

Chapter 3

New Directions in Cochlear Development

Andrew K. Groves and Donna M. Fekete

Abstract The mammalian cochlea is a highly specialized structure in the inner ear whose sensory organ, the organ of Corti, allows for exquisitely precise discrimination of sound frequencies over a huge range of sound amplitudes. The cochlea grows out as a ventral elaboration of the otocyst and is patterned by a variety of extracellular signals that originate both within the cochlear duct itself and from tissues adjacent to the cochlea. In this chapter, we describe the embryonic origins of the cochlear duct and spiral ganglion, the signals that induce the organ of Corti, the precise arrangement of the auditory hair cells and supporting cells, and recent work on how the hair cells become innervated by afferent and efferent neurons before the onset of hearing.

Keywords Bone morphogenetic protein • Cell cycle • Fibroblast growth factor • Hair cell • Inner ear • Morphogen • Otic placode • Patterning • Planar cell polarity • Retinoic acid • Sonic hedgehog • Sensory • Supporting cell • Tonotopy • Wnt

3.1 Introduction

The mammalian cochlea is specialized to detect sound of varying intensities, deconstructing it into its component frequencies and sending this information to the brain through the afferent axons of the spiral ganglion neurons. The brain also feeds

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back to the cochlea via two distinct efferent pathways. Due, in part, to the power of mouse genetics, remarkable progress has been made in the past 10–15 years to reveal molecular-genetic mechanisms of inner ear development in general and cochlear development in particular (see Kelley et al. 2005 for many aspects of inner ear development). This review describes a subset of these new discoveries related to the embryonic origin of the cochlea, the induction of its prosensory domain, the coordination of spatiotemporal gradients in cell cycle exit and differentiation, the fine-grained patterning of cell types in the organ of Corti, and the establishment of cochlear innervation. Some of the unresolved questions remaining in these areas are highlighted to encourage future studies.

3.2 Embryonic Origin of the Mammalian Cochlea

3.2.1 *Defining the Cardinal Axes of the Inner Ear*

The otic placode forms adjacent to the posterior hindbrain as the body plan of the embryo is being laid down after gastrulation and subsequently invaginates to form the otocyst. It is now well established that inducing signals that specify the anteroposterior (A-P) and dorsoventral (D-V) pattern of the embryonic nervous system as a whole are co-opted by the otocyst to define the cardinal axes of the future inner ear. Early in the development of the nervous system, the hindbrain undergoes a precise division into developmental compartments or rhombomeres (Trainor and Krumlauf 2000). Because the otocyst develops adjacent to rhombomeres 5 and 6 of the hindbrain, many developmental biologists hypothesized that these were the source of signals that defined the A-P axis of the ear. However, surgical manipulation of the posterior hindbrain in chicken embryos did not significantly affect otocyst patterning (Bok et al. 2005). Rather, the embryonic ectoderm surrounding the otocyst appeared to specify the A-P axis of the ear, and this activity could be mimicked by retinoic acid (RA; Bok et al. 2011). RA-synthesizing enzymes such as *Raldh2* are expressed in the mesoderm posterior to the developing otocyst, whereas RA-degrading enzymes such as *Cyp26C1* are expressed in the ectoderm anterior to the otocyst (Bok et al. 2011), thus providing a source and sink of RA to establish a gradient of RA responses across the otocyst. Treatment of either chick or mouse embryos with RA resulted in an expansion of posterior markers of the otocyst, such as *Tbx1*, whereas supplying an ectopic anterior source of RA resulted in an otocyst consisting of two mirror image posterior halves (Bok et al. 2011; Fig. 3.1).

Although the hindbrain does not appear to directly regulate the A-P axis of the otocyst, 180° rotation of the hindbrain on its D-V axis causes severe otocyst patterning defects (Bok et al. 2005). Sonic hedgehog (Shh) is a likely candidate for a signal that specifies ventral fate given its expression in the floor plate of the hindbrain and notochord and its demonstrated role in specification of ventral identity in the neural tube (Cohen et al. 2013). The role of Shh in specifying the

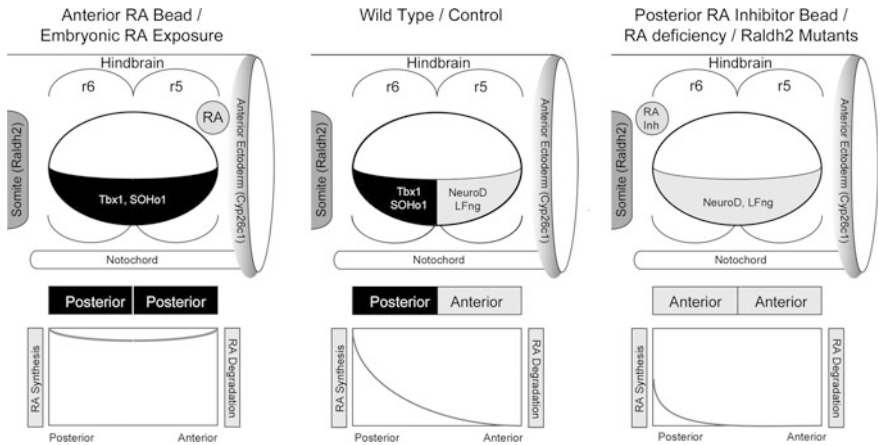


Fig. 3.1 Anteroposterior patterning of the otocyst. The mammalian otocyst is patterned in its anteroposterior axis by a gradient of retinoic acid (RA). The gradient is generated by a region of mesenchyme posterior to the otocyst (r6) that expresses the RA-synthesizing enzyme *Raldh2* and a region of ectoderm anterior to the otocyst (r5) that expresses the RA-degrading enzyme *Cyp26c1*. As a result, the RA concentration is high in the posterior region of the otocyst and lower in the anterior region. This leads to the establishment of distinct gene expression domains (*Tbx1* and *SOHo1* in the posterior half and *NeuroD* and *LFng* in the anterior half). Perturbations of the RA gradient by gain- or loss-of-function approaches lead to a disruption of the anteroposterior character of the otocyst

D-V pattern in the otocyst is supported by gain- and loss-of-function studies in mouse and chicken embryos (Riccomagno et al. 2002; reviewed in Wu and Kelley 2012).

Does *Shh* act directly on the otocyst or indirectly through its patterning of neural tissues? Studies on the transcriptional effectors of *Shh* signaling suggest that both mechanisms function to impart D-V identity to the ear. The *Gli* family of zinc finger transcription factors that mediate *Shh* signaling are expressed in the otocyst, with the transcriptional activators *Gli1* and *Gli2* being restricted to its most ventral regions (Bok et al. 2007). *Gli3* can act as both a transcriptional activator and a repressor; in the absence of *Shh* signaling, it is partially degraded to release an N-terminal domain (*Gli3* repressor [*Gli3R*]) that represses *Shh* targets, whereas in the presence of *Shh*, *Gli3* remains intact (*Gli3* activator [*Gli3A*]) and activates *Shh* targets (Wang et al. 2000). Thus, graded increases in *Shh* signaling gradually reduce *Gli3R* and progressively increase *Gli3A*, with the amount of *Shh* signal received by a given cell being the net output of *Gli3R* and *Gli3A* activities. In the ear, the apical region of the cochlear duct fails to form in *Gli2*^{-/-}; *Gli3*^{-/-} mutants or the *Gli3D699* mutant, which only generates the *Gli3R* form (Bok et al. 2007). This suggests that the apical (ventral) regions of the cochlear duct, which are closest to *Shh* sources (floor plate and notochord), require *Gli3A* function.

Shh levels also affect the development of more dorsal components of the ear. Analysis of the same *Gli* mutants suggests that basal regions of the cochlear duct

and the saccule may be induced by progressively lower exposure to Shh, leading to a balance of Gli3A and Gli3R activities. Indeed, the basal regions of the cochlear duct and saccule are missing in *Shh* mutants (which cannot activate *Gli* genes and therefore have more Gli3R activity) but persist in *Shh*^{-/-}; *Gli3*^{-/-} mice (which lack both Gli3A and Gli3R functions; Bok et al. 2007). More dorsally, *Gli3*^{-/-} mice (in which Gli3A function is compensated by Gli2 and Gli1 but which lack any Gli3R activity) have malformed semicircular canals, which suggested that Gli3R activity is necessary to allow these structures to form properly (Bok et al. 2007). However, a more recent study that conditionally inactivated the Shh receptor Smoothed (Smo) in the otocyst found that the cochlea and saccule were completely absent but that dorsal components of the inner ear (the semicircular canals, endolymphatic duct, cristae, and utricle) appeared to develop quite normally (Brown and Epstein 2011). Reconciling these conflicting studies suggests that Shh acts on the ventral otocyst directly but that the dorsal otocyst is indirectly dependent on Shh through its patterning effects on tissues adjacent to the otocyst, such as the neural tube (Groves and Fekete 2012; Wu and Kelley 2012; Fig. 3.2).

Members of the Wnt family are good candidates to regulate dorsal fates in the otocyst. Within the neural tube, the action of Shh ventrally is opposed by Wnt signals in the dorsal neural tube and ectoderm. In the case of the otocyst, Wnts may play a similar role to specify dorsal identity, although initially they probably act in a paracrine fashion that is then enhanced or replaced by autocrine signaling. *Wnt6* is expressed at the neural plate border immediately adjacent to the otic placode (Jayasena et al. 2008). Both *Wnt1* and *Wnt3a* are later expressed in the dorsal neural tube (Riccomagno et al. 2005) adjacent to the otocyst. *Wnt2b* is expressed in the dorsomedial otocyst (Riccomagno et al. 2005), with another 14 Wnt ligands expressed in the otocyst by embryonic day 11.5 (E11.5; Summerhurst et al. 2008). As the placode invaginates and closes to form the otocyst, Wnt-reporter mice exhibit a D-V gradient of reporter activity, indicated by Lef/TCF-mediated transcriptional activation of transgenes (Riccomagno et al. 2005; Ohyama et al. 2006). Moreover, *Wnt1*; *Wnt3a* double mutants completely lack dorsal inner ear structures, with the remaining cochlea persisting as a rudimentary cyst (Riccomagno et al. 2005). Conversely, activation of Wnt signaling in cultured otocysts causes many dorsal markers to expand into ventral regions, and these same markers are reduced by ablation of the dorsal neural tube in chicken embryos (Riccomagno et al. 2005).

3.2.2 *Transforming the Ventral Otocyst into the Cochlear Duct*

The cochlear duct forms as an elaboration of the ventral otocyst by E11 in the mouse, with a clear protrusion that marks the beginning of the cochlear duct (Wu and Kelley 2012; Brown et al. 2015). The cochlear duct then elongates and coils until it reaches its full one and three-quarter turns in mice, although there is considerable variation in cochlear length and width between mammals (Ekdale 2015).

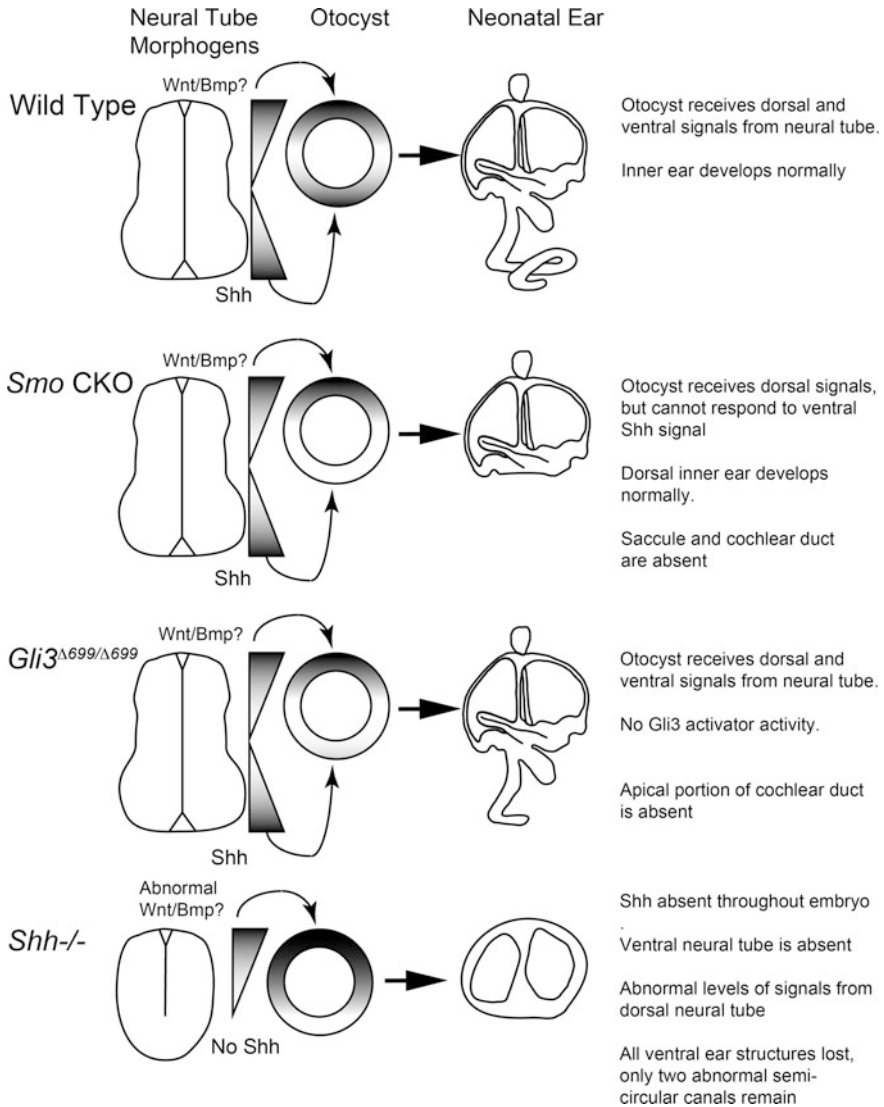


Fig. 3.2 Dorsoventral patterning of the otocyst. Dual morphogen gradients of Sonic hedgehog (Shh) released from the floor plate and Wnts and possibly bone morphogenetic proteins (BMPs) released from the roof plate pattern the dorsoventral axis of the otocyst. If the Shh receptor Smoothed (Smo) is deleted in the otocyst, the dorsal half of the otocyst develops normally in response to Wnt and BMP signals, but the saccule and cochlear duct are absent. In mice carrying a point mutation of *Gli3*, which removes its activator activity, only the most apical part of the cochlear duct is absent. However, in the absence of all Shh activity, the neural tube develops abnormally, and this removes ventral structures and causes dorsal structures to develop abnormally

Fate mapping using a *Neurog1-CreER* transgenic mouse to track *Neurog1*-expressing progenitors that arise in the anteroventral otocyst at E9 shows a significant contribution to the utricle, saccule, and VIIIth ganglion (Koundakjian et al. 2007; Raft et al. 2007). However, only a few descendants of these *Neurog1*-expressing cells are found in the cochlear duct and almost none in the organ of Corti itself (Koundakjian et al. 2007; Raft et al. 2007). Fate mapping using either a CreER line driven by Wnt-responsive *Lef/TCF* binding sites (*TOP-CreER*) or a *Gbx2-CreER* line shows that some Wnt-responsive progenitors located in the medial wall of the otocyst between E9.5 and E11.5 will contribute to the sensory epithelium of the cochlear duct by spreading ventrally (Riccomagno et al. 2005; Brown et al. 2015).

The elongating cochlear duct can be thought of as a tube divided into at least five sectors in cross section (Fig. 3.3). Rotating clockwise from the anterior region, these sectors are the roof of the cochlear duct (becomes Reissner's membrane), the lateral wall (becomes the stria vascularis), the lateral nonsensory floor (becomes the outer sulcus), the prosensory floor (becomes the organ of Corti), and the medial nonsensory floor/wall (becomes the greater epithelial ridge/inner sulcus; Kelley 2007; Wu and Kelley 2012). Several key genes that pattern these cochlear domains are shown in Fig. 3.3 and include *Otx2* (roof; Vendrell et al. 2015), *Pax2* (strial wall; Burton et al. 2004), *bone morphogenetic protein 4* (*Bmp4*; lateral floor; Ohyama et al. 2010), *Sox2* and *Jag1* (prosensory domain; Ohyama et al. 2010), and *Lfn3* and *fibroblast growth factor 10* (*Fgf10*; medial floor/wall; Morsli et al. 1998; Urness et al. 2015). As the cochlear duct grows in length, these domains elongate in the manner of striped toothpaste being squeezed out of a tube, thus preserving their respective territories along the length of the cochlear duct (Fig. 3.3).

3.2.3 Outstanding Issues Concerning the Embryonic Origin of the Cochlea

The cochlear duct likely developed in coordination with the basilar papilla as they arose and enlarged within the sarcopterygian or amphibian lagenar recesses (Fritzscht et al. 2013). As amniotes diverged from the anamniote relatives, the enlarging basilar papilla gradually displaced the lagenar macula to the distal end of what became the cochlear duct (Smotherman and Narins 2004; Fritzscht et al. 2013). The lagena has been lost from the cochlea of all modern mammals with the exception of the egg-laying monotremes (Manley 2012). How were gene regulatory networks co-opted to permit the appearance of the basilar papilla as a distinct sensory organ and the enlargement of the lagenar recess to form a cochlear duct? Evidence from mouse mutants has generated a rapidly growing list of genes that can regulate the growth of the cochlear duct and organ of Corti, including transcription factors or cofactors, signaling ligands and receptors, and regulators of the cytoskeleton. However, it is not known whether any of these genes or the regulatory networks in which they reside were deployed during the derivation of the cochlear

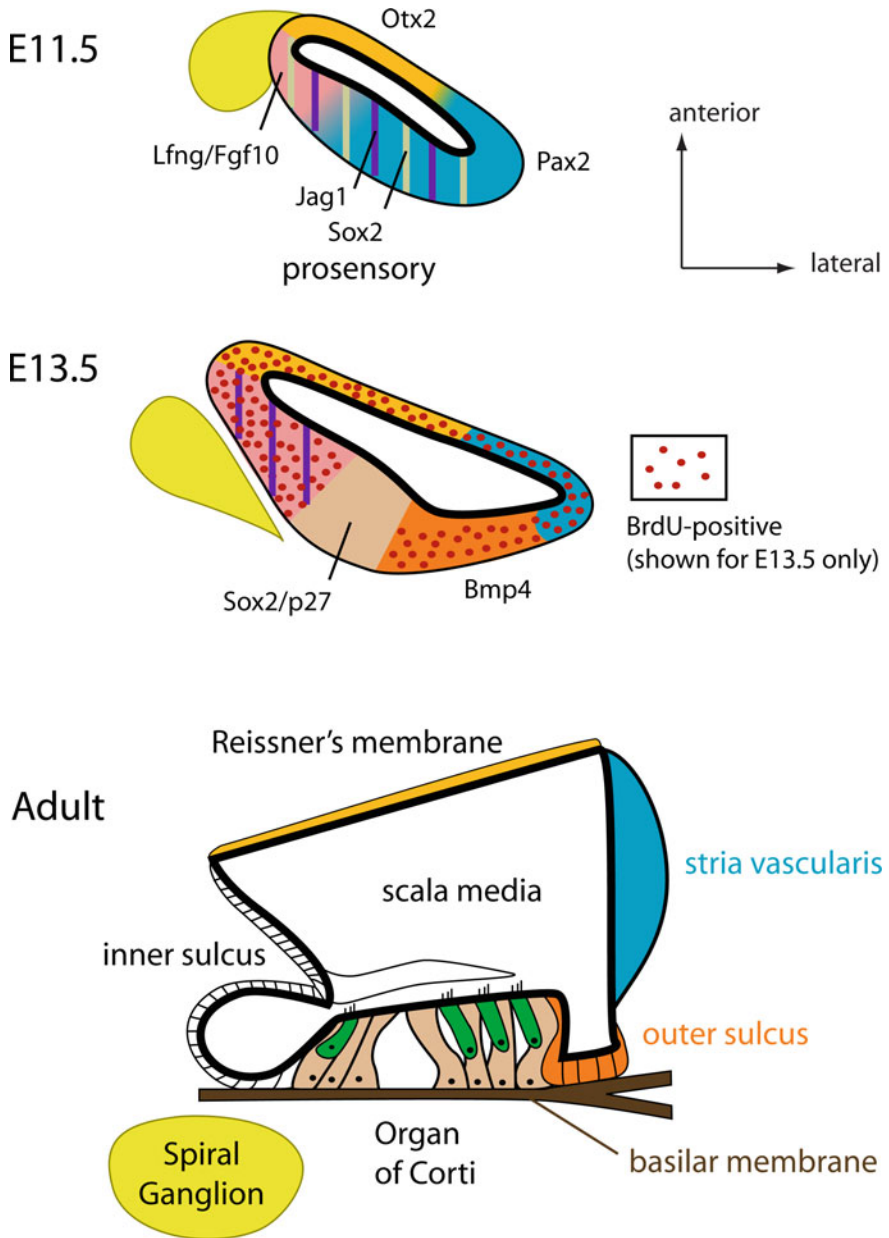


Fig. 3.3 Schematic of regional subdivision of the developing cochlear duct. This schematic highlights a small subset of the genes that gradually emerge as early markers of different cochlear tissues

duct from the lagenar recess or whether the appearance and expansion of the sensory and nonsensory components were coordinated together (perhaps by a cochlear “master gene”) or separately as independent events.

What are the signals that establish the different territories of the cochlea in the ventral otocyst? As discussed in Sect. 3.2.1, conditional deletion of the Shh receptor *Smo* in the otocyst prevents the formation of the cochlear duct without significantly affecting other regions of the inner ear (Brown and Epstein 2011), suggesting that Shh signaling may lie upstream from regional determinants of cochlear identity. Interestingly, the anterior and ventral regions of the otocyst that express *Neurog1* and *LFng* are not strongly affected in *Smo* conditional mutants (Brown and Epstein 2011), although they normally contain some progenitors that can give rise to nonsensory regions of the cochlea in addition to the utricle, the saccule, and their associated maculae (Koundakjian et al. 2007; Raft et al. 2007). It will be of interest to determine whether these cochlear progenitors are diverted to other fates if they are unable to receive Shh signals or whether they simply die.

The signals responsible for the posteroventral stripe of *Bmp4* expression in the otocyst that will become the future outer sulcus of the cochlea are presently unknown, although a recent identified, long noncoding RNA, *Rubie*, that is located upstream from the *Bmp4* locus has been shown to be expressed in an identical pattern as *Bmp4* itself (Roberts et al. 2012) and may play a role in the fine tuning of *Bmp4* expression. The observation that the organ of Corti derives from Wnt-responsive progenitors (Riccomagno et al. 2005; Brown et al. 2015) suggests that Wnt signaling may play a direct or indirect role in the expression or maintenance of early markers of the prosensory domain/future cochlear duct such as *Sox2* and *Jag1* (Munnamalai and Fekete 2013). It is also possible that these signals may act to repress ventrolateral genes such as *Otx2* that will be expressed in the roof of the cochlear duct. A recent study of *Otx2* conditional mutant mice suggests that *Otx2* may repress *Sox2* expression in the nascent cochlear duct, suggesting potential cross-repressive interactions in the division of the medial and lateral territories of the ventral otocyst (Vendrell et al. 2015).

3.3 Formation of the Cochlear Prosensory Domain

3.3.1 Role of Notch Signaling and Sox2 in the Formation of Prosensory Patches

Over the past decade, considerable progress has been made in understanding what regulates the specification and size of the prosensory domains. Each inner ear prosensory domain (organ of Corti, three cristae ampullae, and two maculae) expresses both *Sox2* and *Jag1* (Kiernan 2013). In the vestibular organs of chicken and mice, Jag1-Notch signaling helps specify the size and position of the prosensory patches through a mechanism of Notch-mediated lateral induction (Kiernan 2013; Neves et al. 2013). In this model, activation of Notch receptors by Jag1

ligands leads to the upregulation of *Sox2* in the signal-receiving cell. Evidence for this comes from both loss-of-function studies in which the mutation of either *Jag1* or *Sox2* leads to missing or greatly reduced prosensory patches throughout the ear (Kiernan et al. 2005a; Brooker et al. 2006) or gain-of-function studies in which ectopic expression of *Sox2*, *Jag1*, or the canonical Notch-signaling pathway can induce ectopic prosensory patches in regions of the ear that would normally form nonsensory tissue (reviewed in Neves et al. 2013).

Does this Notch-driven pathway also act within the prosensory domain of the cochlea? To be sure, ectopic activation of either *Sox2* or the canonical Notch-signaling pathway in nonsensory regions of the cochlea can induce the formation of ectopic sensory regions containing hair cells and supporting cells (Kiernan 2013; Pan et al. 2013), and severe *Sox2* hypomorphic mutations also prevent formation of the cochlear prosensory domain and the organ of Corti (Kiernan et al. 2005b). However, although conditional deletion of *Jag1* causes significant loss of cochlear sensory tissue (Kiernan et al. 2006), some prosensory markers still persist in these mutants (Basch et al. 2011). Also, in mice with conditional deletion of *Rbpjk*, the transcriptional mediator of canonical Notch signaling, hair cells continue to form, although these rapidly die (Basch et al. 2011; Yamamoto et al. 2011). Although some of these discrepancies may be due to technical differences in markers used or stages analyzed, it is also important to note that unlike in other prosensory patches, *Jag1* is downregulated from the prosensory domain of the cochlea shortly after outgrowth commences and becomes restricted to the nonsensory epithelium of Kölliker's organ on the neural side of the cochlear duct (Fig. 3.3). This restriction of *Jag1* is regulated in part by *Bmp4*, which is expressed on the opposite, abneural side of the duct (Ohyama et al. 2010). This establishes an asymmetry in *Jag1* expression and in the activation of Notch signaling at the border between the *Jag1* domain and the adjacent *Sox2* domain, where the first inner hair cells will form (Murata et al. 2006). It is possible that this asymmetrical pattern of Notch activation leads to the asymmetrical pattern of the organ of Corti, where its polarized array of inner and outer hair cells contrasts with the more symmetrical arrangement of hair cells seen in the cristae and maculae of the vestibular organs.

3.3.2 Role of Other Signaling Pathways in the Induction of the Cochlear Prosensory Domain: Fibroblast Growth Factors, Bone Morphogenetic Proteins, and Wnts

The current conflicting results of the effect of Notch pathway mutants on the induction of the cochlear prosensory domain raise the possibility that other pathways may also act to induce this region of the cochlea in a manner distinct from other sensory organs of the ear. Fgf signaling is one such candidate pathway

because *Fgfr1* conditional mutants have very few hair cells in the cochlea but apparently normal numbers of hair cells in the vestibular organs (Pirvola et al. 2002). *Fgfr1* conditional mutants also show a decrease in proliferation in some regions of the young cochlea, particularly the greater epithelial ridge (Pirvola et al. 2002), but this study did not directly examine the presence of markers of the prosensory domain. Thus it is not clear whether the defects in these mutants are due to a failure of prosensory induction per se, reduced proliferation of prosensory progenitors, abnormal differentiation of the prosensory region, or a combination of all three effects. Candidate Fgfs, such as Fgf8, -10, or -20, either are not expressed early enough in the cochlea to influence prosensory induction (*Fgf8*; Jacques et al. 2007) or do not compromise the induction of the prosensory domain when mutated (*Fgf10* and *Fgf20*; Huh et al. 2012; Urness et al. 2015).

Bmp4 is expressed on the abneural side of the cochlear duct in the future outer sulcus and establishes a gradient of Bmp signaling across the abneural-neural axis of the duct (Ohyama et al. 2010). Disruption of Bmp signaling of the *Bmpr1a* and *Bmpr1b* receptors in compound conditional mutants causes a complete failure of prosensory domain formation. Instead, the cochlear duct continues to express Jag1 across the entire neural-abneural axis and develops with the molecular identity of Kölliker's organ (Ohyama et al. 2010). The prosensory domain would normally form in the region of the cochlear duct that receives medium doses of Bmp signaling. Thus, it is possible that moderate activation of the Bmp pathway acts to *directly* induce the prosensory domain or, alternatively, simply acts *indirectly* to downregulate Jag1, thereby allowing other inducing signals to function.

Recent evidence also raises the possibility that canonical Wnt signaling may play a role in the induction of the prosensory domain. Wnt-responsive *TCF/Lef* fluorescent reporter mice show a strong activation of the canonical Wnt-signaling pathway across the entire radial (neural-abneural) axis of the cochlear duct at E12.5 (Jacques et al. 2014), and pharmacological or genetic activation of the canonical Wnt pathway in the cochlea leads to an expansion of prosensory markers into nonsensory regions of the cochlea (Jacques et al. 2012; Shi et al. 2014). In contrast, pharmacological or genetic inhibition of the canonical Wnt pathway at E12.5 in these studies did not significantly affect the development of the prosensory domain. However, because the prosensory domain is already present at this time, further experiments will be necessary to determine if canonical Wnt signaling is necessary for induction of the cochlear prosensory domain as opposed to being necessary for maintaining prosensory identity.

3.3.3 Outstanding Issues Concerning the Induction and Radial Patterning of the Prosensory Domain

As discussed in Sect. 3.3.1, the role of Notch signaling in the induction of the prosensory domain of the cochlea has yet to be settled because different studies have used different approaches to disrupt Notch signaling, and to date, none have

resulted in a total and exclusive loss of Notch function. For example, in *Rbpjk*-mutant mice, all canonical Notch signaling is disrupted; however, because this protein acts as a transcriptional repressor in the absence of Notch signaling (Tanigaki and Honjo 2010), some *Rbpjk*-mutant phenotypes may be due to a combination of derepression of genes that are normally repressed by Notch signaling as well as a loss of Notch signaling itself. Although *Jag1* is the only Notch ligand to be strongly expressed in prosensory areas in the ear, different sensory organs are differentially affected in *Jag1* conditional mutant mice, for example, the utricular macula is almost completely absent, whereas the saccular macula remains largely intact (Kiernan et al. 2006). This variable phenotype could be caused by persistence of the Jag1 protein after conditional mutation of the *Jag1* gene or by compensation by another Notch ligand, possibly one of the several “noncanonical” Notch-interacting membrane proteins such as DNER or Dlk-1 (D’Souza et al. 2010). Ultimately, this issue will only be addressed by a complete conditional inactivation of all Notch receptors in the inner ear from as early a stage as possible.

An unresolved question about cochlear development is whether diffusible morphogens underlie radial axis patterning. A defining characteristic of a morphogen is that it is expressed in a gradient, and cells respond differently to different threshold concentrations of the morphogen. Morphogen gradients are often established across an equipotential field of progenitors by the presence of an asymmetric source at one end, which can be enhanced by a sink at the other end. In the developing cochlea, molecules associated with BMP, Wnt, and Fgf signaling are asymmetrically expressed within or adjacent to the presumptive organ of Corti as it segregates into morphologically distinct parts: medial (border cells, inner hair cells, and inner phalangeal cells), middle (pillar cells), and lateral (outer hair cells and Deiters and other supporting cell types). At E11.5–E12.5, *Fgf10* is expressed in the medial nonsensory flank of the organ, *Wnt5a* is expressed in a gradient that is highest on the medial half of the duct, and *Bmp4* is expressed in the lateral nonsensory flank. These three morphogen family members might serve instructive roles in subdividing the radial axis, although further experiments are required to definitely show that any of these has classical morphogen-like activity on cochlear progenitors (discussed further in Groves and Fekete 2012).

3.4 Coordinating Developmental Gradients of Differentiation and Cell Cycle Exit in the Cochlea

3.4.1 The Unusual Pattern of Cell Cycle Exit and Differentiation in the Cochlea

Once the cochlear prosensory domain has been induced, its progenitors begin to exit the cell cycle and differentiate into the distinct hair cell and supporting cell types of the organ of Corti. In contrast to the mammalian vestibular sensory organs,

where cell cycle exit and differentiation appear to initiate in the center of each organ and spread outward radially over an extended period of time (Kirkegaard and Nyengaard 2005; Burns et al. 2012), the prosensory region of the cochlea shows a radically different pattern of cell cycle withdrawal and differentiation (reviewed in Basch et al. 2016). Cell cycle exit commences in the most apical (tip) region of the cochlear prosensory domain on E12.5 in mice and spreads toward the base of the duct over the next few days, although the most basal cells do not complete their final cell division until E15.0 (Lee et al. 2006). Shortly after the onset of cell cycle exit in the apex, cells in the midbasal region begin differentiating into hair cells by expressing the *Atoh1* transcription factor (Cai et al. 2013). *Atoh1* expression then spreads to the base in one direction and in the other direction toward the apex over the next 3–4 days. Thus, the *first* cells to exit the cell cycle at the apex of the cochlea on E12.5 are the *last* ones to differentiate into hair cells. At present, very little is known about how cell cycle exit is initiated in the apex nor how this wave of withdrawal from the cell cycle propagates along the cochlea.

In the last 15 years, a number of cell cycle regulators have been implicated in the cell cycle exit of the cochlear prosensory domain. The G₁ cyclin *CyclinD1* is expressed in proliferating progenitors at this stage and is maintained by canonical Wnt signaling (Jacques et al. 2012). Concomitant with cell cycle exit on E12–E13.5, the cyclin-dependent kinase inhibitor *p27^{kip1}* (*Cdkn1b*) is expressed in the cochlea in an apical-basal gradient (Chen and Segil 1999; Lee et al. 2006). The prolonged period of cell proliferation in the apical regions of the *p27^{kip1}*-mutant cochlea causes an overproduction of both hair cells and supporting cells (Chen and Segil 1999; Lowenheim et al. 1999). In postmitotic supporting cells, *p27^{kip1}* becomes enriched, whereas other cyclin-dependent kinase inhibitors such as *p19^{ink4d}* (*Cdkn2d*) and *p21^{cip1}* (*Cdkn1a*) become upregulated in hair cells and supporting cells. Mutation of these genes causes abnormal cell cycle reentry and death in the organ of Corti (reviewed in Schimmang and Pirvola 2013). Other cell cycle regulators such as the pocket protein family (*Rb1*, *Rbl1/p107*, and *Rbl2/p130*) also maintain the postmitotic state of hair cells and supporting cells, although different members appear to play different roles in the developing and mature cochlea (Rocha-Sanchez and Beisel 2007; Rocha-Sanchez et al. 2011).

In searching for genes that control or coordinate the onset of cell cycle withdrawal and differentiation in the mouse cochlea, investigators studied the mammalian homologs of so-called heterochronic genes that regulate developmental timing in the nematode worm *C. elegans* (Golden et al. 2015). Two of these genes, *Lin28* and *let-7*, oppose each other in controlling the timing of cell lineage decisions. *Lin28* encodes an RNA-binding protein that promotes stemness and prevents differentiation by stabilizing the transcripts of growth-promoting genes. *Lin28* also blocks the biogenesis of mature *let-7* microRNA. Conversely, *let-7* microRNAs repress the translation of *Lin28* as well as other genes that enhance proliferation. These counteracting effects on developmental timing are partly conserved in the mouse cochlea. *Lin28b* transcript levels fall as the prosensory cells pull out of

division between E13 and E16, while the levels of several *let-7* family members rise from E13 to E18 as cells differentiate. Overexpression of human *LIN28B* before E13 delays hair cell differentiation by causing a 16-hour delay in cell cycle exit. This is accompanied by an 80% decrease in *let-7* and significant increases in several *let-7* target transcripts, including *N-myc* and *cyclin D1* that promote cell proliferation in the cochlea. As might be expected, forcing the premature expression of a modified form of *let-7g* that is resistant to negative regulation by *Lin28b* causes precocious cell cycle exit and shortens the cochlea by 40% because of an insufficient number of prosensory cells. However, despite pulling out of division early, hair cells do not immediately differentiate (Golden et al. 2015). That is, the *Lin28b/let-7* network is partly uncoupled in the cochlea compared with the worm. This underscores the cochlea's unusual systematic lengthening of the interval between cell cycle exit and the onset of cell differentiation in progressing from base to apex. These data suggest that the timing of hair cell differentiation is held in check through a *let-7*-independent mechanism, possibly through the activity of *Shh*, as described in Sect. 3.4.2.

3.4.2 How Are Cell Cycle Exit and Differentiation Uncoupled in the Cochlea?

The temporal and spatial uncoupling of cell cycle exit and differentiation in the mammalian cochlea is very unusual because progenitor cells in other systems typically exit the cell cycle and differentiate almost simultaneously, with differentiation signals often triggering cell cycle exit. Mammalian vestibular sensory organs and all nonmammalian sensory organs tend to have a radially symmetrical pattern of hair cells and supporting cells, with a single hair cell surrounded by between 4 and 8 supporting cells (Goodyear and Richardson 1997). This radial pattern appears to be generated by establishing a central region of differentiation and cell cycle exit that expands outward circumferentially. This pattern of cell behavior has been observed in both the mammalian utricle and bird basilar papilla (Katayama and Corwin 1989; Burns et al. 2012). However, one may speculate that the precise and invariant pattern of one inner hair cell, three outer hair cells, and their associated supporting cell types that is repeated serially in the organ of Corti along its length may require the entire progenitor domain to become postmitotic before differentiation commences. In support of this, mouse mutants that delay cell cycle exit in the cochlea have disrupted proportions of hair cells and supporting cells, especially in the apical regions where cell cycle exit normally begins (Chen and Segil 1999).

What delays the onset of hair cell differentiation in the cochlea relative to cell cycle exit? Studies of a number of mouse mutants suggest a role for neurons of the spiral ganglion in holding the differentiation of hair cell progenitors in abeyance.

Prosensory progenitors in *Neurog1*-mutant mice exit from the cell cycle about 24–36 hour earlier than normal, and hair cell differentiation starts in the apical, not the basal, region of these mutants (Matei et al. 2005). A similarly abnormal pattern of hair cell differentiation is seen in *Neurod1*-mutant mice (Jahan et al. 2010). Because *Neurod1* and *Neurog1* are not expressed in the cochlea at detectable levels during differentiation of the prosensory domain, it is unlikely that these transcription factors are regulating hair cell differentiation directly but indirectly by controlling spiral ganglion neurogenesis. Both genes are expressed in neuronal progenitors that delaminate from the anteroventral otocyst earlier in development (Raft et al. 2004) and *Neurod1* or *Neurog1* mutations severely compromise neurogenesis, leading to an absence or severe hypoplasia of the spiral ganglion (Ma et al. 2000; Kruger et al. 2006). These data suggest that signals from the developing spiral ganglion may regulate the timing of differentiation of cochlear hair cells.

Clues as to the identity of the signal released by the spiral ganglion came from studies of mutants in the Shh-signaling pathway. Hypomorphic mutants of the Shh mediator *Gli3* (*Gli3*^{Δ699}), a mouse model of Pallister-Hall syndrome, contain ectopic hair cells (Driver et al. 2008), and treatment of developing cochlear explants with Shh or the Shh inhibitor cyclopamine causes a decrease or increase in the number of hair cells, respectively (Driver et al. 2008). *Shh* is expressed in the spiral ganglion (Liu et al. 2010; Bok et al. 2013) and is downregulated from basal regions of the spiral ganglion at a similar time to the onset of *Atoh1* expression in the corresponding basal regions of the cochlea, providing a circumstantial link between the disappearance of the Shh signal and the initiation of hair cell differentiation. This circumstantial link was independently confirmed by conditional inactivation of *Shh* in the spiral ganglion (Bok et al. 2013) and inactivation of the Shh receptor *Smo* in the cochlear duct (Tateya et al. 2013). Both conditional mutants have a truncated cochlea in which prosensory cells exit the cell cycle and differentiate prematurely. Strikingly, the expression of *Atoh1* and later hair cell markers in these mutants follows an apical-to-basal gradient instead of the normal basal-to-apical gradient seen in the presence of Shh (Bok et al. 2013; Tateya et al. 2013).

3.4.3 Outstanding Issues Concerning Cell Cycle Exit and Differentiation in the Prosensory Domain

The separate waves of cell cycle exit and differentiation of the prosensory domain along the apical-basal axis of the cochlea are without precedent in the mammalian nervous system. It is still unclear how these patterns are initiated and propagated along the cochlear duct. In the case of cell cycle exit, transgenic reporter lines have clearly demonstrated that the apical-basal gradient of *p27^{kip1}* is regulated at both the transcriptional and posttranscriptional levels (Lee et al. 2006). Although cell cycle exit in the mouse cochlea is partially dependent on Shh signaling (Bok et al. 2013),

loss of *Shh* specifically from the spiral ganglion has a far less severe effect on cell cycle exit than on the onset and position of hair cell differentiation (Bok et al. 2013). However, because the progenitors for the future apex of the cochlea are likely to be located in the ventral and medial region of the otocyst, it is possible that *Shh* from the notochord and floor plate of the hindbrain may partially compensate for the loss of *Shh* in spiral ganglion conditional knockouts of *Shh*. Another possibility is that the regulation of cell cycle exit by *Lin28B* occurs independently of *Shh*.

The basal-apical wave of hair cell differentiation does not appear to require an intact cochlear duct or direct contact with the spiral ganglion or underlying mesenchyme because dissected pieces of the mouse cochlear duct are capable of initiating hair cell differentiation in an approximately normal temporal sequence even when cultured separately (Montcouquiol and Kelley 2003). Although loss of *Shh* from the spiral ganglion clearly leads to precocious hair cell differentiation *in vivo*, it is unclear whether there is an additional positively acting hair cell differentiation signal that is normally held in check by *Shh*. *Atoh1* has been shown to be regulated by a variety of other signals, such as *Bmps*, *Fgfs*, and *Wnts* (reviewed in Mulvaney and Dabdoub 2012; Cai and Groves 2014). If these factors are acting in the cochlea *in vivo*, it will be of interest to understand how they initiate hair cell differentiation precisely at the boundary of the prosensory domain and Kölliker's organ (Cai et al. 2013).

An understanding of the mechanisms leading to the acquisition of positional information along the basal-apical axis is another unresolved area of mammalian cochlear development. Once development is completed, there will be numerous biophysical, morphological, and biochemical gradients along the longitudinal axis of the cochlea that correlate systematically with frequency response. As recently as 2011, a review of the development of cochlear tonotopy indicated a remarkable dearth of molecular candidates that might impart basal-apical positional information (Mann and Kelley 2011). Three years later, significant progress was made using the chicken basilar papilla as a model system, where tonotopy is also associated with basal (proximal) to distal (apical) position. Transcriptome comparisons of large longitudinal sectors of the basilar papilla led to the discovery of molecular gradients unrelated to those involved in cell cycle withdrawal (Mann et al. 2014; Thiede et al. 2014). Transcripts for *Bmp7* and a *Bmp* inhibitor, *chordin-like 1*, are expressed in counter-gradients on E6.5 (Mann et al. 2014). Manipulation of *Bmp* signaling (up or down) alters hair cell differentiation in a manner that is consistent with a reprogramming of proximal-distal identity. A parallel study revealed a longitudinal gradient in the transcripts for a RA synthetic enzyme, *Raldh3*, which is highest proximally on E6.5 but reverses direction from E10 onward (Thiede et al. 2014). Based on experimental manipulations where high RA signaling is a powerful inducer of distal hair cell morphologies, the latter gradient appears to be functionally relevant (Thiede et al. 2014). It remains to be determined what sets up the initial *Bmp7* gradient in the bird cochlea, nor is it known whether *Bmp*- and RA-signaling pathways can influence the tonotopic organization of the mammalian cochlea.

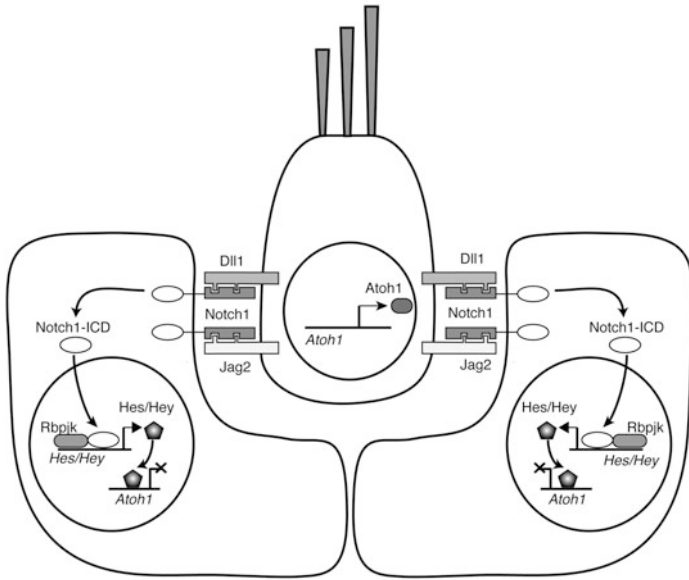
3.5 Fine-Grained Patterning and Cell-Type Specification in the Organ of Corti

As discussed in Sect. 3.3.2, the coordinated activation of a number of signaling pathways in a graded manner across the neural-abneural axis of the prosensory domain leads to the formation of three distinct regions in the organ of Corti defined by inner hair cells, pillar cells, and outer hair cells (Raphael and Altschuler 2003; see also Groves and Fekete 2012). Although other vertebrate species display morphological and functional variation of hair cells in the neural-abneural and basal-apical axes of their hearing organs (basilar papillae; Manley 2000), the separation of inner and outer hair cell regions by pillar cells is an exclusively mammalian innovation that can be observed in even the most basal mammals, the egg-laying monotremes (Ladhams and Pickles 1996). Whatever evolutionary events led to the specialization of the organ of Corti from a basilar papilla, it required the layering of distinct signals and gene regulatory networks to specify distinct cell types on top of the pathways that specified generic hair cell versus supporting cell fate decisions.

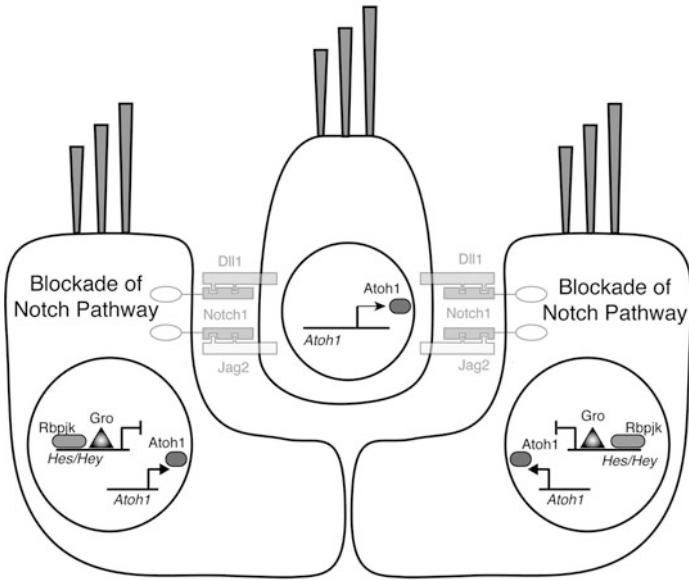
3.5.1 Notch Signaling as a Mechanism to Distinguish Hair Cells from Supporting Cells

The Notch-signaling pathway is an evolutionarily ancient mechanism of cell contact-mediated fate specification that can specify a fine-grained pattern of so-called primary cell fates versus secondary cell fates and can also specify and sharpen boundaries (Artavanis-Tsakonas and Muskavitch 2010). The alternating mosaic of hair cells and supporting cells seen in all vertebrate hearing and balance organs was first suggested as an excellent candidate for regulation by Notch signaling by Julian Lewis over 25 years ago (Lewis 1991). Since then, it has been firmly established that differentiating hair cells express Notch ligands (typically Dll1, Dll3, and Jag2) that signal to Notch receptors expressed by supporting cells (Kiernan 2013). The activation of Notch signaling leads to the repression of hair cell-specific transcription factors such as *Atoh1*, which in turn prevents the expression of hair cell-specific Notch ligands (Fig. 3.4). The resultant lateral inhibition (as distinct from lateral induction of prosensory specification mentioned earlier) has negative feedback that leads to the generation of a stable array of hair cells delivering Notch signals to neighboring supporting cells. Accordingly, genetic or pharmacological disruption of Notch signaling in a variety of vertebrate species leads to the development of extra hair cells at the expense of supporting cells (Kiernan 2013).

Notch Pathway Active



Notch Pathway Blocked



Supporting Cell - Hair Cell Transdifferentiation

◀**Fig. 3.4** A simple model of Notch signaling between hair cells and supporting cells. Hair cells express the Atoh1 transcription factor, which induces the expression of Notch ligands. These signal to supporting cells via Notch receptors, leading to the formation of an activation complex between the Notch intracellular domain (Notch1-ICD) and the coactivator RBPJ κ . This activates expression of Notch downstream target genes (such as the Hes and Hey transcription factors). These suppress Atoh1 expression and hair cell fate in the supporting cells. If Notch signaling is blocked genetically or pharmacologically, the transcriptional activation complex cannot form and a RBPJ κ -Groucho repression complex blocks Notch downstream genes. Atoh1 is thus induced in the supporting cells, driving them to a hair cell fate

Despite the widespread acceptance of the regulation of hair cell and supporting cell fate by Notch signaling, many studies in the past 10 years suggest that this simple model cannot explain all aspects of hair cell and supporting cell patterning in the organ of Corti. First, simple models of lateral inhibition typically generate a radial pattern of a hair cell surrounded by a series of supporting cells of the sort observed, at least to the first approximation, in bird hearing organs and mammalian vestibular organs (Goodyear et al. 1995) rather than the alternating rows of hair cells and supporting cells seen in the organ of Corti (Kelley 2006). Second, although conditional mutants of the *Notch1* receptor or compound mutants of *Jag2* and *Dll1* (*Jag2*^{-/-}; *Dll1*^{hyp/-}) cause many supernumerary hair cells at the expense of supporting cells, some supporting cells appear to be unaffected in these mutants (Kiernan et al. 2005a). Many inner pillar cells persist in these mutants (see Sect. 3.5.2 below), and *Jag2*^{-/-}; *Dll1*^{hyp/-} mutants also appear to retain multiple border and inner phalangeal cells beneath supernumerary inner hair cells (Kiernan et al. 2005a). In other words, the increase in hair cells is not always matched by an expected decline in supporting cells as evidence of a cell fate switch. Moreover, the presence of occasional persistent bromodeoxyuridine (BrdU)-labeled cells suggests that the Notch pathway may also regulate cell cycle exit of at least some organ of Corti progenitors (Kiernan et al. 2005a). It is possible that residual supporting cell differentiation in these mutants reflects a failure to completely abolish all Notch signaling in the cochlea, and a resolution of this issue must await analysis of mice in which all Notch receptors expressed in the ear are completely mutated.

3.5.2 *Specification of Subtypes of Hair Cells and Supporting Cells in the Organ of Corti*

Although Notch signaling is used repeatedly in development to generate fine-grained patterns of alternating cell types, a given strength of Notch activation typically does not discriminate between different variants of a particular class of cells by itself. Thus, in the cochlea, disruption of Notch signaling generates supernumerary hair cells at the expense of some supporting cells, but the identity of these supernumerary hair cells, inner versus outer, is consistent with the location of their normal counterparts in the organ of Corti (e.g., Kiernan et al. 2005b).

Although some genes are expressed specifically in either inner or outer hair cells from an early stage, for example, *Fgf8* in inner hair cells (Jacques et al. 2007) and the transcription factor *Insm1* in outer hair cells (Lorenzen et al. 2015), many genes that distinguish inner from outer hair cells are expressed later as the organ of Corti matures (Liu et al. 2014), and the signals and transcriptional effectors that induce them are largely unknown at present.

The situation is somewhat different for supporting cells, where a number of genes that become restricted to particular classes of supporting cells, such as *Prox1* (Birmingham-McDonogh et al. 2006), *p75^{LN^{GF}R}* (Mueller et al. 2002), *Spry2* (Shim et al. 2005), *Hey1*, and *Hey2* (Benito-Gonzalez and Doetzlhofer 2014), are initially expressed in the prosensory domain before or concomitant with hair cell differentiation. In addition, other members of the *Hes* and *Hey* gene families that are commonly thought of as being Notch responsive show dynamic changes as the cells differentiate, with different combinations of *Hes* and *Hey* genes being expressed in different types of supporting cells (Doetzlhofer et al. 2009). *Hes* and *Hey* genes can be differentially regulated by Notch signaling in a number of ways, for example, the number and arrangement of high- and low-affinity binding sites for cleaved Notch intracellular domains varies between different *Hes* and *Hey* gene promoters (Ong et al. 2006). However, because neither the expression of Notch ligands by inner and outer hair cells nor the expression of Notch receptors by different supporting cells appear to differ significantly, it is likely that other signaling mechanisms collaborate with the Notch pathway to specify distinct supporting cell types.

The best-characterized example of how Notch signaling interacts with other signaling pathways in the cochlea is the specification of pillar and Deiters cell fates by Fgf signaling (Fig. 3.5). Pillar cells are the only supporting cell type that does not transdifferentiate into hair cells in the absence of Notch signaling (Kiernan et al. 2005b; Doetzlhofer et al. 2009). Pillar cells and Deiters cells express the *Fgfr3* receptor and develop in close proximity to inner hair cells, which express *Fgf8* very shortly after they differentiate. Pharmacological blockade of Fgf signaling, conditional deletion of *Fgf8*, or deletion of *Fgfr3* disrupt the differentiation of pillar cells (Jacques et al. 2007; Puligilla et al. 2007), and overexpression of Fgfs in the cochlea can activate pillar cell markers at the expense of markers of outer hair cells and Deiters cells (Jacques et al. 2007; Doetzlhofer et al. 2009). While pillar cells are differentiating, *Hey2* becomes downregulated from the prosensory domain and is restricted to pillar cells (Doetzlhofer et al. 2009). Although *Hey2* is typically thought of as a target of Notch signaling, *Hey2* is also regulated by Fgf signaling, for example, treatment of cochlear cultures with Fgfs upregulates *Hey2* protein in many supporting cells (Doetzlhofer et al. 2009). Moreover, *Hey2* expression and pillar cell fate are unaffected by inhibition of either Notch or Fgf receptors, but simultaneous inhibition of FGF and Notch signaling downregulates *Hey2* and converts pillar cells into hair cells (Fig. 3.5). Finally, genetic deletion of *Hey2* allows pillar cells to convert into hair cells when Notch signaling is blocked (Doetzlhofer et al. 2009).

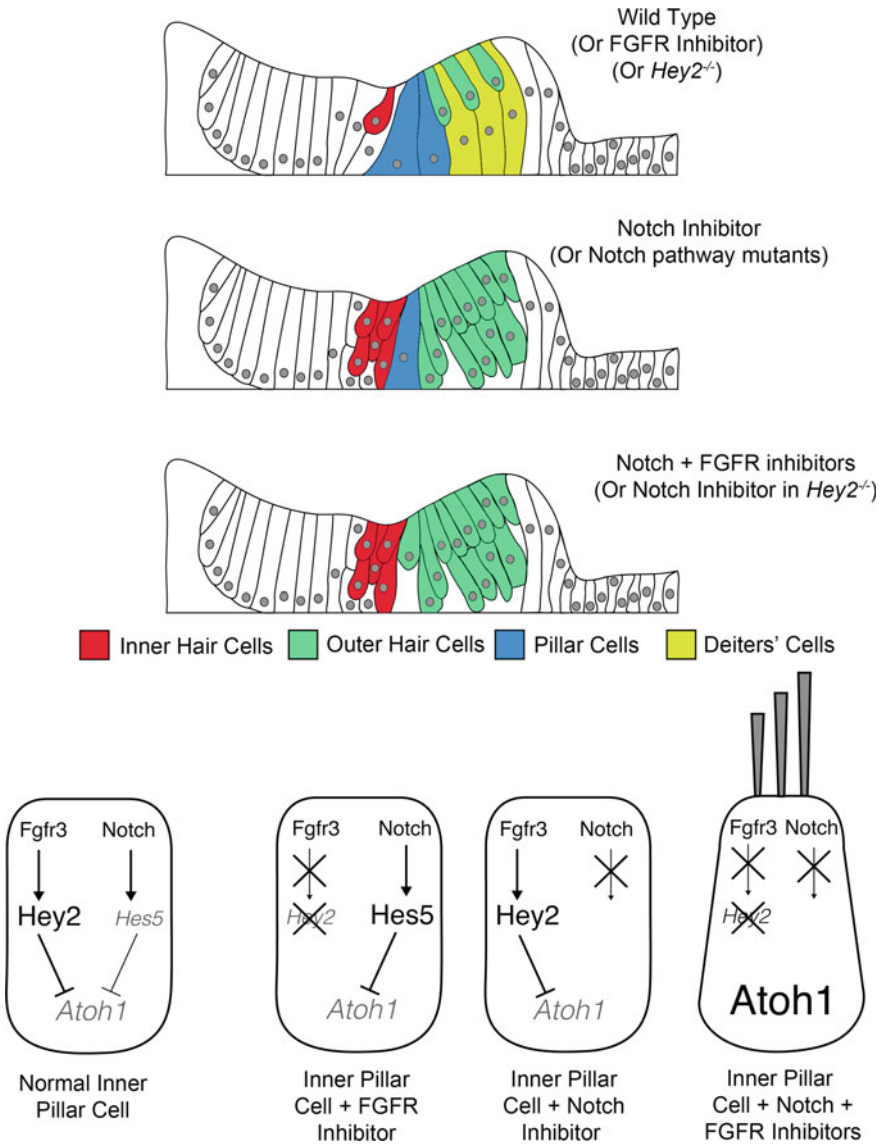


Fig. 3.5 The roles of fibroblast growth factor (FGF) and Notch signaling in pillar and Deiters cell identity. In wild-type embryos, pillar cells separate the inner and outer hair cell regions. If the Notch pathway is blocked, most supporting cells transdifferentiate into hair cells, but outer pillar cells remain because FGF signaling from the inner hair cells maintains *Hey2* expression in these cells. If FGF signaling is blocked or in *Hey2*-mutant embryos, the Notch pathway upregulates *Hes5*, preserving pillar fate. If both pathways are blocked, *Atoh1* expression is activated and the outer pillar cells convert into hair cells

A precise level of Fgf signaling appears to distinguish between pillar and Deiters cells and to maintain their distinct identities in mature animals (Fig. 3.6). The Fgf antagonist *Sprouty2* is expressed in pillar and Deiters cells, suggesting that a gradient of Fgf8 emanating from inner hair cells specifies pillar cells at high levels of Fgf signaling and Deiters cells at lower levels. Indeed, *Sprouty2*-mutant mice show a postnatal transformation of Deiters cells into pillar cells and the *Sprouty2*-mutant phenotype can be rescued by lowering Fgf signaling through the loss of one allele of *Fgf8* (Shim et al. 2005). Moreover, mouse models of the *Fgfr3* mutation found in Muenke syndrome also show a postnatal transformation of Deiters cells into pillar cells (Mansour et al. 2013). Here, however, the mutation in *Fgfr3* allows the receptor to become responsive to another Fgf expressed in the cochlea, Fgf10, thus increasing Fgf signaling in an analogous manner to the loss of *Spry2*. These remarkable results indicate that the fates of pillar and Deiters cells remain plastic even after the onset of hearing.

3.5.3 *Outstanding Issues Concerning the Specification of Hair Cell and Supporting Cell Subtypes*

Our understanding of the signals and gene regulatory networks that distinguish different types of hair cells and supporting cells from each other during development is currently limited by the dearth of unique markers. The difficulty of obtaining pure populations of different cell types and the relatively small number of cells present in the organ of Corti militate against obtaining a good survey of the genes expressed by each cell type. However, with recent technical advances in RNA sequencing from very small numbers of cells (or even single cells; Burns et al. 2015; Waldhaus et al. 2015), a much clearer picture of the gene expression patterns that distinguish cells in the organ of Corti should soon emerge.

Although there clearly are features that are common to all inner ear hair cells, the factors that regulate the development of auditory versus vestibular hair cells or of inner hair cells versus outer hair cells in the cochlea are unknown. *Atoh1* is a generic hair cell transcription factor, but it is not known whether it only regulates genes common to all hair cells or, alternatively, has access to different regions of chromatin in hair cell subtypes that are made differentially available by region-specific signaling pathways. In the case of the cochlea, it is possible that the signaling pathways that help divide the embryonic organ of Corti along its neural-abneural axis may provide a cellular context in which different transcription factors can cooperate with *Atoh1* to specify different types of hair cells. In the case of supporting cells, a generic transcription factor that is expressed in all supporting cell types has yet to be identified. *Hey2* is currently the only transcription factor that singles out one supporting cell type (pillar cells) from other supporting cells, although other supporting cells can be defined by specific combinations of other *Hes* and *Hey* genes.

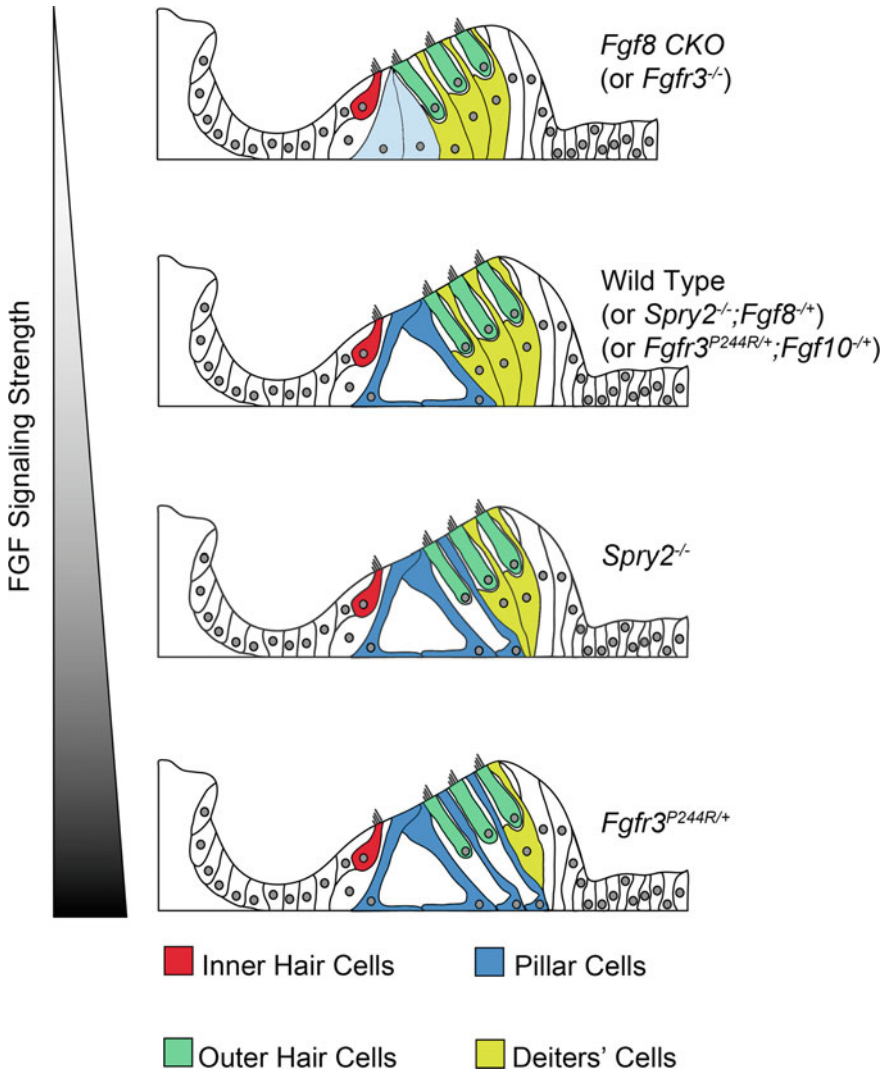


Fig. 3.6 An FGF gradient maintains pillar and Deiters cell fates in postnatal animals. FGF8 from inner hair cells creates a neural-abneural gradient of FGF signaling that allows differentiation of pillar cells and Deiters cells. If FGF8 signaling is reduced or blocked (by mutation of *FGF8* or the *FGFR3* receptor; *top panel*), pillar cells cannot differentiate properly. If the FGF inhibitor *Spry2* is mutated, increased FGF signaling causes some Deiters cells to convert into pillar cells (*third panel from top*). In the Muenke syndrome *FGFR3*^{P244R} mutant (*bottom panel*), the receptor becomes responsive to FGF10 as well as to FGF3, and the further increase in FGF signaling causes more rows of Deiters cells to convert to a pillar cell fate. Further evidence of the dose-dependent effect of FGF signaling is shown by the fact that double mutants of genes that have opposing effects, such as *Fgf8*; *Spry2* mutants or *FGFR3*^{P244R}; *Fgf10* mutants, are phenotypically normal (*second panel from top*)

3.6 Innervation of the Organ of Corti

3.6.1 Neurogenesis of the Spiral Ganglion

The spiral ganglion neurons of the cochlea originate from a neurosensory domain in the ventromedial otocyst (Fig. 3.7). This region expresses several transcription factors (*Eya1*, *Six1*, *Sox2*) considered essential for initiating neurogenesis (reviewed by Appler and Goodrich 2011; Goodrich 2016). An adjacent posteroventral region expresses *Tbx1*, a transcription factor that blocks neurogenesis (Raft et al. 2004). The earliest evidence of neural fate specification in the otocyst is the salt-and-pepper appearance of Neurogenin1 (*Neurog1/Ngn1*)-expressing progenitors within the otic

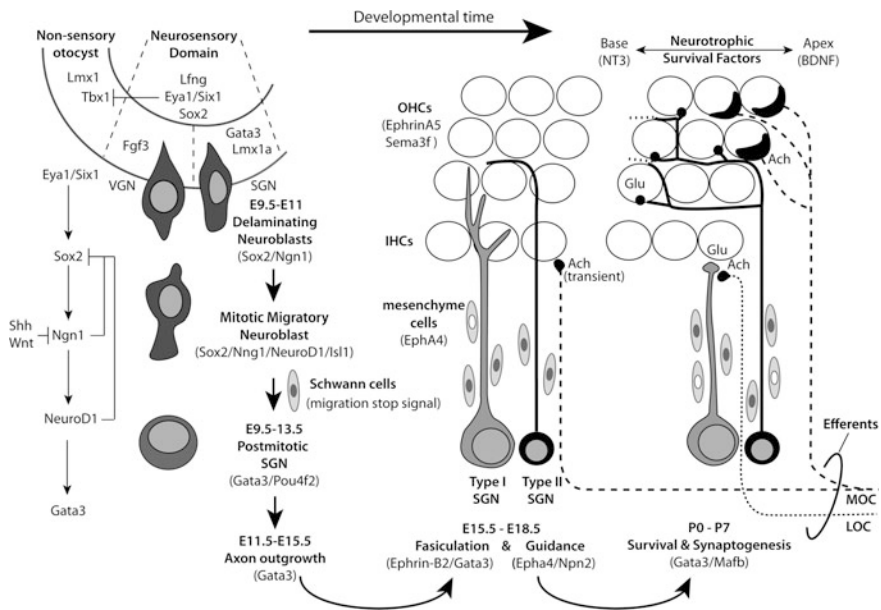


Fig. 3.7 Neurogenesis and the development of cochlear innervation. *Left to right*: neuroblasts are first recognized within the otocyst due to expression of *Neurog1 (Ngn1)*. This initiates a cascade of neurogenic transcription factors. Progression of cells along a neural differentiation pathway requires both negative feedback loops to repress progenitor genes and loss of responsiveness to secreted factors like *Shh* and *Wnt*. Precursors of spiral ganglion neurons (SGNs) maintain expression of a different constellation of genes than those of vestibular ganglion neurons (VGNs), most notably *Gata3*. The final placement of the neuronal cell bodies is influenced by stop signals in the surrounding environment. Radial bundling of afferent neurites is influenced by Eph-to-Ephrin reverse signaling. Type I and type II afferents take distinct pathways across the tunnel of Corti (not shown) as they mingle with inner hair cells (IHCs) and outer hair cells (OHCs). An exploratory phase of axon outgrowth within the cochlea is influenced by Ephrin-to-Eph forward repulsive signaling and Semaphorin-to-Neuropilin repulsive signaling. SGN survival is mediated by the neurotrophins BDNF and NT3. Finally, refinement of connections takes place in the postnatal period for the type I afferents and both medial olivocochlear (MOC) and lateral olivocochlear (LOC) efferents. Ach, acetylcholine; Glu, glutamate

epithelium, some of which also express *Neurod1* and *Isl1* (Raft et al. 2007; Deng et al. 2014). Notch signaling is involved in the specification of neuronal precursors (Abello and Alsina 2007; Abello et al. 2007) and a role for Fgf signaling is also implicated in the chicken (Alsina et al. 2004). Neuroblasts delaminate from the epithelium and gather together as a common statoacoustic ganglion adjacent to the otocyst and the hindbrain. Negative feedback circuits then repress *Neurog1* (Raft and Groves 2015) and *Sox2* (Evsen et al. 2013) in the neuroblasts. *Neurog1* is required for neural specification (Ma et al. 1998, 2000), whereas *Neurod1* is necessary for delamination and survival of postmigratory neuroblasts (Liu et al. 2000; Kim et al. 2001). As the delaminated cells exit the cell cycle, *Neurod1* and *Isl1* are reduced, whereas *Pou4f1* and then *Pou4f2* are increased (Deng et al. 2014). The transition states of premigratory and migratory neuroblasts were identified through single-cell gene expression analysis (Durruthy-Durruthy et al. 2014), which revealed that genes related to Shh and Wnt responsiveness are downregulated as neuronal differentiation progresses. This change appears to be permissive for further neuronal development (Brown and Epstein 2011).

After the statoacoustic ganglion is formed, it then splits to create the vestibular ganglion and the spiral ganglion (Sandell et al. 2014). These two fates may be imposed earlier, while the neuroblasts are still residents of the otocyst, based on their sites of origin and gene expression profiles. The more anterolateral pool expresses *Fgf3*, emigrates earlier, is born first, and becomes vestibular. The more postero-medial pool expresses *Lmx1* and *Gata3*, emigrates later, pulls out of division last, and becomes auditory (Koo et al. 2009). Lineage and fate-mapping experiments in the chicken embryo are consistent with a mostly separate origin of vestibular and auditory neurons (Satoh and Fekete 2005; Bell et al. 2008). In mice, *Gata3* is expressed by neurons in both ganglia by the time they have separated at E12.5. However, within 1 day, it is rapidly downregulated in most vestibular ganglion neurons (Lawoko-Kerali et al. 2004; Lu et al. 2011). A severely reduced statoacoustic ganglion in *Gata3* mutants has been interpreted to mean that spiral ganglion neurons are selectively lost (Karis et al. 2001). Indeed, by delaying the knockout of *Gata3*, a persistent dependence of spiral ganglion neurons on this transcription factor was confirmed; *Gata3* is required for their differentiation, timing of axon outgrowth, and survival (Duncan et al. 2011; Luo et al. 2013). In the absence of *Gata3*, peripheral neurites sprout prematurely, meander excessively, and fail to form radial bundles while en route to the organ of Corti, perhaps because they arrive in advance of or are unable to respond to appropriate guidance cues (see Sect. 3.6.2; Appler et al. 2013). The number of ribbon synapses per inner hair cell is reduced by 50–70% (Yu et al. 2013). Gene expression profiling of *Gata3*-knockout spiral ganglia at E13.5 reveals a requirement for *Gata3* in upregulating the transcription factor *Mafb*. *Mafb* expression by spiral ganglion neurons is itself essential for the elaboration of the postsynaptic density associated with afferent innervation of hair cells at ribbon synapses (Yu et al. 2013). Thus, a cell autonomous defect in the postsynaptic partner, due primarily to the absence of *Mafb*, may explain why there are so few ribbons per inner hair cell after a delayed loss of *Gata3* in the neurons. This is

supported by a gene rescue experiment; restoration of *Mafb* (with an appropriate transgenic allele) in *Gata3*-knockout neurons restored about half of the missing ribbon synapses (Yu et al. 2013). Gene expression profiling also indicates that *Gata3* is required to reinforce the distinction of auditory versus vestibular ganglion neurons, at least for a small fraction of the genes that are differentially expressed across these two populations (Lu et al. 2011; Appler et al. 2013).

As it separates from the vestibular ganglion at E10.5, the spiral ganglion is encased in a sleeve of neural crest cells that also then pile up near the leading edge of the peripheral neurites at E12.5 (Sandell et al. 2014). Some of these cells associate with the spiral ganglion neurons as Schwann and satellite cells. The Schwann cells apparently provide a central stop signal because in their absence, the spiral ganglion neurons drift away from the sensory epithelium into the modiolus (Morris et al. 2006; Mao et al. 2014). An additional peripheral stop signal, mediated by Slit/Robo signaling, confines the neuronal soma to Rosenthal's canal and prevents them from scattering toward the sensory periphery after E14 (Wang et al. 2013). As development progresses, the spiral ganglion acquires its characteristic shape by elongating alongside the coiling cochlea.

3.6.2 *Development of Afferent Innervation*

An understanding of the cellular mechanisms underlying the development of cochlear afferent innervation has been explored for many years, both in vivo and in vitro. Evidence from a variety of studies suggests that the presence of hair cells, even ectopic ones, is sufficient to attract afferents, but hair cells are not strictly necessary for the spiral ganglion to send out processes that reach the sensory periphery (reviewed by Fritzscht et al. 2005b; Fekete and Campero 2007). The laboratory of Bernd Fritzscht championed the use of lipophilic fluorescent dyes in fixed specimens for track tracing of both afferent and efferent neurons into the embryonic mouse cochlea (Yang et al. 2011). Phenotyping a variety of mouse mutants with this technique has yielded a collection of genes that either indirectly (transcription factors) or directly (ligands and receptors) affect cochlear innervation patterns. Among the latter are the *Ntrk2* and *Ntrk3* receptors that bind to two key neurotrophins, brain-derived neurotrophic factor and neurotrophin-3. Although best known for their role in survival (Fritzscht et al. 2016), altering the time and place of their expression through genetic swaps has shown that these neurotrophins can also attract afferents (Tessarollo et al. 2004; Fritzscht et al. 2005a).

Recent work that has revealed additional molecular mechanisms guiding spiral ganglion neurites toward their final targets is summarized in Fig. 3.7 and has been expertly reviewed (Coate and Kelley 2013; Delacroix and Malgrange 2015). Timed induction of Cre recombination in a drug-inducible *Neurog1-CreER^{T2}* driver line permits a fraction of the neurons to be labeled in their entirety, yielding Golgi-like staining patterns. With this method, some spiral ganglion neurons are seen to possess bipolar morphologies by E12.5, with projections directed both centrally and

peripherally (Koundakjian et al. 2007). Peripheral neurites traverse through the mesenchyme toward the sensory epithelium and by E16.5, they are arranged in tight parallel bundles of 50–100 axons. This fasciculation requires *EphA4* expression in the mesenchyme, which is under the transcriptional control of *Pou3f4* (Coate et al. 2012). In the latter study, axonal fasciculation defects were observed in cochleae lacking *Pou3f4*, *EphA4*, or *Ephrin-B2* (the latter specifically absent from neurons). The same group used in vitro coculture assays to interfere with neuron-mesenchymal cell interactions by altering the levels of *EphA4* or *Ephrin-B2*. In summary, the tight fasciculation into radial bundles probably serves to minimize repulsive interactions with surrounding mesenchymal cells, such as those mediated through Eph-to-Ephrin “reverse” signaling. Similarly disorganized radial bundles form in mice when neural crest-derived Schwann cells are missing due to targeted deletion of *Erb2* or *Sox10* (Morris et al. 2006; Mao et al. 2014). In those mutants, it is unclear whether defasciculation is a direct consequence of the missing Schwann cells or whether it is because of the unusual distance between spiral ganglion cell bodies and their peripheral targets. The fasciculation defect found with *Gata3* conditional deletion (Appler et al. 2013) is likely to be due to intrinsic changes in the neurons.

In the next phase of outgrowth, peripheral processes reach the cochlear epithelium and send branches among the hair cells and supporting cells. Crossing the inducible *Neurogl-Cre* driver with mouse lines carrying floxed STOP fluorescent reporters brings a temporal dimension to the analysis because it allows for time-lapse imaging of neurites as they navigate within the sensory epithelium. This has provided a new appreciation of how active, complex, and transient individual axonal branches are as they explore, retract, and then stabilize their connections (Coate et al. 2015; Druckenbrod and Goodrich 2015). Initially in rodents, both type I and type II afferents project beyond the inner hair cell row, cross the tunnel, and travel among immature outer hair cells. By postnatal week (P) 0, type II afferents are spiraling longitudinally between the outer hair cell rows, having first crossed closer to the floor of the tunnel of Corti compared with the presumed type I afferents (Druckenbrod and Goodrich 2015). In mature ears, type II processes invariably turn toward the base (except in the apical turn), travel a long distance, and then build large terminals onto multiple outer hair cells (Nayagam et al. 2011). In contrast, each mature type I radial process will terminate with a simple ending that synapses exclusively with a single inner hair cell (Nayagam et al. 2011). But before this happens, type I neurons enjoy an exploratory phase with clawlike branches that contact a range of cell types on either side of the tunnel of Corti, including outer hair cells (Druckenbrod and Goodrich 2015).

During the first postnatal week in mice, outer hair cells express numerous puncta of presynaptic ribbon components, composed of Ribeye protein that is immunolabeled with Ctip2 antibodies (Huang et al. 2012). These puncta have been interpreted as evidence of transient synapses between type I neurons and outer hair cells because they mostly disappear by P6, and this coincides with the full retraction of type I projections from the outer hair cell domain (Huang et al. 2012).

The remaining puncta on P6 may represent stabilized synapses with type II terminals. However, the presence of transient functional synapses between type I neurons and outer hair cells is still unresolved. First, only 14% and 9% of radial (possible type I) terminals are located among the outer hair cells on P0 and P3, respectively, and these almost never project past the first row of outer hair cells (Druckendrod and Goodrich 2015), whereas the Ribeye puncta are present in all three rows (Huang et al. 2012). Second, on E18.5, the presumed type I processes are highly motile and individual branches frequently and rapidly undergo retractive movements when exploring the outer hair cell domain (Druckendrod and Goodrich 2015). These retractions are likely to be a response to repulsive cues. Indeed, the perinatal outer hair cell region expresses two known axon repellents: secreted Semaphorin-3F and membrane-bound Ephrin-A5 (Defourny et al. 2013; Coate et al. 2015). It is assumed that the Ephrin-A5 ligand on the outer hair cells interacts with the EphA4 receptor expressed by a subset of type I neurites through “forward” signaling (as compared with the earlier role of EphA4-to-EphrinB2 reverse signaling from mesenchymal cells to these same neurites). The presence of these repellents offers a mechanism for why type I afferents fail to find permanent purchase among outer hair cells. This interpretation is bolstered by the phenotypes seen in mutants lacking these repellents or their receptors; an excess of afferent fibers mingles among outer hair cells after the critical period when type I processes should have become restricted to inner hair cells (Defourny et al. 2013; Coate et al. 2015). The Ephrin and Semaphorin repellents may be partially redundant, with Ephrin being more robust, because *Neuropilin2* mutants have only a mild excess of presumed type I projections into the outer hair cell region on E16.5 and this phenotype does not persist postnatally (Coate et al. 2015).

Why are the type II neurons not also subject to repulsion by Semaphorin-3F and Ephrin-A5? In part, this is probably because they do not express the EphA4 receptor thought to bind Ephrin-A5 (Defourny et al. 2013). However, type II neurites do express *Neuropilin2*, the presumed Semaphorin-3F receptor (Coate et al. 2015). It is possible that type II fibers outcompete type I fibers for outer hair cell targets because they find the environment less repulsive.

Additional repulsive, permissive, or attractive cues may guide the type II cochlear afferents. The search for transcriptional regulators of type II guidance cues could begin with *Prox1*, which is present at the right time and place to regulate the expression of axon guidance molecules both in the outer hair cell domain and in the type II neurons that project there. By birth, type II spiral fibers lacking *Prox1* fail to form tight bundles between Deiters cells, their projections are disorganized, and many axons turn toward the apex instead of toward the base (Fritzsch et al. 2010). This misguidance must be cell autonomous to the type II neurons because it is evident even when the outer hair cell domain (but not the spiral ganglion neurons) has normal *Prox1* gene expression. An analysis of the genes misregulated in the organ of Corti versus the spiral ganglion of *Prox1* mutants may identify the relevant guidance factors and receptors, respectively.

3.6.3 Development of Efferent Innervation

In mammals, efferent axons that project into the cochlea originate from two separate pools of neurons in the brainstem called the medial olivocochlear and the lateral olivocochlear neurons (Brown 2011). In the mature cochlea, these pools maintain separate projections on each side of the tunnel of Corti: medial olivocochlear neurons target outer hair cells and lateral olivocochlear neurons target the terminals of type I afferents beneath inner hair cells. However, during development, a more promiscuous phase of synaptic connections is seen for the medial olivocochlear efferents, as discussed in the following paragraph (Fig. 3.7).

Relatively little is known about the molecules guiding cochlear efferent axons from the periolivary complex in the brainstem through the VIIIth cranial nerve and into the organ of Corti. Efferent neurons express *Gata3* and in its absence on E12.5 in the mouse, they show midline-crossing defects and their axons are misrouted into the facial nerve (Karis et al. 2001). It is generally assumed that efferent axons track along the VIIIth nerve to reach the cochlea (Ma et al. 2000; Huang et al. 2001), with the pioneers arriving at the spiral ganglion by E12.5 (Fritzsche and Nichols 1993). After passing through the ganglion, at least some efferent fibers depart from afferents to create the intraganglionic spiral bundle at the periphery of the ganglion. The formation of this spiral bundle requires the presence and normal localization of the spiral ganglion cell bodies (Kim et al. 2001; Morris et al. 2006). Efferent fibers then travel along the afferent radial bundles to reach the organ of Corti by E16.5 (Simmons et al. 2011).

The first two weeks after birth are a period of synaptic plasticity in the cochlea; some synapses are retracting as others are consolidating (Pujol et al. 1998; Katz and Elgoyhen 2014). Most notable are transient axosomatic contacts between medial olivocochlear efferent fibers and inner hair cells observed from birth to postnatal day 14 (Simmons 2002). The final targets for medial olivocochlear fibers are not inner but outer hair cells, which they contact via large axosomatic terminals. As the medial olivocochlear efferents depart from the inner hair cells, they are replaced by afferents. Finally, late-arriving lateral olivocochlear efferents make small axodendritic synapses onto type I afferents beneath inner hair cells. Interestingly, functional synapses between inner hair cells and medial olivocochlear axons can reappear in aging cochleae concomitant with the loss of outer hair cells and of the afferent innervation of inner hair cells (Lauer et al. 2012; Zachary and Fuchs 2015).

3.6.4 Unresolved Questions in Afferent and Efferent Innervation of the Cochlea

Time-lapse imaging of cochlear afferents in the early postnatal period may help to resolve the question of whether type I neurites stop exploring long enough to form synapses with outer hair cells. If so, physiological recordings are necessary to

determine if any of these transient synapses are functional. There are still many unknown aspects of type II pathfinding within the cochlea, including what molecular mechanisms instruct them to make a characteristic sharp turn as they reach the outer hair cells or why they spiral basally for 100–200 μm before establishing synapses with a cluster of outer hair cells. The development of the cochlear efferents is also in need of further study. Selective and temporal deletion of *Gata3* in the efferent neurons, followed by transcriptomic analysis of the mutant cells, may be a fruitful strategy to catalog and then test potential guidance factors that direct these neurons through their complex trajectories to reach first their transient targets and then their final targets.

3.7 Concluding Thoughts

This chapter has summarized recent research on cochlear development. Although this review may appear to focus excessively on the organ of Corti, this is a fair reflection of the focus of research on the functional sensory tissue, with far fewer development studies devoted to nonsensory regions. This final section considers a few other aspects of cochlear development that have received less attention and suggests that some of these areas are ripe for investigation in the future.

3.7.1 *How Does the Cochlea Grow and Coil?*

The first area in which research on nonsensory areas of the cochlea has lagged behind studies of the development of the organ of Corti is, ironically, the development of the coiled shape that gives the cochlea its name (see Manley, Chap. 2). Others have described how the planar cell polarity pathway regulates cell rearrangements of convergent extension and radial intercalation that thin the prosensory domain into an array of hair cells and supporting cells (Ezan and Montcouquiol 2013). Although this extension clearly contributes to the thinning and lengthening of the prosensory domain and organ of Corti (Chacon-Heszele and Chen 2009), it is unlikely to be the only mechanism involved in cochlear outgrowth and coiling. Indeed, the wide range of cochlear lengths and morphologies seen across mammals and differences in cochlear dimensions, volumes, and thickness of the basilar membrane (Ekdale 2015) suggest that cochlear cell shape, cell division, and cell polarity are likely to be regulated at many levels and by many gene regulatory networks. At present, the field lacks even basic quantitative measurements of how the cochlea grows out from the otocyst and undergoes coiling. The ability to localize fluorescent proteins to cell nuclei and membranes in a cell- and tissue-specific manner, however, together with recent advances in high-resolution imaging of large volumes of cleared tissue samples (Kopecky et al. 2012) suggest that it will be possible to develop a complete picture of cochlear growth and coiling,

at least in mice. The large number of mouse mutants in which the shape and length of the cochlear duct are altered offers a rich resource that can be combined with imaging technology to understand the cellular and genetic regulation of cochlear morphology.

The epithelium of the mature cochlear duct is suspended between two chambers, the scala vestibuli and scala tympani. These contain the perilymph fluid responsible for conduction of sound waves along the cochlear duct. These two chambers are carved out of the mesenchyme surrounding the cochlear duct through the local death or dispersion of mesenchymal cells. Interestingly, this process proceeds from base to apex with approximately the same timing as the differentiation of the organ of Corti (Kopecky et al. 2012). It is tempting to speculate that signals regulating the basal-apical gradient of differentiation in the cochlea, such as *Shh*, may also regulate the behavior of the surrounding mesenchyme, although there are a variety of other signals exchanged between the otic epithelium and mesenchyme that may also affect mesenchymal cell behavior and transcription.

3.7.2 *Reissner's Membrane and the Stria Vascularis: The Importance of Nonsensory Cochlear Development*

The perilymph of the scala vestibuli is separated from the endolymph of the scala media of the cochlea by Reissner's membrane. This delicate membrane is composed of two cell populations: one derived from a single layer of cochlear epithelium and the other from two layers of periotic mesenchymal cells (Slepecky 1996). As expected from a membrane that separates two extracellular fluid compartments of very different ionic compositions, the cells of the mature Reissner's membrane express a variety of different ion channels and exchangers (Lang et al. 2007). The epithelial cells of Reissner's membrane are derived from *Otx1*- and *Otx2*-expressing cells in the ventrolateral region of the otocyst that will contribute to the roof of the cochlear duct (Wu and Kelley 2012; Vendrell et al. 2015). Conditional mutants of *Otx2* completely lack Reissner's membrane, with an ectopic prosensory region and organ of Corti developing in its place (Vendrell et al. 2015). The association between the epithelial and mesenchymal layers of Reissner's membrane is promoted by *Fgf9* expressed in nonsensory otic epithelium. This source of *Fgf* signaling is necessary for the correct formation of Reissner's membrane because *Fgf9*-mutant mice have disorganized mesenchymal cells loosely attached to the epithelial layer (Pirvola et al. 2004). Reissner's membrane is also missing in *Fgf10*-mutant mice (Urness et al. 2015). However, there are very few other studies that have investigated the process that leads to the thinning of the epithelium of Reissner's membrane and the development of the association between its epithelial and mesenchymal cell layers.

The development and morphology of Reissner's membrane can also be affected indirectly by defects in the development and composition of endolymph fluid (e.g., Somma et al. 2012), which is regulated by another component of the cochlear duct, the stria vascularis (see Wangemann and Marcus, Chap. 9). Like Reissner's membrane, different components of the stria vascularis are derived from several embryonic sources. The marginal cells that line the wall of the cochlear duct and contact the endolymph are derived from the cochlear epithelium (Ohyama and Groves 2004). The pigmented intermediate cell layer is derived from migrating cranial neural crest cells that differentiate into melanocyte-like cells (Steel and Barkway 1989). Fibrocytes of the mesenchymal spiral ligament form a basal layer that interdigitates with marginal and intermediate cells (Spicer and Schulte 1991). These three cell populations are invaded by a dense network of capillaries that give the stria vascularis its name. Although genes have been identified that influence the development of particular components of the stria, little is known about the developmental events that orchestrate the integration of these different cell types. For example, several transcription factors such as *Tbx18*, *Sox9*, and *Pou3f4* are expressed in the periotic mesenchyme and later in the basal cells of the stria, and mutation of these genes all cause strial defects. As expected, genes involved in the differentiation of neural crest melanocytes (such as *Pax3*, *Sox10*, and *c-kit*, whose mutation causes Waardenburg syndrome) can also affect the formation and function of the stria vascularis (e.g., Kim et al. 2014). The orphan nuclear receptor *estrogen-related receptor β /Nr3b2* is expressed in the developing marginal cells of the stria, and conditional mutants of this gene cause severe cochlear defects, including collapse of Reissner's membrane and the loss of multiple ion channels and transporters from the marginal cells (Chen and Nathans 2007). These mutants also have secondary defects in the adjacent capillaries and marginal cells. Given the importance of the stria vascularis in the formation and maintenance of the endolymph and the role of strial degeneration in age-related hearing loss, understanding how the different cells of the stria develop and integrate with each other requires continued scrutiny.

Highlighting the developmental plasticity of the cochlear epithelium is evidence that the anlagen of the stria vascularis and Reissner's membrane have at least a transient ability to form ectopic prosensory tissue and an ectopic organ of Corti containing hair cells and supporting cells. This is suggested by results from gain-of-function experiments where *Sox2* or the active intracellular domain of the Notch1 receptor are overexpressed in the otocyst (Pan et al. 2010, 2013). Moreover, *Otx2* conditional mutants also have ectopic prosensory/hair cell-containing regions in place of Reissner's membrane (Vendrell et al. 2015). It will be of interest to carefully map the duration for which the progenitors for these two structures remain competent to differentiate into sensory tissue and to determine whether the mechanism of this competence involves the upregulation of *Sox2* to act as a multipotency or proneural factor (Raft and Groves 2015).

3.7.3 *Postmitotic Maturation of the Organ of Corti*

As can be seen from the preceding sections, current research on cochlear development tends to focus on the events that regulate the pattern and orientation of hair cells and supporting cells in the organ of Corti. However, once the numbers and disposition of cells in the organ of Corti have been fixed and before the onset of hearing, both hair cells and supporting cells undergo a period of morphological and functional maturation, which in mice encompasses a two-week period after birth. In the case of the inner and outer hair cells, much of this maturation centers on the development and growth of their stereociliary bundles and the expression of protein complexes and ion channels that will support mechanotransduction and synaptic transmission (Schwander et al. 2010). The morphological maturation of supporting cells has received much less attention, which is somewhat surprising given the extraordinary changes in cell morphology exhibited by these cells. Pillar cells and Deiters cells elongate dense actin-rich processes and produce large phalangeal processes at their apical extremity. These interconnect with the apical surfaces of hair cells to form the reticular lamina of the organ of Corti. With the exception of some of the adhesion molecules that organize the apical surfaces of hair cells and supporting cells (Togashi et al. 2011), very little is known about the molecular composition of these specialized support structures. As discussed in Sect. 3.6, the observation of plasticity between pillar cell and Deiters cell phenotypes in the postnatal mouse (Shim et al. 2005; Mansour et al. 2013) suggests that the mature phenotype of these cells may be maintained in the adult through ongoing signaling. Understanding the signals and transcriptional effectors that drive and maintain the differentiated state of hair cells and supporting cells is highly relevant to the questions relating to the inherent regenerative capacity of the organ of Corti. However, the small amount of tissue that can be isolated from the organ of Corti has made biochemical analysis of mature hair and supporting cell specializations difficult, although recent success in using mass spectrometry to identify components of stereociliary bundles (Shin et al. 2013) suggests that it may also be possible to apply these techniques to the reticular lamina if a suitable method of isolating it can be found.

Acknowledgements We dedicate this chapter to the memory of Julian Lewis (1946–2014), a visionary scientist whose hypothesis about the role of Notch signaling in patterning the cochlea was just one of his many contributions to the field of developmental biology.

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