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Dissecting the molecular basis of organ of Corti development: where are we now?

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Abstract

This review summarizes recent progress in our understanding of the molecular basis of cochlear duct growth, specification of the organ of Corti, and differentiation of the different types of hair cells. Studies of multiple mutations suggest that developing hair cells are involved in stretching the organ of Corti through convergent extension movements. However, *Atoh1* null mutants have only undifferentiated and dying organ of Corti precursors but show a near normal extension of the cochlear duct, implying that organ of Corti precursor cells can equally drive this process. Some factors influence cochlear duct growth by regulating the cell cycle and proliferation. Shortened cell cycle and premature cell cycle exit can lead to a shorter organ of Corti with multiple rows of hair cells (e.g., *Foxg1* null mice). Other genes affect the initial formation of a cochlear duct with or without affecting the organ of Corti. Such observations are consistent with evolutionary data that suggest some developmental uncoupling of cochlear duct from organ of Corti formation. Positioning the organ of Corti requires multiple genes expressed in the organ of Corti and the flanking region. Several candidate factors have emerged but how they cooperate to specify the organ of Corti and the topology of hair cells remains unclear. *Atoh1* is required for differentiation of all hair cells, but regulation of inner versus outer hair cell differentiation is still unidentified. In summary, the emerging molecular complexity of organ of Corti development demands further study before a rational approach towards regeneration of unique types of hair cells in specific positions is possible.

Keywords

cochlea; development; organ of Corti; cell fate decision

Introduction

The mammalian inner ear develops from a flat area of ectoderm, the otic placode. The placode invaginates and forms a vesicle, which then undergoes a complex morphogenetic process to generate a labyrinth of ducts and recesses, including the cochlear duct. The sensory epithelium of the cochlear duct is the organ of Corti, the hearing organ of mammals including human. The organ of Corti is a masterpiece of cellular micro-architecture. A radial cellular unit of the organ of Corti consists of two sets of hair cells, a single row of inner and three rows of outer hair cells, and multiple types of supporting cells strategically positioned

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on the basilar membrane. The organization of these cells maximizes the extraction of sound energy by amplifying sound-induced basilar membrane motion and transmitting those movements, via the help of the tectorial membrane, to inner hair cells. The basic radial organization varies along the length of the cochlea, displaying a graded modification in width and stiffness to enhance frequency specific sound perception along a tonotopic axis: high frequency in the narrower, stiffer base and low frequency in the wider, more flexible apex.

The development of the organ of Corti requires several crucial steps. First, a cochlear duct has to form and grow. Second, the organ of Corti has to develop between the scala tympani and scala media. Third, the differentiation of inner and outer hair cells at appropriate positions has to occur. Fourth, the topology and specific differentiation of the supporting cells needs to be specified to ensure the mechanical properties needed for sound-induced movements. Certain molecular decision making processes revealed thus far have been adopted in strategies aimed at generating hair cells from stem cells (Groves, 2010; Oshima et al., 2010). However, we are currently unable to generate inner and outer cochlear hair cells in the topographically specific configuration needed to reconstitute a functional organ of Corti after congenital or acquired loss of hair cells using these approaches.

An excellent example for the level of understanding needed to guide attempts to reconstitute an equally complex organ of Corti is provided by the *achaete-scute* complex of the fruit fly (Garcia-Bellido and de Celis, 2009). The four genes of this complex (*achaete*, *scute*, *lethal of scute*, *asense*) encode proneural basic Helix-Loop-Helix (bHLH) proteins and guide formation of ‘bristles’ and ‘hairs’ in specific configurations on the thorax. Topographically restricted expression of these genes occurs in clusters destined to develop into sensory cells. This process is regulated by position-specific enhancers of these bHLH genes that are, in turn, regulated by three sets of transcription factors: the iroquois proteins, the GATA factor containing protein pannier, and the Zn-finger spalt proteins (Garcia-Bellido and de Celis, 2009; Ghysen and Dambly-Chaudiere, 1988). The developing sensory organs can be viewed as a landscape of transcription factors acting in a combinatorial manner to confer genetic identity to each region (Garcia-Bellido and de Celis, 2009), resulting in a pre-pattern that bestows competence for neural differentiation. Despite over 70 years of research, ‘more work is needed to understand how these processes relate to the singling out of individual cells in constant positions’ (Garcia-Bellido and de Celis, 2009). Refinement of the initial prosensory cluster is achieved by lateral inhibition with the neurogenic genes of the Notch signaling pathway, ensuring that only the cell with the highest level of proneural gene expression escapes from the lateral inhibition to develop as a sensory cell.

While it is now clear that the development of the organ of Corti uses transcription factors closely related to certain proneural and neurogenic genes also found in flies (Doetzlhofer et al., 2009; Fritzscht et al., 2010a), the details of their interactions are less clear. For more than a decade we have known that only one proneural bHLH gene, *Atoh1* (previously *Math1*) is required for all hair cell development (Bermingham et al., 1999). However, the evidence provided by knocking out *Atoh1* has not provided an understanding of how differential formation occurs between the two distinct hair cell types in each sensory epithelium of the inner ear (Type I and Type II in vestibular epithelia, inner and outer hair cells in the organ of Corti). Clearly, reconstituting a functional organ of Corti cannot be achieved by regenerating vestibular hair cells or only outer or inner hair cells in the organ of Corti. Comparable to the single bHLH gene *Atoh1*, initial work revealed a set of two neurogenic genes involved in lateral inhibition, *Hes1* and *Hes5* (Zine et al., 2001). However, *Hes1* and *Hes5* cannot account for the formation of seven distinct types of supporting cells in the organ of Corti. Only recently has the complexity of proneural bHLH genes and neurogenic bHLH genes in the developing organ of Corti reached a level proportionate for the differential regulation of

the multiple cell types of the organ of Corti (Doetzlhofer et al., 2009; Fritzscht et al., 2010a). As with the *Ac/Sc* complex in fly development, relating these transcription factors to stereotyped formation of the specific hair cells and supporting cells requires additional work. Below we summarize current progress in the understanding of this process and highlight many open questions beyond discussions provided by several recent reviews (Kelley et al., 2009; Puligilla and Kelley, 2009).

The organ of Corti evolved from the basilar papilla

The basilar papilla is found in all tetrapods, except for a few derived species that have secondarily lost it (Fritzscht and Wake, 1988; Lewis et al., 1985). This sensory epithelium may have evolved in the bony fish ancestors of tetrapods when the lagena, a gravistatic sensory organ, segregated from the sacculle (Fritzscht, 1992). All basilar papillae, whether consisting of only a few hair cells or several thousand hair cells, are always near the opening of the lagenar recess. In contrast, the tip of the lagenar recess is occupied by a gravistatic sensory organ, the lagena (Fig. 1). Among mammals, only the monotremes have retained that tetrapod configuration with the lagena sensory epithelium at the tip of the lagenar recess (Jorgensen and Locket, 1995). In contrast to the typical basilar papilla, the organ of Corti of monotremes is already composed of inner and outer hair cells, a feature shared by all mammals. However, only the basal tip of the organ of Corti shows the eutherian and marsupial pattern of one row of inner and three rows of outer hair cells (Ladhams and Pickles, 1996). The apex of the monotreme organ of Corti has multiple rows of inner and outer hair cells. The transition from the monotreme to the eutherian/marsupial organization entails the loss of the lagena, turning the lagenar recess into a cochlear duct, and the extension of the organ of Corti and the cochlear duct to form the coiled cochlea (Fig. 1), which are eutherian/marsupial novelties. How the loss of the lagena sensory epithelium relates to the evolutionary expansion and coiling of both the cochlear duct and the organ of Corti is currently unclear (Luo et al., 2011).

Growing a cochlear duct

Since the initial description of cochlear defects in the *Fgf3* (Mansour et al., 1993) and *Pax2* mutants (Favor et al., 1996; Torres et al., 1996), we know that specific genes are necessary for growth and development of the organ of Corti. The list of genes has since grown rapidly and includes multiple genes identified as being defected in several human hearing mutations (Dror and Avraham, 2009; Friedman et al., 2007; Hilgert et al., 2009). In addition, microarray data have added hundreds of genes that may need to interact during development to form a single sensory epithelium (Chen and Corey, 2002; Sajan et al., 2007). Among these genes, some are known to affect cochlear duct and organ of Corti growth and patterning, such as *Sox2* (Kiernan et al., 2005), *Jag1* (Kiernan et al., 2006), *Pax2* (Bouchard et al., 2010; Burton et al., 2004), *Gata3* (Karis et al., 2001; Lillevall et al., 2006; Milo et al., 2009), *Eya1* (Zou et al., 2008), *Lmx1a* (Nichols et al., 2008), *Nr2f1* (Tang et al., 2005), *Fgfr1* (Pirvola et al., 2002), *Foxg1* (Hwang et al., 2009; Pauley et al., 2006), *Bmp4* (Ohshima et al., 2010), *Rac1* (Grimsley-Myers et al., 2009) and several micro-RNAs (Dror and Avraham, 2009; Soukup et al., 2009). These genes play roles in specifying the position of the cochlear duct, ensuring its growth and defining the competence that allows hair cell formation only in the organ of Corti. How these factors interact in these processes is not yet clear. Below, we first outline vestibular before cochlear canal development, the former being better understood than the latter.

In the vestibular system, sensory epithelia formation governs the development of canals. Mutations in genes such as *Fgf10* (Pauley et al., 2003), *Otx1* (Fritzscht et al., 2001; Morsli et al., 1999), *Foxg1* (Hwang et al., 2009; Pauley et al., 2006), *Bmp4* (Chang et al., 2008) and *Jag1* (Brooker et al., 2006; Kiernan et al., 2006) affect primarily crista development but also

canal formation. This suggests that the initial formation of a canal crista as a prosensory patch expressing multiple genes is needed to induce the upregulation of genes necessary for canal growth (Bok et al., 2007). This process ensures proper canal growth leading to endolymph flow during head rotation, thus allowing appropriate angular acceleration mediated stimulation of the canal cristae. The cochlear duct seems to grow by two processes: proliferation of cells and intercalation of existing cells (convergent extension) to extend the duct into the two or more turns as found in mammals. Like in the vestibular system, the growth of the cochlear duct is regulated by the growth of the organ of Corti, which apparently requires convergent extension movements of the developing hair cells (Chen et al., 2008; Hwang et al., 2010; Yamamoto et al., 2009). Disruptions of several genes that affect the appropriate alignment of hair cells result in a shortened and wider organ of Corti. For example, loss of *Foxg1* shortens the cochlea to a half turn and expands the rows of hair cells in the apex to approximately ten or more [(Pauley et al., 2006); Fig. 2]. The shape and organization of the hair cells in these mutant cochleae closely resemble those of monotremes (Fig. 1, 2). In addition, absence of *Foxg1* also disturbs the polarity of many hair cells (Fig. 2C), which is consistent with the idea that convergent extension movements of developing hair cells relate to planar polarity signals (Chen et al., 2008; Kelly and Chen, 2009) but may not be needed for the monotreme organ of Corti (Fig. 2D). Several other mouse mutants that have a shortened cochlea also display multiple rows of hair cells (Fig. 3), such as *Neurog1* null mice [(Ma et al., 2000); Fig. 3A–A'''], *Neurod1* null mice [(Jahan et al., 2010b); Fig. 3B–E'] and *Jxc1* null mice (Chen et al., 2008) whereas *Rac1* mutants have a shortened cochlea without extra rows of hair cells (Grimsley-Myers et al., 2009). These data suggest that morphogenetic movements related to convergent extension elongate the organ of Corti (Hwang et al., 2010) possibly be acting via planar polarity (Grimsley-Myers et al., 2009; Kelly and Chen, 2009) to initiate collective cell movement (Kim et al., 2010; Wallingford, 2010).

Studies of some other mutants support the notion that the numbers of organ of Corti precursor cells may determine the overall extension of the cochlear duct. For example, deletion of *Shh* derails cochlear formation entirely through massive reduction of proliferation (Riccomagno et al., 2002). Later in development, *Shh* is released from the forming sensory neurons and disrupted *Shh* signaling through a mutated *Gli3* results in aberrations of organ of Corti organization (Driver et al., 2008), including formation of vestibular-like hair cells in the greater epithelial ridge (GER, also referred to as Koelliker's organ). *Neurog1* null mice also lack sensory neurons and thus *Shh* signaling from them. Like *Gli3* mutants, *Neurog1* mutant mice have a shortened organ of Corti with multiple rows of hair cells (Ma et al., 2000) and formation of ectopic hair cells (Fig. 3A') in the GER (Matei et al., 2005) and *Rac1* null mice have fewer hair cells and a shortened cochlea (Grimsley-Myers et al., 2009).

However, some mutations require modifications of this basic idea. For example, *Atoh1* null mice do not differentiate hair cells and lose many organ of Corti cells (Chen et al., 2002), but show a near normal extension of the cochlear duct (Fritzsche et al., 2005; Pan et al., 2010a). Such data could be reconciled with the convergent extension hypothesis by assuming that the apparently limited degree of development and viability of organ of Corti precursors nevertheless provide the convergent extension movement. In contrast to the data on *Atoh1* mutant mice, a shortened cochlear duct forms in *Sox2* null mice (Kiernan et al., 2005). *Sox2* protein is essential for the specification of hair cell precursors that never differentiate to hair cells. In contrast to *Atoh1*, which is upregulated after hair cells have exited the cell cycle (Lee et al., 2006; Matei et al., 2005; Ruben, 1967), *Sox2* expression precedes cell cycle exit of hair cells (Mak et al., 2009; Nichols et al., 2008). The *Sox2* transcription factor may affect proliferative expansion of organ of Corti precursors and their concomitant convergent extension movements. A correlation of aberrant proliferation with

reduction of convergent extension of the organ of Corti has been claimed for *Foxg1* and *Neurog1* null mice (Matei et al., 2005; Pauley et al., 2006), but there has been no investigation of this possibility in *Sox2* null mice.

Shh and *Fgf*'s regulate an important gene for cochlear development, *Pax2*. Earlier work has shown that deletion of *Pax2* blocks initial formation of a cochlear duct (Burton et al., 2004; Torres et al., 1996), but others have suggested the formation of a cochlear sac either within the otic capsule (Favor et al., 1996) or extruding into the cranial cavity in *Pax2* null mice (Bouchard et al., 2010). The later sizable growth of the cochlear duct happens in the absence of sensory epithelia formation, implying that cochlear duct growth can be uncoupled from organ of Corti development (Bouchard et al., 2010). *Gata3* is yet another gene that is tightly involved in cochlea formation. Absence of *Gata3* arrests the ear development at the otocyst stage (Karis et al., 2001), but more recent work shows that some degree of cochlear growth without sensory or neuronal development is possible in the absence of *Gata3* (Duncan et al., 2011). These defects in the organ of Corti are consistent with the hearing defects found in *Pax2* and *Gata3* haplo-insufficient patients (Favor et al., 1996; van Looij et al., 2005). It is possible that *Gata3* affects the development of the organ of Corti at multiple levels: competence, proliferation and convergent extension. Another set of factors that affect cochlear duct growth and hair cell formation are micro-RNAs. These 21–22 nucleotides long RNA molecules are essential for organ of Corti differentiation and may lead to patchy loss of hair cells (Soukup et al., 2009) or complete absence of all hair cells (Kersigo et al., 2011), but nevertheless form a cochlear duct.

Combined, these data suggest that some cochlear duct growth is possible even without any sensory epithelia formation. This uncoupling of cochlear duct growth from the organ of Corti is consistent with the presence of a cochlear/lagenar duct without hair cells in certain amphibians (Fritzsche and Wake, 1988). However, full extension of the cochlear duct and the organ of Corti requires convergent extension movements of organ of Corti precursors. Reduced proliferation of organ of Corti precursors or disrupting their convergent extension movements will affect overall organ of Corti growth and patterning. Thus, while not as clear at the molecular level as the regulation of canal growth by the canal cristae, there is a connection between the elongation of cochlear duct and the organ of Corti: the proper elongation to a coiled cochlea is proportional to the organ of Corti precursor formation and their intercalation to achieve convergent extension.

Specifying the position and the molecular competence to form an organ of Corti

Interactions between the developing cochlea and the surrounding (periotic) mesenchyme define the position and coiling of the cochlea. This initial insight (Van de Water, 1983) has been characterized at the molecular level. Expression studies and deletion of several genes such as *Tbx1*, *Pou3f4/Brn4* (Braunstein et al., 2008; Braunstein et al., 2009; Phippard et al., 1999), *Fgf9* (Pirvola et al., 2004) and *Sox9* (Mak et al., 2009; Trowe et al., 2010) suggest a critical interaction between periotic mesenchyme and proper cochlear development. Interestingly, *Sox9* has been described in *Xenopus* but seems to have no effect on basilar papilla (Park and Saint-Jeannet, 2010), the non-coiled evolutionary precursor of the organ of Corti (Fritzsche, 2003). In frogs, the basilar papilla sits on thick periotic mesenchyme instead of a basilar membrane on top of the scala tympani (Lewis et al., 1985). This indicates that the extension and coiling of the organ of Corti might in part be related to the transformation of the periotic mesenchyme underneath the basilar membrane into the scala tympani, one of the least understood aspects of cochlear development in terms of its molecular basis (Montcouquiol and Kelley, 2003). Beyond these observations, there is no molecular causality established that directs the formation of the organ of Corti only in that particular area of the cochlear duct adjacent to the scala tympani.

Similar to the unresolved issues around the positioning of the organ of Corti, the molecular basis that guides that the inner and outer spiral sulcus brackets the organ of Corti for appropriate mobility remains unclear. This process can be broken down into the early prosensory specification to develop competence in the otic epithelium and the upregulation of *Atoh1* gene to translate this competence into hair cell differentiation. Mutations of *Eya1*, *Pax2*, *Jag1*, *Sox2* and *Gata3* apparently disrupt the sensory competence, resulting in limited formation of hair cells in the cochlear duct. This implies that the proteins produced by these genes are necessary for sensory development in the cochlea duct and do so through a stepwise refinement and restriction of sensory specification and competence to specify the organ of Corti (Zou et al., 2008). They also interact with other factors expressed adjacent to or within the developing organ of Corti such as *Bmp4* (Hwang et al., 2010; Ohyama et al., 2010), *Fgf10* (Pauley et al., 2003), *Lmx1a* (Nichols et al., 2008), *Prox1* (Birmingham-McDonogh et al., 2006; Fritzsche et al., 2010b), *Fgf8* (Jahan et al., 2010b), *Fgf20* (Hayashi et al., 2008). Together, these genes provide the context to allow prosensory cells, after cell cycle exit, to appropriately respond to *Atoh1* expression and differentiate as hair cells. If true, defining the cochlea prior to its *Atoh1* mediated differentiation would be equivalent to defining the minimal essential (spatial and/or temporal) co-expression of genes that allows this process to work. Mutations in the genes expressed in or adjacent to the organ of Corti prior to onset of hair cell differentiation (*Sox2*, *Jag1*, *Gata3*, *Fgfr3*, *Fgf20*, and *Bmp2/4*) result in disruption of the organ of Corti differentiation (Fritzsche et al., 2010b; Hayashi et al., 2008; Hwang et al., 2010; Karis et al., 2001; Kiernan et al., 2006; Kiernan et al., 2005; Nichols et al., 2008; Ohyama et al., 2010; Pirvola et al., 2002; Puligilla et al., 2007). Unfortunately, most work conducted on these mutants has not tested the effects of a given null mutation on the expression of all these prosensory genes, simply because many were not known at the time of initial descriptions of the mutants.

The onset of *Atoh1* mediated hair cell differentiation causes expression changes in several prosensory genes, relegating them to supporting cell expression (Dabdoub et al., 2008), and ultimately shutting down their expression over time (Kwan et al., 2009; Pan et al., 2010a; White et al., 2006). Suppression and possible re-activation of the embryonic transcription factors could be of crucial importance in the transformation of the “flat epithelium” that remains after hair cell loss into proliferating and subsequently *Atoh1* induced differentiating hair cells. Without such “priming”, the flat epithelium seems to have only a very limited, if any, capacity to proliferate and transform into hair cells in response to *Atoh1* action (Batts et al., 2009; Izumikawa et al., 2008; Wang et al., 2010).

Molecular development of prosensory domains and differentiation into hair cells and supporting cells

One of the least understood mechanisms in cochlear development is how the upregulation of *Atoh1* is accomplished and what the molecular basis is for the differentiation of distinct hair cell and supporting cell types. bHLH proteins, like *Atoh1*, are positive regulators for cell fate determination and differentiation of sensory neurons and hair cells in the vertebrate ear, much like the proneural genes that drive sensory development in flies (Garcia-Bellido and de Celis, 2009). Clearly, a single gene is unable to specify the multitude of hair cells and supporting cells. Recent data show multiple proneural and neurogenic bHLH genes in ear development (Doetzlhofer et al., 2009; Fritzsche et al., 2010a), suggesting a high degree of similarity to fly sensory development (Garcia-Bellido and de Celis, 2009). The complexity of neurogenic bHLH factors has recently been reviewed in the context of hair cell regeneration (Groves, 2010). Below we focus on *Atoh1* and two other proneural bHLH genes that affect cochlear hair cell development, *Neurog1* and *Neurod1*.

Atoh1 is in many respects an unusual bHLH transcription factor (Bertrand et al., 2002; Guillemot, 2007). It is the only proneural bHLH transcription factor that is expressed in

proliferating precursors to guide their expansion while maintaining their fate specification [the cerebellum is a prime example for this expression (Bermingham et al., 2001; Pan et al., 2009)]. Alternatively, it is expressed in both premitotic proliferative precursors and in the postmitotic differentiating cells [the endothelial precursors that give rise to both paneth cells and enteroenteric neurons are a major example (Shroyer et al., 2007)]. In contrast to the neuronal expression, in the ear *Atoh1* is only upregulated in postmitotic hair cells (Fig. 4, 6) to guide their differentiation (Bermingham et al., 1999; Fritzscht et al., 2005; Lee et al., 2006; Matei et al., 2005). The molecular selection mechanism of these cells to upregulate *Atoh1* for hair cell differentiation in a highly stereotyped pattern is unknown. The well-known Delta/Notch system of lateral inhibition is unlikely for this selection process, as this would result in a random and not a stereotyped pattern of hair cells, a process that has been well demonstrated in fly development (Garcia-Bellido and de Celis, 2009). Prime candidates for *Atoh1* upregulation would be the six mammalian *Iroquois* genes. Iroquois proteins bind to the enhancers of bHLH genes to initiate bHLH gene expression (Garcia-Bellido and de Celis, 2009; Gomez-Skarmeta et al., 2003). However, only limited expression of iroquois genes has been reported in the developing mouse ear, leaving the question open about the involvement of these factors in the regulation of *Atoh1* expression. *Prox1* has been proposed as a possible selector of hair cell fate (Bermingham-McDonogh et al., 2006; Kirjavainen et al., 2008), but *Prox1* null mice show little to no defects in hair cell development except for a reduction in hair cells in the canal cristae (Fritzscht et al., 2010b). Fgfs and Fgfrs could certainly specify bHLH gene upregulation but typically show a wider expression. They also can diffuse away from the cells and thus are difficult to direct the stereotyped *Atoh1* upregulation in postmitotic hair cells by themselves. In the cochlea, Fgfs may interact with Bmp4 signaling for hair cell specification (Ohyama et al., 2010) in agreement with such interactions in neuronal specification of epidermis (Fritzscht et al., 2006; Gaspard and Vanderhaeghen, 2010). *Sox2*, while essential for hair cell differentiation (Kiernan et al., 2005), is expressed prior to *Atoh1* (Mak et al., 2009) and could thus determine specification of the organ of Corti (Fig. 6). However, *Sox2* is expressed much wider than *Atoh1* and thus cannot be the factor that specifies *Atoh1* upregulation. *Jag1* null mice have only partial loss of cochlear hair cells indicating that it is not essential for hair cell differentiation (Brooker et al., 2006; Kiernan et al., 2006). Interestingly, misexpression of *Jag1* (Pan et al., 2010b) and deletion of *Lmo4* (Deng et al., 2010) result in formation of extra hair cells, indicating that the competence to express *Atoh1* is widely distributed in the cochlear duct. Most interesting is the formation of only inner hair cells in some areas of the developing cochlea of *Jag1* null mice, suggesting a possible convergence of *Neurod1/Neurog1* mutation defects (see below) with *Jag1* signaling. In older embryos *Pax2* is expressed in hair cells and seems to be essential for cochlear hair cell formation (Bouchard et al., 2010), but in early embryos its expression appears to be much wider than the cochlea (Burton et al., 2004; Lawoko-Kerali et al., 2002) and could not be the determining factor for *Atoh1* upregulation.

Another transcription factor that is expressed in the organ of Corti is *Gata3*, an ortholog of the fly Gata zinc finger factor *pannier* that plays a role to topographically restrict bHLH gene activation in flies (Garcia-Bellido and de Celis, 2009; Gomez-Skarmeta and Modolell, 2002). *Gata3* is essential for ear morphogenesis (Lillevali et al., 2006) and has been suggested to play the role of an upstream regulator of early neurosensory specification (Karis et al., 2001). The expression of *Gata3* in the cochlea and the progressive hearing loss found in *Gata3* haploinsufficient mice (van Looij et al., 2005) seem to indicate that *Gata3* protein is needed for neurosensory development (Fig. 6). However, a direct connection of *Gata3* with hair cell development requires conditionally knocking out the floxed *Gata3* gene using a cre that is expressed as soon as hair cells start to differentiate, such as *Atoh1-cre* (Matei et al., 2005; Yang et al., 2010).

Atoh1 protein regulates directly, or through unknown intermediates, other crucial genes for hair cell development and maintenance such as *Pou4f3* (previously *Brn3c*), *Gfi1* (a homologue of the fly *senseless* gene) and *Barhl1*. Loss of each of these genes results in delayed loss of hair cells (Chellappa et al., 2008; Hertzano et al., 2004; Li et al., 2002; Pauley et al., 2008; Wallis et al., 2003). In flies, *senseless* directly interacts with bHLH proteins to regulate subtype specification (Acar et al., 2006) and can compensate for lost bHLH proteins. It is unclear if *Gfi1* plays similar function in mammalian hair cell development.

Atoh1 activates the Delta/Notch system of lateral inhibition, forcing adjacent cells to assume a non-hair cell phenotype. Mis-regulation of *Atoh1* through changes in the lateral inhibition of the Delta/Notch system (Zine et al., 2001) or simple mis-expression in the GER (Gao, 2003; Gubbels et al., 2008; Zheng and Gao, 2000) can result in hair cell differentiation. This suggests that the competence to respond to *Atoh1* with hair cell differentiation is much broader than the final upregulation of *Atoh1*. Indeed, the enhancer region of *Atoh1* shows consistent expression in supporting cells such as the inner pillar cells (Matei et al., 2005) and transcription of *Atoh1* seems to occur as well in inner pillar cells (Yang et al., 2010). This suggests a more widespread pattern of low-level expression of *Atoh1*, comparable to developing fly sensory organs (Garcia-Bellido and de Celis, 2009).

To understand how the hair cell specific upregulation of *Atoh1* is accomplished and how it relates to different hair cell types, we will briefly outline the multitude of bHLH genes and their interaction in neurosensory development of the ear (Fritzscht et al., 2000; Fritzscht et al., 2010a).

Neurogenin1 (*Neurog1*, formerly *Ngn1*) expression can be demonstrated by *in situ* hybridization at embryonic day (E) 8.75 (Ma et al., 1998) prior to upregulation of any genes of the Delta/Notch signaling pathway (Brooker et al., 2006; Fritzscht et al., 2006). In *Neurog1* null mice, there is no formation of sensory neurons and the initial activation of the Delta/Notch system as shown by *Rbpj* upregulation never happens (Ma et al., 1998). A follow-up study showed that absence of *Neurog1* not only eliminates neuronal formation in the ear but also has massive effects on hair cell development (Ma et al., 2000). Saccular hair cells nearly completely disappear in *Neurog1* null mice and the cochlea is significantly shortened with multiple rows of inner and outer hair cells in the apex (Ma et al., 2000). In addition, the appearance of ectopic hair cells in the GER and the Ductus reuniens (Fig. 2A 'A'), indicate that *Neurog1* can suppress *Atoh1* upregulation and truncate sensory hair cell precursor populations, possibly by changing the cell cycle exit and spatiotemporal expression of other bHLH genes such as *Atoh1* (Matei et al., 2005). Studies using a tamoxifen inducible *Neurog1-cre* transgene construct showed that hair cells in the utricle and saccule could be labeled after the induction of the *Neurog1-cre*, indicating that many utricular and saccular hair cells have a lineage relationship to *Neurog1* positive cells (Koundakjian et al., 2007; Raft et al., 2007). Interestingly, little evidence for such a lineage relationship was revealed in canal cristae and none in the cochlea, which also show a profound reduction in hair cells in *Neurog1* null mice (Matei et al., 2005). In addition, *Neurog1* mutant mice show changes in sensory/non-sensory specification such as transformation of the non-sensory cruciate eminence of the anterior and posterior canal crista into hair cells (Jahan et al., 2010b; Ma et al., 2000). One of the possible explanations for these differences is that only some hair cells have lineage relationship to non-hair cells while others do not (Fritzscht et al., 2006; Raft et al., 2007). Alternatively, there is an early and near complete segregation of sensory and neuronal precursors in some epithelia such as the cochlea, which precludes the discovery of the lineage tracing with current techniques. Clearly, more work is necessary to understand how absence of *Neurog1* mechanistically

affects hair cell formation in the developing organ of Corti and upregulates hair cell formation in the GER.

Neurod1 is the second bHLH gene to be expressed in the developing ear and this early expression depends on *Neurog1* in neurons (Ma et al., 1998). *Neurod1* null mutants lose most sensory neurons, generate hair cells inside the ganglia and show defects in hair cell development in the organ of Corti (Jahan et al., 2010a; Jahan et al., 2010b; Kim et al., 2001; Liu et al., 2000). *Neurod1* seems to be able to suppress *Atoh1* in differentiating hair cells and neurons (Jahan et al., 2010b; Pan et al., 2009). Previous work using an *Atoh1-cre* mediated upregulation of LacZ suggested a transient and weak expression of *Atoh1* in delaminating sensory neurons. However, the *Atoh1* expression as revealed by the Lac-Z marker system seems to become more profound with age (Matei et al., 2005). Recently we demonstrated that this early expression is more prominent in *Neurod1* null mice and leads to the formation of cells that express hair cell markers, including *Atoh1*, in the remaining ganglia (Jahan et al., 2010b). Not only do hair cells differentiate in these ganglia, but also these hair cells have the ability to organize surrounding cells to form vesicles (Fig. 5). What exactly the cellular source of these hair cells is in terms of lineage relationship remained somewhat unclear (Fig. 5). Using *Pax2-cre* to knock-out the floxed *Neurod1* and simultaneously activate a *Rosa-26 LacZ* reporter, we show that many hair cells that form in these ganglia also express *LacZ* (which transcribes the enzyme β -galactosidase) and *Myo7a*, a hair cell marker (Fig. 5C').

Neurod1 is not only suppressing *Atoh1* and the expression of other transcription factors in the developing neurons but also in the developing cochlea. One of the most puzzling aspects of cochlear development has been the apex to base progression of cell cycle exit (Lee et al., 2006; Matei et al., 2005; Ruben, 1967) followed, with a delay, by the base to apex upregulation of *Atoh1* (Fig. 6) and subsequent hair cell differentiation (Chen et al., 2002). Our data in *Neurod1* null mice show that every gene associated with hair cell differentiation that was tested showed an apex to base upregulation, opposite to the normal base to apex progression (Jahan et al., 2010b). These data demonstrate that *Neurod1* affects gene upregulation in postmitotic hair cells and this altered expression changes the fate acquisition of hair cells.

Certain correlations of non-transcription factors such as the motor proteins *Myo6* and *Myo7a* have been found with inner and outer hair cells (Cotanche and Kaiser, 2010). However, *Neurod1* is the only transcription factor thus far recognized to be required for outer hair cell development. In *Neurod1* null mice, outer hair cells in the apex acquire either an inner hair cell phenotype or do not develop at all (Jahan et al., 2010b).

Like *Neurod1* null mutants, *Neurog1* null mutants have a shorter cochlea (Jahan et al., 2010b) and disorganization of hair cells in the apex (Fig. 2A–A''): they develop multiple rows of inner and outer hair cells near the apex (Ma et al., 2000) that end in a single row of inner hair cells as in *Neurod1* null mice (Jahan et al., 2010b). These data indicate that the *Neurog1/Neurod1/Atoh1* interactions in the cochlea are profound but not yet understood mechanistically. This is reminiscent of the *Ac/Sc* interactions that control the specification of subsets of sensory cells (Garcia-Bellido and de Celis, 2009). What other transcription factor (s) is (are) complementary to *Neurod1/Neurog1* and support outer hair cell differentiation in the base and middle turn remains unclear. Further work will have to include other bHLH genes now known to be expressed in the ear (*Nhlh1*, *Nhlh2*) that may mediate in part the apparent cross-regulation of the currently investigated bHLH genes (Fritzsch et al., 2010a).

Given the multitude of genes thus far identified to disrupt organ of Corti development in the respective null mutants, we like to propose that it is not a single factor that ultimately defines upregulation of *Atoh1* in future hair cells of the organ of Corti. In parallel to data

generated in flies (Garcia-Bellido and de Celis, 2009), we propose that a yet-to-be fully defined combinatorial code of partially overlapping expression of transcription factors, including multiple proneural bHLH proteins, is interacting with *Atoh1* to differentiate specific types of hair cells in defined positions.

In conclusion, progress has been made towards understanding how the organ of Corti grows, is molecularly specified and differentiates, but even more questions about genetic interactions abound. The next few years will require going beyond appreciating the complexity of the molecular interactions and should aim to establish the quantitative aspects of these interactions using newly developed tools, such as next generation sequencing. This will establish expression profile changes of all genes in reasonably well understood mutant lines in order to generate testable models of gene networks (Roy et al., 2010). Given the emerging complexity of molecular interactions during development, and the stochastic interaction of genes in a given cell, it is possible that our current approaches to verify the functions of critical genes one or two at a time will not allow elucidation of such complex interactions. A better understanding of the interactions of transcription factors during development is needed to reconstitute an organ of Corti that has lost all hair cells and has transformed into a ‘flat epithelium’ with little to no cellular differentiation. Clearly, such epithelia have lost the ability to respond to *Atoh1*, a powerful transcription factor that drives hair cell differentiation during development and regeneration (Bermingham et al., 1999; Izumikawa et al., 2008). Deconstructing the molecularly guided development that establishes cochlear duct formation, positioning of the organ of Corti and bestowing competence to allow *Atoh1* to differentiate appropriate types and numbers of hair cells in the correct position may provide such information. Translating the current rudimentary insight without such in depth understanding may lead to short-lived success stories, but is not useful for a consistent effective treatment of patients with complete loss of hair cells. In essence, we may be able to re-activate the developmental program so that *Atoh1* expression will be driven in the organ of Corti position by the same transcription factors that guide that process during development.

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Fig. 1.

The evolution of the basilar papilla/organ of Corti is depicted in this scheme. A basilar papilla at the orifice of the lagenar recess harboring the gravistatic lagenar organ is a common feature of all tetrapods. The most parsimonious explanation would be that a single evolutionary event has led to the formation of a lagena, a lagenar recess and the basilar papilla (left). Data on the sarcopterygian fish, *Latimeria*, suggests that this evolutionary event dates back to the aquatic ancestor of tetrapods. Monotremes have retained the original configuration of tetrapods and have a lagenar recess with a lagena sensory epithelium (middle). However, while the organ of Corti of monotremes is in the same position as the basilar papilla of tetrapods, it has evolved the cellular architecture of the organ of Corti with inner and outer hair cells. The coiling of the cochlear duct and extension of the organ of Corti throughout the cochlear duct in modern mammals (marsupials, eutherians) is somehow related to the evolutionary and developmental loss of the lagena (right). Adopted from (Jorgensen and Locket, 1995; Pauley et al., 2003)

Fig. 2. Lack of *Foxg1* results in a shortened and wider organ of Corti

Absence of *Foxg1* results at E18.5 (A,B,C) in cellular disorganization of the organ of Corti. Multiple rows of inner and outer hair cells form in the apex (B,B') with near normal organization in the base (A,A'). Despite the presence of multiple rows of hair cells, their innervation is severely disrupted with overshooting fibers towards the lateral wall of the cochlea (A,B). The cross section through the apex and the base of the cochlea of the *Foxg1* null ear show near normal orientation of inner and outer hair cells in base (A''), but the apex displays ten rows of outer hair cells with multiple supporting cells (B''). The SEM demonstrates apical specializations of the multiple rows of disorganized hair cells (C). The polarity of hair cells is distorted in the apex of the *Foxg1* null cochlea (C) indicating that *Foxg1* is not only needed for convergent extension movements but also plays a role in planar polarity formation. The apex of *Foxg1* null mice resembles the spiny anteater (a monotreme) cochlea. The extant monotremes (spiny anteater and platypus) are the only mammals that have multiple rows of hair cells in a shortened cochlea (D). GER, greater epithelial ridge; IHC, inner hair cells; OHC, outer hair cells; TM, tectorial membrane; SC, supporting cells. C, modified after (Pauley et al., 2006); D, modified after (Ladhams and Pickles, 1996). Bar indicates 100 μ m in A,A',B,B' and 10 μ m in A'',B'',C,D.

Fig. 3. Absence of *Neurog1* and *Neurod1* leads to truncation of cochlear elongation

Deletion of *Neurog1* stunts cochlear growth (A), results in multiple rows of inner and outer hair cells near the apex (A') and the formation of hair cells in the GER and ductus reunions (A', A''). The apical tip is characterized by 1–2 rows of only inner hair cells and complete absence of outer hair cells (A'''). Note the near complete loss of saccular hair cells (S in A'', insert in A'''). Floxed *Neurod1* conditional knockout (CKO) using *Pax2-cre* also results in a shorter cochlea (B) and the formation of two rows of inner and four or more rows of outer hair cells in apical half of the organ of Corti (C,D). *Neurod1* CKO mice are viable and studying the postnatal hair cells reveals developmental deviations with ectopic inner hair cell formation (C,D) in positions that are topographically equivalent to outer hair cells. Some Deiter's cells show pillar cell like differentiation (C'). As with *Neurog1* null mice (A'''), *Neurod1* CKO mice show only inner hair cells in an irregular row in the apical tip (E,E') that receive an unusual pattern of innervation of the greatly diminished spiral neurons (E). DR, ductus reunions; GER, greater epithelial ridge; OC, organ of Corti; S, saccule; U, utricle. Bar indicates 100 μm except in C–E' where it indicates 10 μm.

Fig. 4. An organ of Corti consisting of undifferentiated cells develops in *Atoh1* null mice
 Absence of the bHLH gene *Atoh1* results in agenesis of hair cells. However, using either the hair cell marker *Bdnf* (A,B) or the LacZ reporter knocked into the *Atoh1* locus (C) reveals expression of these markers suggesting some degree of hair cell precursor specification in mice lacking *Atoh1* (B,C). Wild type mice show upregulation of *Bdnf* in all hair cells throughout the cochlea (A, A', A''). In contrast, there is no expression of *Bdnf* in the base of the cochlea (B) whereas it is expanded in the apex without any recognizable rows of inner and outer hair cells (B, B'). Sections show distribution of the *Bdnf* marker throughout all cells in the undifferentiated organ of Corti (B''). The LacZ reporter inserted to replace the coding frame of the *Atoh1* gene shows expression along the entire length of the organ of Corti (C). However, this expression is reduced to 1–2 irregular rows of cells in the base (C') and much wider in the apex (C). Radial sections through the organ of Corti show the β -galactosidase reaction product throughout the height of the organ of Corti (C''). Occasionally, cells in the correct topology of outer hair cells may be found (arrows in C''). IHC, inner hair cells; OHC, outer hair cells. Bar indicates 100 μ m except in A'', B'', C'' where it indicates 10 μ m.

Fig. 5. Lack of *Neurod1* results in formation of hair cells in the remaining ganglia

Mice with conditional deletion of *Neurod1* (*Pax2-cre::Neurod1^{fl/fl}*; A–C) have a reduction in innervation as shown with anti-tubulin (A, green) but also show an unusual distribution of Myo7a positive cells in the remaining ganglia in addition to the hair cells of the sensory epithelia. These cells persist in adult animals and form hair cell-like tufts extending into multiple large and small vesicles that form inside the ganglia (C,D). *Pax2-cre* mediated Rosa 26 LacZ expression (B, C') shows that neurons and hair cells have mostly β -galactosidase positive nuclei. This level of co-localization suggests that hair cells and neurons of ganglia derive from the otocyst, and that hair cell may derive from neuronal precursors that alter their fate in the absence of *Neurod1* into hair cells. AC, anterior crista; HC, horizontal crista; U, utricle; V, intraganglionic vesicle; Vgl, vestibular ganglion. Bar indicates 100 μ m except in B', which indicates 10 μ m.

Fig. 6.

This scheme displays the time of cell cycle exit and some of the transcription factors known or suspected to play a role in organ of Corti and hair cell development. Note that Sox2 and Gata3 are expressed throughout the organ of Corti prior to, during and after cell cycle exit. They could promote cell cycle progression but are unlikely to be causal for either defining alone the organ of Corti or expression of hair cell specific bHLH genes. In contrast to the apex to base progression of the cell cycle exit, expression of bHLH genes is from upper middle turn ('base') to apex. Indeed, expression of bHLH genes reaches the first hair cells that exit the cell cycle in the apex around E12.5 only around birth. This delay indicates that Atoh1 (and other bHLH transcription factors) play no role in cell cycle exit but act as differentiation factors in the organ of Corti. When exactly first expressions occur will depend on the techniques used. Some factors (Neurod1) have early expression in general followed much later by specific expressions. Modified after (Fritzsche et al., 2010a; Karis et al., 2001; Matei et al., 2005; Nichols et al., 2008; Ruben, 1967).