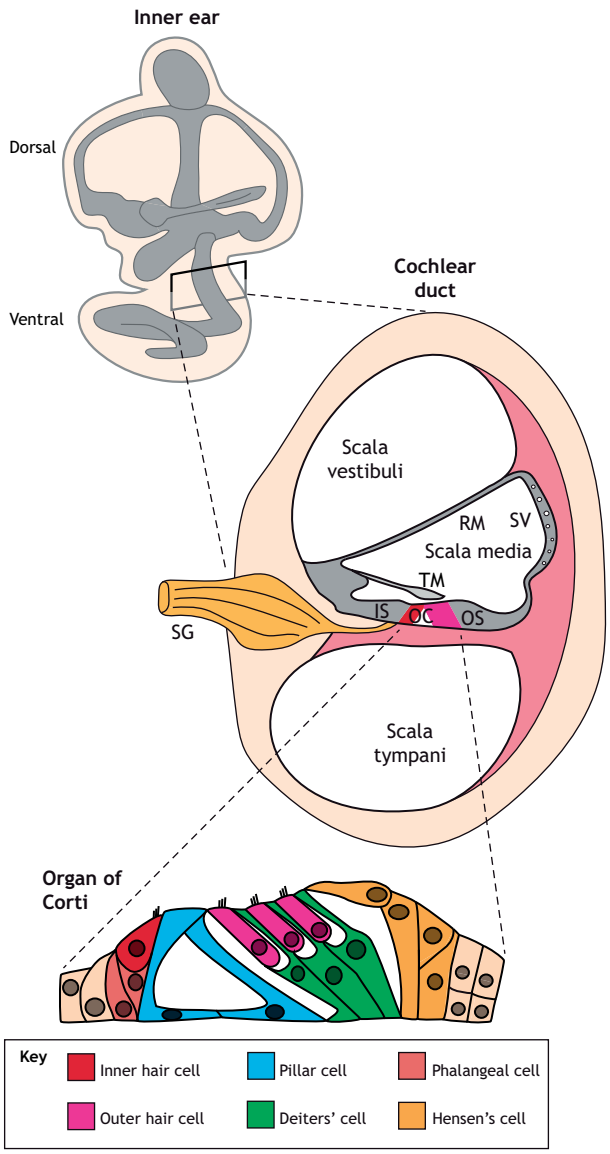

Fig. 1. The inner ear and cochlea. The bony and membranous labyrinth of the inner ear includes both dorsal/vestibular structures, related to perception of balance and motion, and the ventral cochlear duct, which transduces sound. The membranous labyrinth (gray) is composed of epithelial cells and is surrounded by the bony labyrinth (beige), which is derived via the condensation of periotic mesenchyme. The cochlear duct includes three canals (or 'scala'): the scala vestibuli, the scala media and the scala tympani. The scala media, part of the membranous labyrinth, is comprised of three walls: Reissner's membrane (RM), the stria vascularis and spiral ligament (SV), and the cochlear floor, which contains the sensory organ of Corti (OC) flanked by two regions of non-sensory cells, the inner sulcus (IS) and outer sulcus (OS). The organ of Corti contains two types of hair cells (inner hair cells and outer hair cells) and several different types of unique supporting cell types, including inner phalangeal cells adjacent to the inner hair cells, pillar cells separating the inner and outer hair cells, Deiters' cells interdigitated among outer hair cells, and Hensen's cells lateral to the organ of Corti. Hair cells and supporting cells are arranged in a precise cellular mosaic. Hair cells have characteristic stereocilia bundles on their lumenal surface, and the stereocilia of the outer hair cells are in contact with the tectorial membrane (TM) within the scala media. Bipolar neurons of the spiral ganglion (SG) synapse with hair cells and project centrally to the cochlear nucleus.

et al., 2002, 2005), whereas the A-P axis is regulated through a gradient of retinoic acid (Bok et al., 2011). Initial M-L axis specification is not as well understood but is regulated, at least in part, through a source of FGF3 in the hindbrain (Basch et al., 2016; Choo et al., 2006; Lin et al., 2005). Soon after the specification of the three primary axes, the otocyst begins an elaborate process of morphogenesis that converts a simple sphere into the complex structures of the inner ear. One of these processes, the emergence and outgrowth of the cochlear duct, beginning at around E11 in the mouse, is also dependent on Shh; however, whether this is a direct effect of Shh on outgrowth of the duct or a secondary result of changes in D-V patterning is difficult to determine.

The cochlea contains three ducts, however only one of these – the scala media – is derived from the otocyst. The overlying scala vestibuli and scala tympani arise through condensations of the periotic mesenchyme that also gives rise to the bony labyrinth that surrounds the membranous inner ear. As the cochlear duct develops into the scala media, the epithelial cells that line it initially develop as five different regions. The roof of the duct gives rise to both Reissner's membrane (medial half) and the stria vascularis (lateral half), whereas the floor is initially divided into three domains: a central region that contains the precursors of the organ of Corti (the prosensory region), a cellularly dense medial domain (Kölliker's organ) and a less dense lateral domain (the lesser epithelial ridge, LER). The inner and outer sulci are derived from Kölliker's organ and LER, respectively, which undergo significant remodeling before the onset of hearing. Anatomical and genetic evidence suggest that the domains discussed above are already specified to varying extents at the time of initial outgrowth (Groves and Fekete, 2012; Muthu et al., 2019) (Fig. 2). For example, the thickness of the epithelium is heterogeneous, with the floor containing a much higher density of cells by comparison with the roof. Further, within the epithelial cells that make up the floor, expression of Sox2, which is necessary for formation of the prosensory domain, is restricted to a band of cells in the medial half of the floor of the duct, corresponding to both Kölliker's organ and the prosensory domain (Dabdoub et al., 2008; Gu et al., 2016; Kiernan et al., 2005b; Steevens et al., 2019). Flanking regions, which develop into Reissner's membrane and the LER, express *Otx2* and *Bmp4*, respectively, whereas some cells in the lateral roof, which give rise to the stria vascularis, express *Lmo4* (Chang et al., 2008; Deng et al., 2014; Morsli et al., 1999; Ohyama et al., 2010; Vendrell et al., 2015). Additional genes, such as *Jag1*, *Lfng* and *Fgf10*, are expressed in overlapping or adjacent domains (Alsina et al., 2004; Morrison et al., 1999; Morsli et al., 1998; Ohyama et al., 2010; Urness et al., 2015; Zhang et al., 2000). Overall, these studies suggest that as the duct begins to extend at E11.0, it is already divided into five regions: the prospective Reissner's membrane, the stria vascularis, the LER, the prosensory domain and Kölliker's organ (Fig. 2).

Although the early cochlear duct is clearly divided into distinct regions, the overall degree of lineage restriction within any of these domains is less clear. *Bmp4*, which encodes a secreted morphogen, is restricted to the lateral third of the floor of the duct as early as E11.5 (Morsli et al., 1998). Elimination of three of the four alleles of the Bmp4 receptors *Alk3* (also known as *Bmpr1a*) and *Alk6* (*Bmpr1b*) leads to a lateral shift in the expression of genes that mark the medial (future Kölliker's organ) and central (the prosensory domain) thirds of the duct (Fig. 2), demonstrating plasticity between the three domains located along the floor of the duct, and suggesting that Bmp4 may create a gradient that specifies regional cell identities (Ohyama et al., 2010). Similarly, *Fgf10*, which encodes another secreted factor, is expressed in Kölliker's organ as early as E11.5 (Urness et al., 2015; Wright and Mansour, 2003). Considering the established antagonistic roles of Fgfs and Bmps in other systems, a role for interactions between these factors in medial-lateral patterning seems likely.

Development (2020) 147, dev162263. doi:10.1242/dev.162263

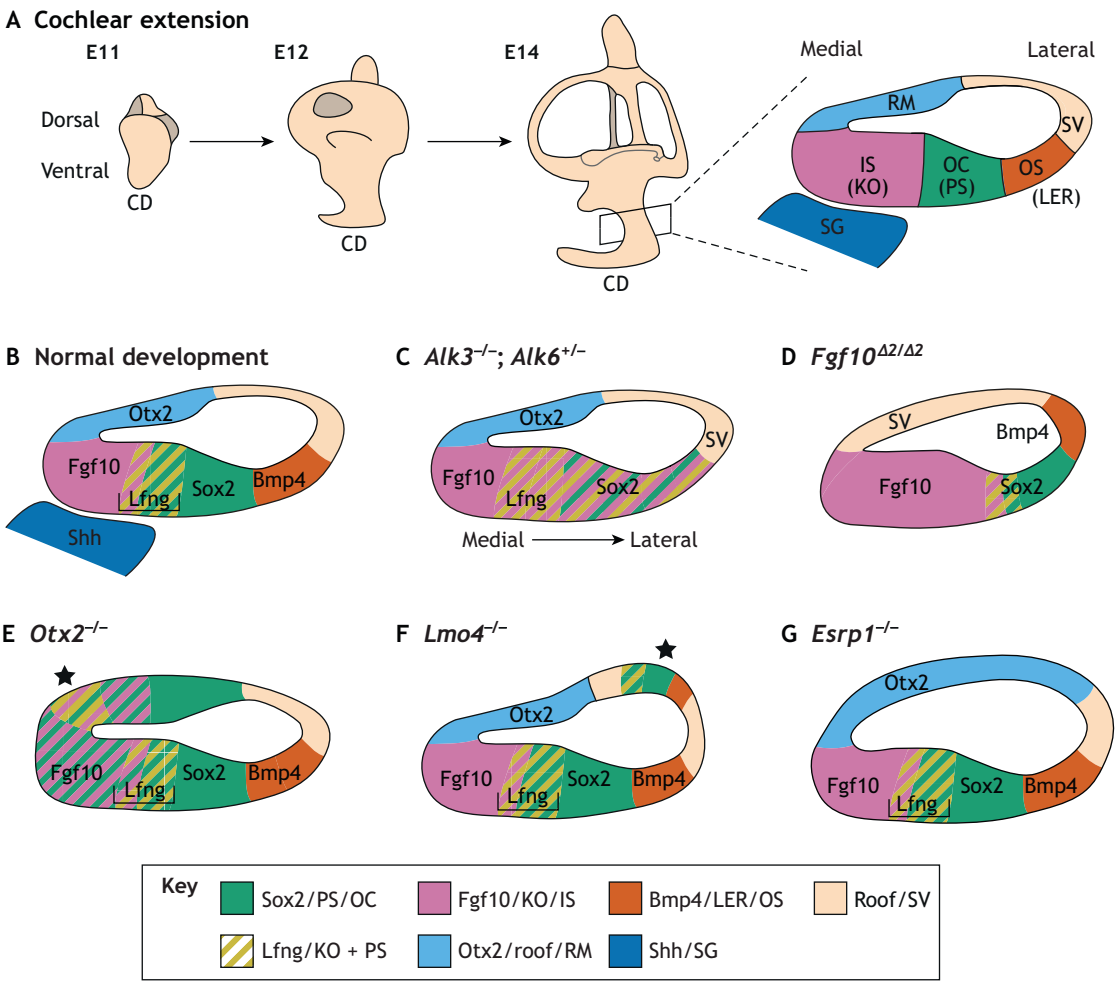

Fig. 2. Cochlear extension and patterning of the duct along the medial-to-lateral axis. (A) Diagram illustrating extension of the cochlear duct (CD) from the ventral region of the otocyst beginning at E11 through E14. A cross section through the cochlear duct at ~E14 (shown on right) highlights the mature structures that arise from each region, with the transient embryonic structures indicated parenthetically. (B) Examples of gene/protein expression patterns in the cochlear duct during normal development. Overlapping expression is indicated by hatched colors. SG, though still present, is not shown in panels C-G. (C) Bmp4 expressed in the future OS potentially creates a morphogen gradient along the lateral-to-medial axis that regulates cell fates. Reduction of Bmp signaling (i.e. in the case of *Alk3/6* conditional deletion) leads to a lateral shift of medial regions and the absence of lateral cell fate markers. (D) Loss of Fgf10 signaling in $Fgf10^{\Delta 2/\Delta 2}$ cochleae leads to the absence of RM and changes in the shape of the cochlear duct. KO is still present, as indicated by continued expression of *Fgf10*. (E) *Otx2*, expressed in the developing RM, is necessary for its formation. Absence of *Otx2* results in expanded expression of KO and PS genes (*Fgf10*, *Sox2*) and formation of an ectopic PS domain, indicated by *Lfng* expression (star). (F) *Lmo4*, normally expressed in two regions just medial and lateral to the PS domain at E14, inhibits sensory cell fates. Loss of *Lmo4* leads to formation of an ectopic mirror-image PS domain in the lateral cochlear duct (star). (G) In *Esrp1* mutants, splicing defects in *Fgfr2* result in disruption to FGF signaling, causing expansion of the RM domain at the expense of the SV. IS, inner sulcus; KO, Kölliker's organ; LER, lesser epithelial ridge; OC, organ of Corti; OS, outer sulcus; PS, prosensory domain; RM, Reissner's membrane; SG, spiral ganglion; SV, stria vascularis.

However, unlike the apparent 'medialization' of the duct that occurs in response to decreased Bmp signaling, deletion of *Fgf10* does not lead to an over-representation of lateral cochlear phenotypes (Urness et al., 2015). One caveat is that *Fgf3* is also expressed in the cochlear duct, so it could act to compensate for the loss of *Fgf10*. Alternatively, or additionally, Wnt signaling may play a role in medial cochlear identity. Indeed, using an *in vitro* approach, inhibition of glycogen synthase kinase 3β (GSK3β), a key antagonist of the canonical Wnt signaling pathway, was shown to increase medial cochlear cell fates at the expense of lateral fates (Munnamalai and Fekete, 2016). However, because GSK3β can modulate multiple signaling pathways (Patel and Woodgett, 2017), the downstream mechanisms mediating its effects on medial fates are unclear. Furthermore, although strong inhibition of GSK3β at early stages leads to an increase in the expression of Wnt target genes (Munnamalai and Fekete, 2016), a similar study using a lower dose of the GSK3β inhibitor showed a medialized phenotype but without an increase in expression of Wnt target genes (Ellis et al., 2019). In both studies, downregulation of *Bmp4* was observed, and Ellis et al. showed that addition of exogenous BMP4 was sufficient to induce a partial rescue of the medialized phenotype.

Several studies have demonstrated similar levels of plasticity in cells within the cochlear roof. Overexpression of *Atoh1*, *Sox2* or activated *Notch1* induces ectopic regions of HCs and SCs in Reissner's membrane or the stria vascularis, demonstrating sensory potential in these regions (Fig. 2) (Kelly et al., 2012; Pan et al., 2013). Similarly, deletion of *Otx2*, which is expressed in the medial half of the cochlear roof, or *Lmo4*, which is expressed in the lateral half of the roof, also results in the formation of ectopic sensory structures in Reissner's membrane or the stria vascularis (Deng et al., 2014; Vendrell et al., 2015). In addition, deletion of *Esrp1*, which encodes a splicing regulatory protein, causes a splicing defect in *Fgfr2* that results in an

Development (2020) 147, dev162263. doi:10.1242/dev.162263

increase in the size of Reissner's membrane at the expense of the stria vascularis (Rohacek et al., 2017; Urness et al., 2015). Overall, these results demonstrate that, although the early duct is divided into several broad regions, a significant degree of plasticity exists. The findings also reveal that suppression of a sensory fate is a key step in cochlear patterning.

Sox2 specifies prosensory identity

The transcription factor Sox2 is both necessary and sufficient for the formation of prosensory cells, and is expressed in the prosensory region of the cochlea during normal development (Dabdoub et al., 2008; Kiernan et al., 2005b; Pan et al., 2013; Puligilla and Kelley, 2017). However, both antibody labeling and Cre-mediated lineage tracing for *Sox2* indicate that it is expressed throughout the entire ventral half of the otocyst at E9.0, including in cells that give rise to the four non-prosensory regions of the mature cochlear duct (Gu et al., 2016; Mak et al., 2009; Steevens et al., 2019; Wood and Episkopou, 1999). These results are consistent with the idea that the entire cochlear duct is initially competent to develop as prosensory cells. As development continues, Sox2 expression is progressively lost from different regions of the cochlear duct. By E12.5, Sox2 is restricted to the prosensory domain plus Kölliker's organ (Dabdoub et al., 2008; Gu et al., 2016) and, as development continues, a medial-to-lateral downregulation occurs such that, by ∼E16, strong Sox2 expression is restricted to the prosensory domain and the lateral region of Kölliker's organ. As Sox2 is sufficient for the formation of prosensory cells, it is unclear why cochlear duct cells that initially express Sox2 do not uniformly develop as prosensory cells. One possibility is that other factors may act to inhibit the prosensory effects of Sox2. For instance, Otx2 has been shown to directly repress Sox2 in both the CNS and optic cup (Nishihara et al., 2012; Omodei et al., 2008). Similarly, disruption of Shh signaling, which also suppresses inner ear sensory development, leads to changes in the pattern of Sox2 (Muthu et al., 2019).

An additional consideration is that the ability of Sox2 to induce a prosensory fate may be dependent on the duration or level of its expression in a particular cell. Within the cochlear duct, Sox2 continues to be expressed in cells within Kölliker's organ through at least E18.5. If the duration of Sox2 expression is important, then these cells may possess increased or prolonged prosensory potential. Several studies have demonstrated that forced expression of positive regulators of prosensory identity, including *Atoh1*, *Eya1*, *Six1* or the Notch1 intracellular domain (Notch1-ICD), is sufficient to induce the formation of prosensory patches in Kölliker's organ, and in other regions of the duct (Ahmed et al., 2012; Hartman et al., 2010; Kelly et al., 2012; Pan et al., 2013). Similarly, inhibition of Shh signaling leads to the formation of prosensory patches in Kölliker's organ (Driver et al., 2008), and ablation of the SCs surrounding IHCs in early postnatal mice initiates a regenerative response in which Kölliker's organ cells migrate into the prosensory domain and develop as replacement SCs (Mellado Lagarde et al., 2014). In addition to expressing Sox2, Kölliker's organ cells express other markers of the prosensory domain, including *Jag1* and *Lfng* (Gu et al., 2016; Hume et al., 2007; Morrison et al., 1999; Morsli et al., 1998; Zhang et al., 2000). Overall, these studies suggest that at the outset of cochlear elongation, a large number of cells within the duct possess some degree of prosensory potential. However, as development continues, that potential (and the expression of Sox2) is progressively lost in cells outside of the final sensory domain.

There are two intriguing implications that arise from the idea that the tissues that give rise to the inner sulcus and the organ of Corti – Kölliker's organ and the prosensory domain – have similar development potentials during a significant portion of cochlear formation. First, from an evolutionary perspective, given the degree of prosensory potential that exists in Kölliker's organ, it would be useful to understand why Kölliker's organ cells typically do not develop as HCs and SCs. The most likely explanation is the existence of inhibitory factors that suppress prosensory formation. This hypothesis suggests that the sensory epithelium in the ancestral mammalian cochlear duct may have included Kölliker's organ, and subsequent selective pressures have resulted in the conversion of most of these cells to the non-sensory inner sulcus. The process through which this occurred is unknown, but the existence of a somewhat larger sensory structure in the auditory organs of monotremes (Ladhams and Pickles, 1996; Schultz et al., 2017), as compared to therian mammals, suggests a gradual transition over evolutionary time. Second, the prosensory potential of Kölliker's organ also has implications for the development of regenerative strategies. Different cochlear pathologies result in varying degrees of cell death along the basilar membrane. When damage is mild and relatively acute, HCs are lost, but the remaining cells retain their SC morphology. In conditions of more severe long-term damage, the cell types that remain on the basilar membrane assume a flattened-cuboidal shape that is difficult to attribute to any particular cell type (Raphael et al., 2007). These cells could be SCs that have undergone a phenotypic change over time, or they could be inner or outer sulcus cells that have expanded to cover the area of the basilar membrane after the death of both HCs and SCs. As inner sulcus cells are derived from Kölliker's organ, it is possible that they might retain a greater degree of prosensory potential, which could be leveraged to induce a regenerative response.

Regulation of cochlear outgrowth

By the onset of extension, which in mice occurs at ∼E11, a broad regional pattern has been laid out within the cochlear duct. The duct continues to extend until approximately postnatal day (P)3, roughly 11 days, and once outgrowth is completed it will traverse a total of 1¾ turns from base to apex (McKenzie et al., 2004). Most of the cells that line the duct continue to proliferate throughout the period of elongation, suggesting that the generation of new cells may contribute to growth. In contrast, all of the cells within the prosensory population are generated fairly early, with prosensory cell cycle exit completed by E14 (Chen and Segil, 1999; Ruben, 1967). As the organ of Corti extends the full length of the cochlea, prosensory cells must move towards the apex as extension continues (Driver et al., 2017). At E14, the prosensory cells comprise a dense pseudostratified epithelium (Fig. 3) (Driver et al., 2017; McKenzie et al., 2004). Between E14 and E16, this region of the duct narrows along the medial-lateral axis (via convergent extension) and thins along the basal-lumenal axis (via radial intercalation) to eventually form a bilayer, with HCs located lumenally and SCs spanning the basal-lumenal axis (Chen et al., 2002; Driver et al., 2017; McKenzie et al., 2004; Tateya et al., 2019). Convergent extension was long considered to be the primary driving force for prosensory outgrowth (Chen et al., 2002; McKenzie et al., 2004). However, although some degree of intercalation does occur along the medial-lateral axis, time-lapse imaging and morphometric modeling suggest that radial intercalation also has a large impact on outgrowth (Driver et al., 2017). Moreover, beyond E16, physical growth of the HCs contributes significantly to continued extension.

The factors that regulate cochlear outgrowth remain poorly understood. Extension has been shown to be reduced or even eliminated in *Shh* and *Wnt* mutants (Bok et al., 2007; Brown and Epstein, 2011; Riccomagno et al., 2002, 2005), but the roles of these signaling pathways are complex and likely include effects on both the

Development (2020) 147, dev162263. doi:10.1242/dev.162263

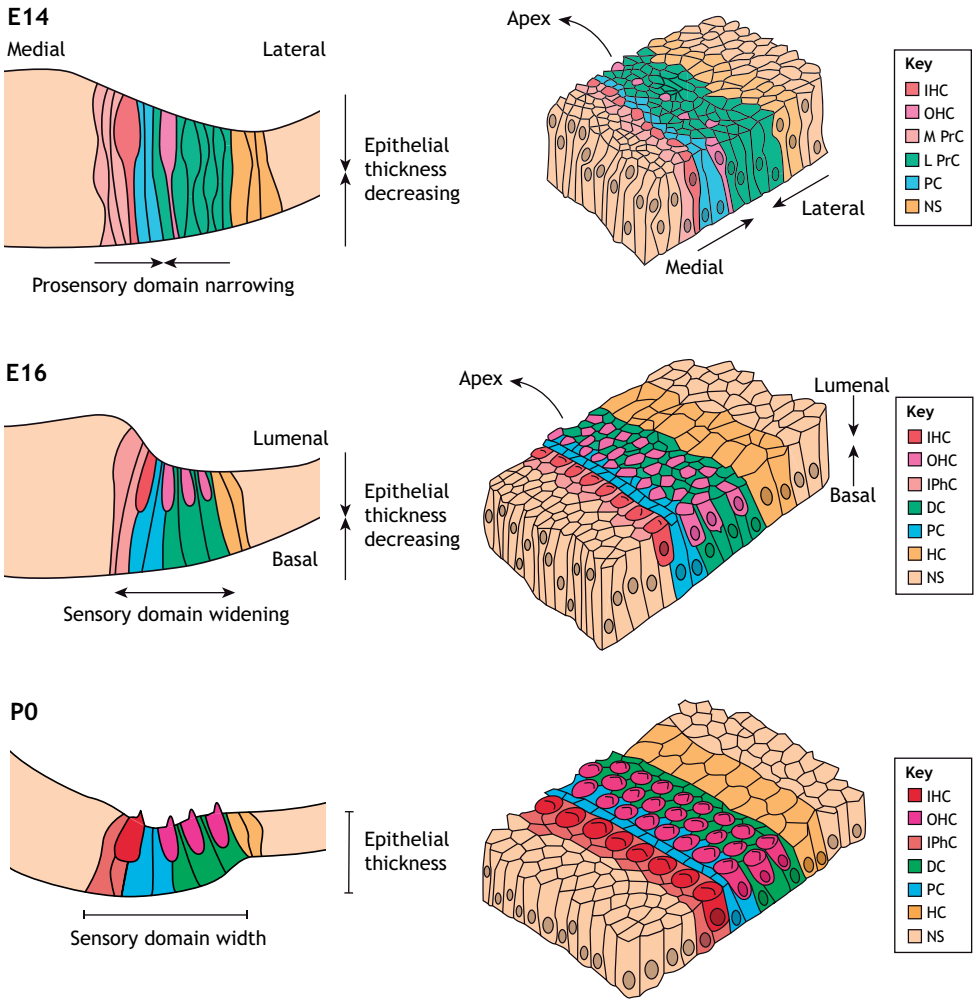

Fig. 3. Convergent extension and radial intercalation regulate cochlear extension. At E14, post-mitotic cells within the prosensory domain, which form a pseudostratified epithelium, extend towards the apex of the duct as the cochlea grows. Extension is driven by both convergence of cells located at the medial and lateral edges of the prosensory domain towards the middle, and by radial intercalation-thinning of the epithelium as pseudostratified cells intercalate and shorten along the lumenal-to-basal axis. At E16, radial intercalation continues to generate movement of the sensory population towards the apex, but the width of the sensory domain along the medial-lateral axis expands as cell size increases. Between E16 and P0, cell growth, especially of hair cells, also contributes to ongoing extension. DC, Deiters' cell; HC, Hensen's cell; IHC, inner hair cell; IPhC, inner phalangeal cell; L PrC, lateral prosensory cell; M PrC, medial prosensory cell; NS, nonsensory cells; OHC, outer hair cell; PC, pillar cell.

specification of the ventral region of the otocyst and outgrowth directly. In particular, before E11, SHH originating in the floor plate and notochord is required to specify ventral identity in the otocyst, but subsequent SHH signaling arising in the developing spiral ganglion neurons (SGNs) is required for normal cochlear extension (Bok et al., 2007; Driver et al., 2008). However, *Shh* expression in SGNs is progressively downregulated from base-to-apex (Bok et al., 2013; Liu et al., 2010) suggesting SHH could create a directional gradient for migrating prosensory cells. As loss of *Shh* from developing SGNs also leads to premature HC differentiation, which could similarly alter outgrowth, it is not clear which of these mechanisms, or if both of them, underlies cochlear truncation.

Within the prosensory cells themselves, components of the planar cell polarity (PCP) pathway and non-muscle myosin II have been shown to be required for normal outgrowth. Mutations in several PCP pathway genes, such as *Vangl2* and *Dvl1/2*, result in cochleae that are ~25% shorter than normal, with patterning defects near the apex, including multiple rows of IHCs and OHCs, a phenotype that is consistent with a failure in outgrowth (Mao et al., 2011; Montcouquiol et al., 2003; Qian et al., 2007; Saburi et al., 2012; Wang et al., 2006a). Similarly, forced expression of a dominant-negative form of *Myh10* (MyoIIB) leads to shortened cochleae and comparable patterning defects (Yamamoto et al., 2009). Exactly how these two pathways modulate cellular outgrowth remains to be determined, but the PCP pathway has been shown to influence both convergent extension and radial intercalation in the inner ear and elsewhere (Ossipova et al., 2015; Skoglund and Keller, 2010; Xu et al., 2018).

Patterning along the tonotopic axis

The physical extension of the cochlear duct creates an elongated spiral structure. In the mature animal, different auditory frequencies stimulate HCs located in specific regions along the cochlea, an organizing principle referred to as tonotopy (Fig. 4) (Mann and Kelley, 2011). This spatiofrequency code is achieved as a result of physical differences in the width, thickness and stiffness of the basilar membrane, which lead to frequency-specific changes in the distance over which sound-induced traveling waves are propagated. Similar graded changes in the overlying tectorial membrane along the tonotopic axis also exist (Gavara and Chadwick, 2009; Teudt and Richter, 2014). Finally, HCs demonstrate subtle physiologic and phenotypic variations, including differences in stereocilia length and ion channel expression, corresponding to their position along the tonotopic axis (Beurg et al., 2018; Pujol et al., 1991). The structural components of both the basilar and tectorial membranes are produced by different populations of precursor cells within the cochlear duct, beginning as early as E12.5 (Goodyear and Richardson, 2018; Mann and Kelley, 2011; Rau et al., 1999). These results suggest that positional identity along the apical-basal axis of the cochlea is specified before full extension; however, as the timing of the development of tonotopic specializations within either the basilar or tectorial membranes has not been determined, it has been difficult to draw conclusions regarding when positional identity is determined.

Recently, it was demonstrated that SHH originating from the notochord and floor plate appears to play an early role in

Development (2020) 147, dev162263. doi:10.1242/dev.162263

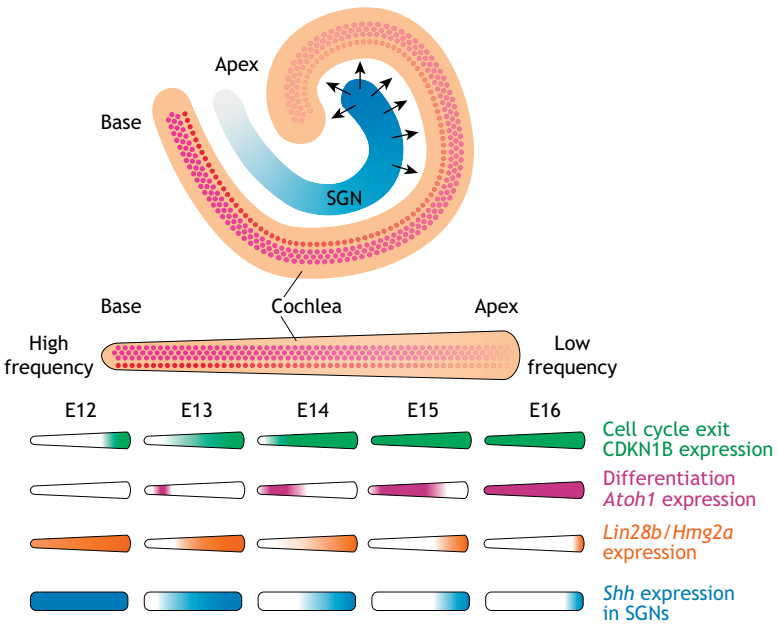

Fig. 4. Regulation of cellular differentiation along the cochlear basal-to-apical (tonotopic) axis. Patterning of the long, tonotopic, axis of the spiral cochlea occurs via several events, which occur in gradients along its basal-to-apical axis. The upper panel shows the spiraled cochlea, highlighting its association with the spiral ganglion. The middle panel illustrates the duct 'unrolled', with the narrow base representing the high frequency-responding end and the wider apex representing the low frequency-responding end. The lower panel depicts some of the events that occur during various stages of cochlear development. Cochlear prosensory cells exit the cell cycle (in response to CDKN1B expression) in a gradient that initiates in the apex and extends towards the base of the cochlear duct between E12 and E14. In contrast, cellular differentiation (marked by *Atoh1* expression) begins near the base at E13 and extends to the apex by E16. As a result, cells in the apex of the cochlea are maintained in a post-mitotic but uncommitted state for approximately 4 days. Timing of the onset of differentiation is mediated, at least in part, through a base-to-apex gradient of downregulation of *Lin28b*/*Hmga2*. The gradient of differentiation, and potentially downregulation of *Lin28b*/*Hmga2*, is regulated through a temporal base-to-apex decrease in *Shh* signaling from adjacent spiral ganglion neurons (SGNs).

determining positional identity in the auditory organs of both mammals and birds (Son et al., 2015). In both organisms, early extension of the auditory duct is initially oriented toward the midline. Therefore, SHH could create a gradient along the apical-basal axis of the cochlea, and the corresponding distal-proximal axis of the avian auditory organ (the basilar papilla). Consistent with this hypothesis, it was shown that genetic activation of the Hedgehog pathway in mice leads to an overexpression of genes associated with the distal (apical) region of the duct, which is initially located closer to the midline. Similarly, beads soaked in SHH cause transcriptional and phenotypic changes in the basilar papilla that are consistent with a more distal (low frequency) identity.

Although the downstream effectors of Shh signaling in mammals have not been fully determined, both Son et al. (2015) and a separate study by Mann et al. (2014) demonstrated the presence of a tonotopic gradient of *Bmp7* along the developing basilar papilla. Moreover, *Bmp7* is among the genes that are induced in response to the introduction of an SHH-soaked bead within the basilar papilla (Son et al., 2015). Directly increasing Bmp7 activity, either *in vitro* or *in ovo*, induces more distal fates along the tonotopic axis of the chick basilar papilla, whereas inhibition of BMP7 using chordin-like 1, which is expressed in a counter gradient to *Bmp7*, promotes more proximal (high frequency) fates (Mann et al., 2014), indicating that Bmp7 acts to direct tonotopic identity in the chick. However, *Bmp7* is not expressed along the tonotopic gradient in the mouse cochlea (Son et al., 2015), suggesting that although the role of Shh in establishing initial tonotopic identity has been conserved, the downstream effectors have not. Additional studies will clearly be required to identify the factors that regulate tonotopic identity within the mouse cochlea, but the demonstration that signals from the midline act to establish an initial spatial map suggests this process occurs quite early in development.

Regulation of terminal mitosis and cellular differentiation in the cochlea

As discussed, all of the prosensory cells within the cochlear duct are post-mitotic by E14. However, cell cycle exit is not uniform. Terminal mitosis begins at the apical end of the duct on E12 and proceeds in a gradient that reaches the base by E14 (Fig. 4) (Chen and Segil, 1999; Ruben, 1967). Cellular differentiation, in contrast, begins near the base of the cochlear duct around E14 and extends apically. As a result, apical prosensory cells exist in an undifferentiated, post-mitotic state for up to 96 h in the mouse. The reasons for these opposing gradients of cell cycle exit and cell differentiation are not clear. However, we are beginning to gain a better understanding of how these opposing gradients are regulated.

The cell cycle inhibitor *Cdkn1b* (formerly $p27^{kip1}$) is expressed in an apex-to-base gradient that precedes cell cycle exit, and deletion of *Cdkn1b* extends proliferation by 24-48 h and reverses the gradient of terminal mitosis (Chen and Segil, 1999; Lee et al., 2006). SHH expressed in SGNs also plays a role in regulating this gradient, as SGN-specific deletion of *Shh* also causes a reversal in the gradient of cell cycle exit (Bok et al., 2013; Tateya et al., 2013). Whether *Shh* regulates CDKN1B expression directly remains to be determined. For prosensory cells located near the base of the cochlea, differentiation begins almost immediately following terminal mitosis (Rubel, 1978), with expression of *Atoh1* being one of the earliest markers of this process (Chen et al., 2002; Lanford et al., 2000; Tateya et al., 2019; Woods et al., 2004). Over the next several days, differentiation proceeds as a gradient that extends both apically and over the shorter distance to the extreme base of the cochlea. This process has also been shown to be dependent on *Shh* signaling, although in this case the mechanism is clearer, as expression of *Shh* in SGNs is downregulated in a temporal gradient from base to apex, preceding sensory differentiation. SHH has also been shown to inhibit HC formation (Driver et al., 2008) so this base-to-apex decrease in expression could act to derepress differentiation along the length of the cochlea.

Within the cochlear prosensory cells, the RNA binding genes *Lin28a* and *Hmga2*, shown to regulate heterochronic cellular differentiation in *C. elegans* (Faunes and Larraín, 2016; Tsialikas and Romer-Seibert, 2015), are broadly expressed before differentiation (Golden et al., 2015). Both genes are downregulated in a temporal gradient from base to apex, consistent with a role in regulation of differentiation (Fig. 4), and overexpression of *Lin28a* acts to delay, but not entirely prevent, HC differentiation (Golden et al., 2015). The effects of *Lin28a* are mediated through both the stabilization of other cell cycle exit-promoting transcripts and by inhibition of *let-7* miRNAs, which negatively regulate *Lin28a* through a double-negative feedback loop. Consistent with these observations, *let-7*

Development (2020) 147, dev162263. doi:10.1242/dev.162263

overexpression causes prosensory cells to exit the cell cycle prematurely. However, premature differentiation does not occur, so this aspect of *Lin28a* function may be regulated through other factors. *Shh* has recently been shown to positively regulate *Lin28a* expression in the cochlea (Muthu et al., 2019), suggesting a likely mechanism for Shh-mediated regulation of the timing and pattern of HC differentiation.

Once differentiation begins, a mosaic of HCs and SCs forms. This process is largely mediated through Notch-mediated lateral inhibition, in which developing HCs upregulate the expression of the Notch ligands Dll1 and Jag2, leading to activation of the Notch pathway in adjacent cells (Fig. 5) (Brooker et al., 2006; Doetzlhofer et al., 2009; Driver et al., 2013; Kiernan et al., 2005a; Lanford et al., 1999; Mizutari et al., 2013; Murata et al., 2006). Notch activation then induces expression of *Hes1* and *Hes5*, which antagonize *Atoh1* expression, diverting neighboring cells from an HC fate (Lanford et al., 2000; Zheng et al., 2000; Zine et al., 2001). In addition to producing these inhibitory signals, HCs generate signals that induce surrounding cells to develop as SCs (Woods et al., 2004). The factors that mediate these signals are not well understood; however, activation of Notch signaling has been shown to induce the formation of generic SCs (Campbell et al., 2016).

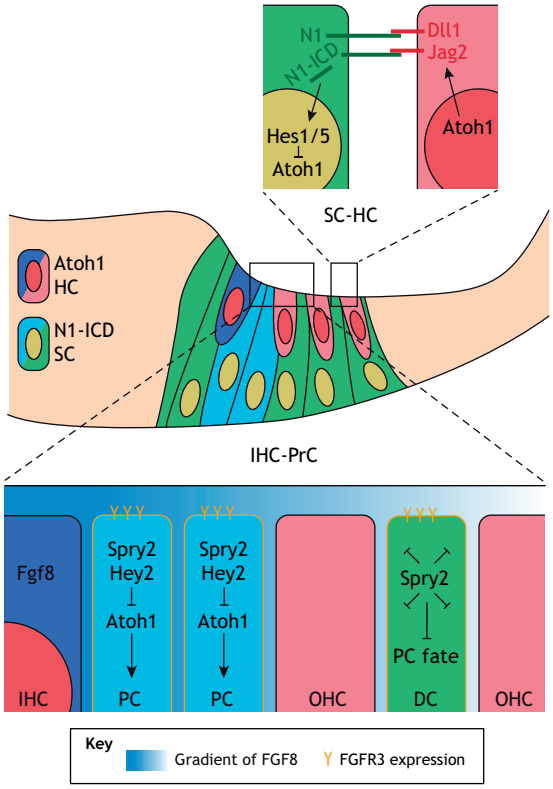

Fig. 5. The Notch and Fgf signaling pathways regulate cell fates in the organ of Corti. Developing hair cells (HCs) express the Notch ligands delta-like 1 (Dll1) and jagged 2 (Jag2), which bind to Notch1 (N1) in adjacent supporting cells (SCs), leading to the generation of Notch1 intracellular domains (N1-ICD) that translocate to the nucleus. N1-ICDs then induce the expression of *Hes1/5*, which act to inhibit *Atoh1* activity and expression, diverting cells from an HC fate. Inner hair cells (IHCs) express *Fgf8*, whereas adjacent prosensory cells (PrCs) express *Fgfr3* and the Fgf antagonist *Spry2*. Diffusion is thought to create a gradient of Fgf8 that extends away from the IHC. Exposure to a high concentration of Fgf8 is sufficient to activate Fgfr3 to levels that overcome the antagonistic effects of Spry2 and induce a pillar cell (PC) fate. In contrast, in SCs located more distant to the Fgf8 source, such as Deiters' cells (DCs), activation of Fgfr3 is not sufficient to overcome the effects of Spry2, and cells are not induced to develop as PCs. Developing OHCs downregulate Fgfr3 and, as a result, are not affected by the gradient of Fgf8. An additional effect of Fgfr3 activation is to upregulate expression of the Notch target Hey2, which inhibits *Atoh1*, preventing the formation of ectopic HCs in the PC region.

How the specialized SC types of the organ of Corti are specified remains largely, although not completely, unknown. The inner and outer pillar cells represent two such types of specialized SCs. Mutations in *Fgfr3*, which is expressed in developing pillar cells, OHCs and Deiters' cells, lead to defects in pillar cell formation and deafness (Colvin et al., 1996). The FGFR3 ligand FGF8 is expressed in the row of developing IHCs located directly adjacent to the pillar cells, and the inner ear-specific deletion of *Fgf8* leads to a similar pillar cell phenotype (Fig. 5) (Jacques et al., 2007). In contrast, the Fgf antagonist sprouty2 (*Spry2*) is expressed in an overlapping domain with *Fgfr3*, and deletion of *Spry2* results in a conversion of some Deiters' cells into ectopic pillar cells (Shim et al., 2005). Overall, these results suggest that all SCs in the lateral region of the organ of Corti may initially have the potential to develop as either Deiters' cells or pillar cells in response to FGFR3 activation. During normal development, FGF8-induced FGFR3 activation is limited to only those cells that are exposed to the highest level of FGF8 as a result of ligand diffusion and SPRY2-mediated antagonism.

Finally, two recent studies have identified transcription factors that regulate different aspects of OHC development. *Insm1*, which encodes a zinc-finger transcription factor that is known to play a role in specifying cell fate in both nervous and neuroendocrine tissues (Lan and Breslin, 2009), is transiently expressed only in developing OHCs from ~E16.5 to P2 (Lorenzen et al., 2015). Inner ear-specific deletion of *Insm1* generates an intriguing phenotype in which approximately half of OHCs convert into cells with characteristics of IHCs, and the remaining OHCs retain OHC characteristics (Wiwatpanit et al., 2018). Despite the presence of seemingly normal OHCs, these mice are deaf and have defects in OHC function. Similarly, a separate study showed that *Ikzf2* (helios), another zinc-finger transcription factor unrelated to *Insm1* (Grzanka et al., 2013), is also exclusively expressed within the organ of Corti in OHCs, but not before P4 (Chessum et al., 2018). Mice with an ENU-induced inactivating mutation in *Ikzf2* have progressive hearing loss and defects in OHC function. Moreover, forced expression of *Ikzf2* is sufficient to induce some OHC characteristics in IHCs. These studies suggest that OHC development may be an extended process that begins with inhibition of an IHC fate, followed by a gradual specification of an OHC phenotype.

Hair cell polarization

As discussed, all cochlear HC stereociliary bundles are shaped like chevrons, with the vertex oriented towards the duct's lateral edge. The orientation of the bundles is functionally relevant, as only deflections in the direction of the vertex induce channel opening. This directional sensitivity is directly related to the orientation of the tip links, which only form connections between individual stereocilia adjacent to one another along the medial-to-lateral axis. Stimulation of the cochlea by incoming sounds creates vibrations that deflect stereocilia along this axis. As a result, appropriate orientation of stereociliary bundles is crucial for normal function (Montcouquiol and Kelley, 2020). Previous studies have demonstrated that the control of overall (or epithelial) alignment involves members of the core PCP pathway, including *Vangl2/1*, *Fz3/6* (*Fzd3/6*), *Celsr1* and *Dvl1/2* (Curtin et al., 2003; Montcouquiol et al., 2003; Wang et al., 2005, 2006b).

Development (2020) 147, dev162263. doi:10.1242/dev.162263

Mutations in these genes lead to differing levels of rotated or misoriented bundles. However, regardless of the extent of misorientation, each individual HC still develops a stereociliary bundle that is polarized to one side of the cell. This result indicates that, although polarization across the epithelium is disrupted in these mutants, individual polarization remains intact, suggesting that cell-autonomous asymmetry must be regulated by other signaling molecules.

Work from several laboratories has identified a G-protein-associated complex that plays a crucial role in autonomous bundle orientation and development (Bhonker et al., 2016; Ezan et al., 2013; Tarchini et al., 2013). These studies focused on a group of molecules known to play a role in the polarization of *Drosophila* sensory organ precursors (SOPs), which are progenitor cells that undergo a physically oriented cellular division to asymmetrically distribute cell fate determinants (Carvajal-Gonzalez et al., 2016). Before the mitotic division of an SOP, the core PCP pathway polarizes two complexes – one containing G-protein alpha I ($G\alpha_i$)/inscuteable(insc)/partner of inscuteable (pins) and another containing par3(baz)/par-6/atypical (a)PKC – to opposite sides of the cell (Chen and Zhang, 2013; Culurgioni and Mapelli, 2013). As both complexes interact with centrosomes, their localization to each side of the SOP directly orients the subsequent cell division. The interaction of these complexes with centrosomes in *Drosophila* suggested that they could also play a role in HC stereociliary bundle polarization, as the first indication of bundle orientation is the migration of the developing kinocilium across the HC lumenal surface from its center towards its rim (Cotanche and Corwin, 1991; Dabdoub and Kelley, 2005; Montcouquiol et al., 2003). As every cilium, including the kinocilium, is anchored to an underlying basal body/centrosome, it follows that kinocilial migration may be linked to the positioning of the basal body.

As HC kinocilium migration is initiated, $G\alpha_{i3}$, Gpsm2 (a mammalian homolog of Pins) and Insc form an asymmetric crescent along the lateral edge of each HC (Fig. 6) (Ezan et al., 2013). This module antagonizes aPKC, leading to progressive restriction of aPKC to the medial half of the lumenal HC surface (Fig. 6). As development proceeds, the $G\alpha_{i3}$/Gpsm2/Insc complex expands to cover the entire microvilli-free area lateral to the kinocilium (the bare zone) of each HC, such that by P0 the lumenal surface has a medial region expressing aPKC and Par6b (Pard6b), and a lateral region expressing $G\alpha_{i3}$/Gpsm2/Insc. Analysis of cochleae from *Gpms2* or *Insc* mutants, or those in which $G\alpha_{i3}$ activity is inhibited, indicated defects in both cell-autonomous and epithelial polarity (Ezan et al., 2013; Tarchini et al., 2013). To confirm a direct link between bundle orientation and localization of these complexes, asymmetry was examined in *Vangl2*$^{Lp/Lp}$ mutants (Ezan et al., 2013), revealing that an asymmetric crescent of $G\alpha_{i3}$ is located lateral to the stereociliary bundle of each HC regardless of the overall cell orientation, which suggests that each HC maintains its intrinsic cellular polarity even when tissue-level polarity is disrupted. Finally, although stereociliary bundles in $G\alpha_{i3}$-inhibited explants are misoriented, the localization of Vangl2 is largely unaffected, suggesting that $G\alpha_{i3}$ acts downstream of or in parallel with the core PCP pathway. These results suggest that, in developing HCs, a molecular mechanism that acts to orient mitotic spindles has been co-opted to localize the basal body associated with the developing kinocilium, and consequently the stereociliary bundle, to one side of the cell.

Although the discoveries discussed above provide insights into the cellular mechanisms that direct cell-autonomous polarization, the uniform orientation of sterociliary bundles requires a link between this cell-autonomous process and tissue-level PCP signals. A recent study has suggested that the dishevelled-binding protein Daple (Ccdc88c) can mediate this link (Siletti et al., 2017). Daple can bind to both Dvl and $G\alpha_i$, and is asymmetrically localized to the lateral side of developing cochlear HCs prior to bundle formation (Siletti et al., 2017). In addition, *Daple* mutants have polarization phenotypes consistent with disruption of both tissue-level and cell-intrinsic polarity and, significantly, show defects in the localization of $G\alpha_i$/Gpsm2 and aPKC, while core PCP proteins are unaffected. These results suggest that Daple could act as bridge between the core PCP and cell intrinsic polarization machinery, although further studies are clearly required.

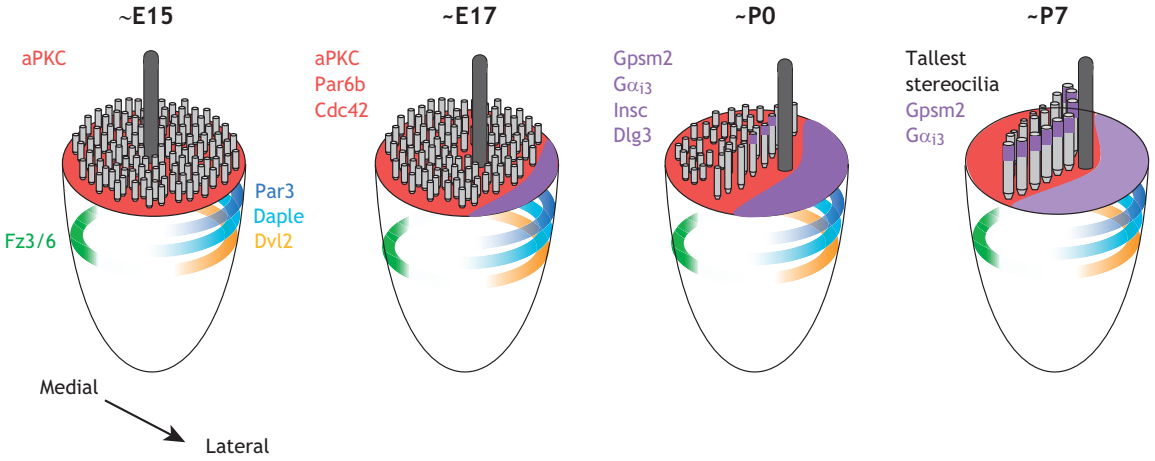

Fig. 6. Polarization of stereociliary bundles. At ~E15, the lumenal surfaces of immature hair cells have a centrally located true cilium surrounded by many short microvilli. aPKC is localized throughout the lumenal surface of the cell, whereas Par3 is restricted to the lateral side. The core PCP molecules Dvl2 and Fz3/6 are asymmetrically localized to the medial (Fz3/6) and lateral (Dvl2) sides of the cell. Daple is localized to the lateral half of each cell. At E17, the developing kinocilium begins to migrate towards the lateral edge of the cell. Par6b and Cdc42 are co-localized with aPKC, whereas Gpsm2, $G\alpha_{i3}$, Insc and Dlg3 become localized to the lateral edge at the lumenal surface, leading to exclusion of aPKC/Par6b/Cdc42 from the lateral region. By P0, expression of Gpsm2/$G\alpha_{i3}$/Insc/Dlg3 has expanded concomitant with the formation of the fonticulus (the microvilli-free region between the kinocilium and the lateral edge of each HC), lateral to the developing stereociliary bundle. An accumulation of Gpsm2 and $G\alpha_{i3}$ also appears at the tips of the centrally located tallest stereociliary bundles. Finally, at P7, Gpsm2/$G\alpha_{i3}$ is localized to the tips of all stereocilia in the tallest row. A corresponding decrease in GPSM2/$G\alpha_{i3}$ is observed in the fonticular region.

Development (2020) 147, dev162263. doi:10.1242/dev.162263

The control of stereocilia elongation and length

The development of a staircase pattern within each stereociliary bundle is crucial for HC mechanotransduction. Stereocilia develop from a subset of the population of short microvilli that cover the lumenal surfaces of immature HCs (Tilney et al., 1986). As each HC begins to mature, individual microvilli located adjacent to the kinocilium elongate and thicken. Microvilli closer to the kinocilium grow taller, with neighboring microvilli growing to progressively shorter heights, leading to the formation of the staircase pattern (Krey et al., 2020; Tilney and Saunders, 1983; Tilney et al., 1986). As discussed, $G\alpha_{i3}$, Gpsm2 and Insc, become localized to the lateral side of developing HCs and play a crucial role in bundle orientation. However, in the studies that initially defined this novel role for the $G\alpha_{i3}$/Gpsm2 complex, a secondary accumulation of $G\alpha_{i3}$ and Gpsm2 was noted in the tips of developing stereocilia in the row closest to the kinocilium (Mauriac et al., 2017; Tarchini et al., 2016), indicating that these factors may play a role in stereocilia elongation and positioning of the tallest row. Moreover, Tarchini and colleagues noted a decrease in $G\alpha_{i3}$/Gpsm2 at the lateral edge of HCs that coincided with the appearance of the same proteins in developing stereocilia, suggesting that the protein complex may be shuttled from the cell body into the bundle (Fig. 6). The factors that regulate such a transition are not clear, but Insc, which was not observed in stereocilia, could play a role; in *Insc* mutants, HCs show premature localization of $G\alpha_{i3}$/Gpsm2 in stereocilia, indicating a possible disruption in the anchoring of this complex in the cell body (Tarchini et al., 2016).

Exactly how Gpsm2 and $G\alpha_{i3}$ might regulate elongation is not yet known. Deletion of *Gpsm2* or inhibition of $G\alpha_{i3}$ leads to HCs with stunted or shortened stereocilia (Mauriac et al., 2017). Although the molecular basis for these defects is not completely understood, it has been shown that Gpsm2 interacts with whirlin, a PDZ scaffolding protein required for normal HC function (Mburu et al., 2003). Gpsm2 localization to the tips of stereocilia is dependent on both whirlin and myosin 15 (Myo15; also known as Myh15), a motor protein required for hearing that regulates the movement of proteins to the tips of stereocilia (Belyantseva et al., 2005; Delprat et al., 2005). Moreover, in the tallest row, Gpsm2 and $G\alpha_{i3}$ stabilize a higher level of Myo15 and the actin-regulatory protein Eps8, suggesting that stereocilia height may be directly related to the amount of Myo15-Eps8 at the tip, and that the proximity of the first row of stereocilia to the bare zone is a key determinant of the location of the tallest row of stereocilia (Tadenev et al., 2019). As stereocilia elongation and thickening are dependent on the formation and stabilization of filamentous actin, these phenotypes suggest a possible novel role for Gpsm2 in the modulation of actin assembly or retrograde flow. The results also provide insights into the molecular etiology of Chudley-McCullough syndrome, which is caused by mutations in *Gpsm2* and is characterized by early onset deafness (Diaz-Horta et al., 2012; Doherty et al., 2012).

Development of the staircase pattern of stereocilia is also perturbed in mice with mutations that affect mechanotransduction, or following a pharmacological block of transduction channels *in vitro* (Beurg et al., 2018; Krey et al., 2020; Vélez-Ortega et al., 2017). Localization of Gpsm2, whirlin, Myo15 and Eps8 in mechanotransduction mutants suggests that the specification of the tallest row of stereocilia is disrupted, implicating a component of activity in bundle maturation (Krey et al., 2020). Influx of Ca^{2+}, which occurs following the opening of mechanotransduction channels, may act as a signal that inhibits accumulation and/or function of Myo15-Eps8 in shorter stereocilia. As transduction channels are not present on the tallest stereocilia (Kurima et al., 2015), Ca^{2+} influx should be minimal, and may contribute to the higher level of Myo15-Eps8 in the tallest row. However, two different mechanotransduction mutants (*Tmie* versus *Tmc1/2*) show subtle differences in changes in stereocilia lengths, even though Ca^{2+} entry should be equivalently disrupted in both mutants. In particular, the length of first row stereocilia appears to be more adversely affected in *Tmie* versus *Tmc1/2* double mutants (Krey et al., 2020). These results suggest that changes in Ca^{2+} influx, although important for regulating stereocilia length, do not explain all of the observed changes in stereocilia length in mechanotransduction mutants.

The results above again highlight a key functional link between epithelial and intrinsic polarity. However, key questions regarding how these polarities are coordinated remain unanswered. In particular, the external factors that direct tissue-level polarity across the organ of Corti remain to be determined. While a gradient or gradients of secreted Wnts have been suggested (Dabdoub and Kelley, 2005; Qian et al., 2007), genetic data to support this hypothesis are still lacking. In other HC sensory epithelia, such as the mammalian utricle and zebrafish lateral line neuromasts, deletion of the transcription factor gene *Emx2*, which may be negatively regulated by Notch signaling (Jacobo et al., 2019), causes a reversal in the overall plane of bundle orientation (Jiang et al., 2017). In the cochlea, however, deletion of *Emx2* leads to more severe developmental disruptions that prevent a clear identification of any possible roles for Emx2 in polarity (Holley et al., 2010; Jiang et al., 2017). Moreover, the direction of bundle orientation changes in different regions of the cochlear epithelium relative to the body axis, suggesting that a global external signal may not exist. Several studies have suggested that activation of the PCP pathway through cell-cell signaling is sufficient to achieve uniform orientation, but those same studies have indicated that an initial polarization signal must be present (Peng and Axelrod, 2012). Considering that the plane of polarization within the cochlear duct appears to switch at the border between the Kölliker's organ and the IHCs (Goodyear et al., 2017), the identification of factors that regulate or are localized to this boundary might provide insights regarding initial polarization cues.

Conclusions

The cochlea and the organ of Corti are fascinating structures. Within the organ of Corti, a minimum of six distinct cell types are specified in defined ratios from a common progenitor pool, and are arranged in a rigorous cellular mosaic that extends along the length of the cochlear spiral. Invariant and asymmetric cellular patterns are generated along all three structural axes (tonotopic, medial-lateral, lumenal-basal), while both the entire epithelium and individual cells become uniformly polarized. The precision required to assemble this structure suggests an incredible level of developmental organization. Over the last 20 years, remarkable strides have been made in our understanding of this process. It is clear that the organ of Corti derives from a population of uniquely specified prosensory cells that sort themselves into HCs and SCs, while simultaneously migrating to create the tonotopic axis. Although initial cues for the specification of at least some axial identities are derived from structures outside the ear, the coiled nature of the cochlea suggests internal patterning signals rapidly become paramount. Careful genetic dissections of several different aspects of the development of the cochlea, the organ of Corti and HCs have provided valuable insights into many different aspects of this developmental process. However, many features of cochlear development remain poorly understood. In particular, we have only recently begun to identify the factors that regulate the specification and number of the many

specialized cell types. The first steps have been taken to understand how OHCs are differentiated from IHCs, and pillar cells from other SCs, although the generation of other unique SC types is far less well understood. Similarly, although the factors that lay out the primary tonotopic axis have been identified, how that initial plan is leveraged to generate tonotopic gradients in the basilar membrane, tectorial membrane or HCs has not been determined. Nonetheless, with the advent of additional molecular and genetic tools, such as single cell transcriptomics and CRISPR/Cas9-based genome editing, it appears likely that the pace of discovery will increase.

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