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Understanding the evolution and development of neurosensory transcription factors of the ear to enhance therapeutic translation

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Abstract

Reconstructing a functional organ of Corti is the ultimate target towards curing hearing loss. Despite the impressive technical gains made over the last few years, many complications remain ahead for the two main restoration avenues: in vitro transformation of pluripotent cells into hair cell-like cells and adenovirus-mediated gene therapy. Most notably, both approaches require a more complete understanding of the molecular networks that ensure specific cell types form in the correct places to allow proper function of the restored organ of Corti. Important to this understanding are the basic helix-loop-helix (bHLH) transcription factors (TFs) that are highly diverse and serve to increase functional complexity but their evolutionary implementation in the inner ear neurosensory development is less conspicuous. To this end, we review the evolutionary and developmentally dynamic interactions of the three bHLH TFs that have been identified as the main players in neurosensory evolution and development, Neurog1, Neurod1 and Atoh1. These three TFs belong to the *neurogenin/atonal* family and evolved from a molecular precursor that likely regulated single sensory cell development in the ectoderm of metazoan ancestors but are now also expressed in other parts of the body, including the brain. They interact extensively via intracellular and intercellular cross-regulation to establish the two main neurosensory cell types of the ear, the hair cells and sensory neurons. Furthermore, the level and duration of their expression affect the specification of hair cell subtypes (inner hair cells vs. outer hair cells). We propose that appropriate manipulation of these TFs through their characterized binding sites may offer a solution by itself, or in conjunction with the two other approaches currently pursued by others, to restore the organ of Corti.

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Keywords

Inner ear; Development; Hair cell; Restoration; Transcription factor

Introduction

Hearing loss of various forms afflicts over 200 million people, including half of individuals over the age of 65, making sensorineural hearing loss one of the most frequent neurosensory disorders worldwide. Many forms of severe sensorineural hearing loss result from the progressive and near-complete loss of all hair cells in the organ of Corti (OC). A cochlear implant, the only treatment currently at hand, incompletely restores hearing by providing a limited set of stimulation points to the remaining spiral ganglion cell processes, which may be lost over time in humans as they are in animal models (Alam et al. 2007; Shibata et al. 2010). While a cochlear implant is, for a growing number of elderly patients, the only way to restore at least some level of communication ability, the functional OC is clearly “the world's best hearing aid” (Puligilla and Kelley 2009). Therefore, numerous attempts to fully restore hearing are currently centered on two principal approaches:

A) In vitro transformation of embryonic or induced stem cells into hair cell-like cells (Kopecky and Fritzsch 2011; Oshima et al. 2010; Ronaghi et al. 2012), followed by seeding them into the hair cell-depleted cochlea to differentiate as hair cells.

B) In vivo manipulation of proliferation of adult ear cells to increase the number of supporting cells, followed by converting them into hair cells through regulated differentiation with genes inserted into adenoviral vectors (Batts et al. 2009; Brigande and Heller 2009; Izumikawa et al. 2005, 2008; Kopecky and Fritzsch 2011).

Impressive progress in these two areas elevates hair cell regeneration into the realm of possibility as far as generating in vitro hair cell-like cells (Oshima et al. 2010) or transforming in vivo supporting cells into hair cells (Izumikawa et al. 2005, 2008). Both approaches are in line with the state of the art in other systems where simple replacements, such as the trachea coated with induced stem cells of a given patient, are possible (Jungebluth et al. 2011; Jungebluth and Macchiarini 2011). However, in contrast to other systems, the more complex OC has a highly ordered distribution of two different subtypes of hair cells (inner hair cells, IHCs; outer hair cells, OHCs) surrounded by seven different subtypes of supporting cells. This sophisticated organization is functionally meaningful for the perception of sound ranging from 2 to 20 kHz in human and to over 70 kHz in mouse. While restoring only IHCs may provide improvement over no hair cells, restoration of both subtypes of hair cells in specific positions is likely necessary to restore normal hearing. Simply speaking, specific subtypes of hair cells have to be regenerated in a highly stereotyped pattern to ensure function of the restored OC. To achieve such topologically restricted cell type differentiation requires recapitulating the differentiation process during development in therapeutic regeneration.

Multiple developmental studies have made it clear that the transformation of the naïve otic placode epithelium into the neurosensory components of the ear is a stepwise process (Ahmed et al. 2012a; Nichols et al. 2008; Ohyama and Groves 2004; Zou et al. 2008). At the end of this process, topographically restricted expression of transcription factors (TFs) “prime” the otic epithelium to respond to locally expressed diffusible factors such as Fgfs and Bmps to develop the sophisticated microanatomy of the OC (Dabdoub et al. 2008; Fritzsch et al. 2011; Ohyama et al. 2011; Puligilla and Kelley 2009). Existing evidence

indicates that the specification of hair cell subtypes depends on certain gene regulations as mutants have been described that specifically lose most IHCs or most OHCs (Ahmed et al. 2012a; Brooker et al. 2006; Deol 1981; Holley et al. 2010; Huh et al. 2012; Pan et al. 2012), though the molecular mechanism remains unclear. Furthermore, we have just begun to understand the emerging feedback loops between different diffusible factors (Huh et al. 2012) and TFs within (Jahan et al. 2012; Pan et al. 2012) and between cells of the OC (Basch et al. 2011) and their roles in regulating cell differentiation. Obviously, a much deeper understanding of the sequential activation of genes and the interactions between them during development of the OC is needed before we can attempt to recapitulate the formation of specific hair cell subtypes in specific places with either of the two above-indicated approaches.

Studies in the mouse suggest that the expression of many differentiation factors is progressively downregulated in aging animals, in particular, following disruption of the OC development (Groves 2010; Pan et al. 2011, 2012), leaving only a small number of those factors to be permanently expressed, such as *Foxg1* (Pauley et al. 2006) and *Gata3* (Duncan et al. 2011; Karis et al. 2001). It is therefore possible that the molecular guidance for topologically correct differentiation of hair cells is insufficient in the adult cochlea, making cell-specific targeting seemingly unresolvable at our current level of understanding. Indeed, treatment with TFs that effectively regenerate hair cells in embryos cannot achieve the same effect in the adult cochlea devoid of an OC (Izumikawa et al. 2008). However, in analogy to the TFs and microRNAs needed to reprogram cells to form inducible pluripotent stem cells (Rosa and Brivanlou 2011), it may be possible to upregulate a limited set of TFs and microRNAs (Ahmed et al. 2012a; Soukup et al. 2009) to “prime” the epithelium to respond with differentiation upon expression of cell type-specific TFs such as Atoh1, something the adult “flat epithelium” is incapable of doing on its own (Izumikawa et al. 2008). Precisely which TFs and ear specific microRNAs and other factors are necessary for “priming” remains to be fully elucidated.

Alternatively, a third approach to restore a functional OC is to directly transform existing non-sensory epithelial cells of the “flat epithelium” (Izumikawa et al. 2008; Pan et al. 2011) into a new OC by activating the necessary set of TFs and other factors (Ahmed et al. 2012a; Fritzsch et al. 2011). As a proof of principle, recent data suggest that direct transformation of skin-derived cells into neurons is possible (Lujan et al. 2012; Pang et al. 2011) and neuron-specific microRNAs can transform fibroblasts into neurons (Yoo et al. 2011). Obviously, this approach is at the moment still far removed from translation into restoration of the OC but could embody the ultimate solution. Again, molecular dissection of the interactions and cross-regulation and a reasonable understanding of transcriptional regulations of the critical TFs and microRNAs for OC development are required for the elucidation of the right combination of factors needed to accomplish this goal.

Ultimately, no matter where we start, the task remains the same: ensuring that specific subtypes of hair cells develop in specific positions and drive topologically correct differentiation of supporting cells to restore a functional OC. Unfortunately, defining these specific cell types and subtypes seems to depend on a growing set of TFs and diffusible morphogens (Ahmed et al. 2012a; Basch et al. 2011; Fritzsch et al. 2011; Groves and Fekete 2012; Huh et al. 2012; Ohyama et al. 2011) with as yet mostly unclear interactions and hierarchies. Below, we will review the best-characterized TFs that execute the neurosensory differentiation in the ear, the basic helix-loop-helix (bHLH) TFs and their roles in cell type specific differentiation. We will introduce the evolution of these factors and how bHLH TF evolution ties into hair cell evolution. This analysis will be followed by an assessment of the TFs' molecularly dissected functions to direct the development of specific hair cell subtypes of the OC. We will then explore the possibility to regulate the expression of genes

downstream to these factors using the emerging knowledge of the binding of these TFs to specific promoter regions of molecularly distinct E-boxes. Finally, we will provide a novel perspective on how to use recently generated complex mutant mice to understand the molecular tuning of specific cell types independent of the topological information.

The evolution of bHLH proteins predates the evolution of inner ear neurosensory cells

bHLH TFs belong to ancestral pro-metazoan TFs that are already found in single-celled ancestors of metazoans such as fungi and choanoflagellates, the latter being the likely ancestor of metazoans (Degnan et al. 2009; Gazave et al. 2009; Young et al. 2011). What role the bHLH TFs played in the single-celled metazoan ancestors remains speculative but the function of some bHLH genes such as the *Myc* genes appears highly conserved from yeast to the mammalian ear (Kopecky et al. 2011; Schuldiner et al. 1996; Young et al. 2011). Other conserved metazoan bHLH TFs such as Twist1 mediate transitions from proliferation to differentiation, including migration (Lee and Yutzey 2011). Such variable function from proliferation to differentiation is also found in proneural bHLH TFs (Ali et al. 2011). Among metazoans, bHLH genes have diversified by an order of magnitude (from about 10 in choanoflagellates to 118 in humans) but the small number of bHLH TFs in choanoflagellates could also represent a secondary reduction (Sebe-Pedros et al. 2011).

It appears that a specific group of bHLH TFs, the group A bHLH TFs (Simionato et al. 2008) that contain factors essential for cell fate specification, evolved together with a range of other TFs in metazoan ancestors (Galliot and Quiquand 2011). Several members of these group A bHLH TFs such as Atoh1, Neurog1 and Neurod1, as well as members of other TF families such as Foxg1 (Pauley et al. 2006), Pax2/5/8 (Bouchard et al. 2010), Pou4f3 (Xiang et al. 2003), Lmx1a (Nichols et al. 2008) and Eya1/Six1 (Ahmed et al. 2012a; Ahmed et al. 2012b; Zou et al. 2008), are now known to be essential for the ear or other organ development. Furthermore, the diversification of bHLH TFs was accompanied by a diversification of their E-box DNA binding sites (between 600 and 1,000 partially overlapping genes; Klisch et al. 2011; Seo et al. 2007) and an increase in the number of cell types (from 1 to over 200 distinct cell types recognized in humans). In essence, the correlated changes in DNA binding properties and DNA binding sites resulted in co-evolution and diversification of the roles of the bHLH TFs and their binding sites (Degnan et al. 2009; Yang et al. 2011).

In single-celled organisms, the 10–31 bHLH TFs identified seem to play a role in regulating proliferation and possibly in transitioning from one phase of the life cycle into another, such as the shift between mitosis and growth (Sebe-Pedros et al. 2011). The metazoan group A bHLH TFs play an apparently similar role in the developing cortex (Ali et al. 2011), cerebellum (Klisch et al. 2011), cochlear nuclei (Maricich et al. 2009) and intestine (Li et al. 2011; Shroyer et al. 2007). Unlike non-group A bHLH TFs, which have retained and are mostly limited to the function of regulating mitosis (Young et al. 2011), group A bHLH TFs evolved the additional function to regulate topographically distinct cell differentiation in metazoans. It is mostly unclear how exactly the topographically restricted expression of these factors in only a subset of precursors is achieved but numerous mutant analyses have demonstrated that restricted expression of bHLH TFs provides a crucial developmental step that cannot be replaced by other TFs. It is possible that the large number of downstream genes regulated by each of these factors create non-redundant pathways (Klisch et al. 2011; Seo et al. 2007). However, this cannot be the only reason for bHLH diversification as some other TFs such as the Pax genes regulate even larger numbers of downstream genes and are typically not recruited for cell fate but rather organ fate determination (Bouchard et al. 2010; Kozmik et al. 2003; O'Brien and Degnan 2003). Nonetheless, it is likely that the group A

bHLH TF family evolved into their diverse assortment of cell fate determination roles because of the ancestral function of bHLH genes in regulating various stages of a cell's life cycle. Indeed, in most metazoans, there is a life-long balance between cell cycle and cell differentiation, with the mammalian ear and central nervous system being on the extreme end of differentiation without any overt ability to regenerate hair cells and neurons via reinitiating proliferation. In this context, it is noteworthy that the function of one group A factor, *atonal/Atoh1*, can be regulating proliferation (Klisch et al. 2011), differentiation (Bermingham et al. 1999), or both (Fritzsch et al. 2010; Shroyer et al. 2007).

If this idea is correct, we would need to study in more detail the expression of different bHLH genes of the same group or of different groups, using modern RT-qPCR approaches to monitor expression changes over time (Brar et al. 2011) in very short intervals in the same cells as a prerequisite towards evaluating the complexity of cross-regulation. This knowledge would be necessary to understand why in certain species of vertebrates all precursors are depleted through differentiation, while, in other cases, precursors are set aside as adult stem cells to repopulate lost cells when needed or even continue to proliferate throughout life.

bHLH transcription factors that are involved in proliferation in the ear

In the ear, two bHLH proto-oncogenes, *N-Myc* and *L-Myc*, are mostly co-expressed (Kopecky et al. 2011), whereas a third gene of the same family, *C-Myc*, seems to play little if any role (Dominguez-Frutos et al. 2011). Conditional deletion of *N-Myc* in the ear shows a massive reduction of ear growth, apparently through reduced proliferation (Kopecky et al. 2011). Consistently, overexpression of *N-Myc* shows increased ear size through increased proliferation (Dominguez-Frutos et al. 2011). However, *N-Myc* is not only expressed in the developing ear but is also expressed later on in differentiated hair cells. It is puzzling that a proto-oncogene is expressed in terminally differentiated cells that are unable to undergo induced mitosis but will die through apoptosis if so induced (Mantela et al. 2005). At a molecular level, *N-Myc* could alter the bHLH signaling through interactions with ID proteins and/or alter the microRNA content of differentiating hair cells (Kopecky and Fritzsch 2011; Kopecky et al. 2011) possibly through interactions with other TFs such as *Eya1-Six1* (Ahmed et al. 2012a). Notably, the initially differentiating hair cells of the OC but not the vestibular system, eventually die in the *N-Myc* conditional knockout (CKO) mutant, indicating that *N-Myc* plays a yet to be defined unique role in the OC hair cells. Early expression studies of bHLH genes associated with neurosensory differentiation in the ear (*Neurog1*, *Neurod1*, *Atoh1*, *Nhlh1*, *Nhlh2*) in the absence or presence of *N-Myc* will help to elucidate its additional function in regulating ear neurosensory development. Moreover, comparable work on *L-Myc* CKO mutants is needed to verify the possible functional redundancy of these two proto-oncogenes.

Understanding the molecular regulation of cell cycle control and the balance between proliferation and differentiation is essential for the attempts to restore hearing. For restoration along avenue “A”, which requires the initial formation of stem cells followed by seeding these cells into their topologically restricted positions along the OC, it is necessary to precisely control the cell cycle to not only acquire the correct number of physiologically normal hair cells but also to be able to inhibit extended proliferation when cell numbers are met and differentiation must occur (Jeon et al. 2011; Kopecky and Fritzsch 2011). Uncontrolled proliferation would create a cancer-like state whereas a lack of profound proliferation would create too few cells that may eventually die, as seen in the *N-Myc* CKO mice (Kopecky et al. 2011). The task may be even more daunting for restoration avenue “B”, as the in vivo post-mitotic cells to be manipulated have long since exited the cell cycle and are not normally responsive to proliferation cues, unlike the stem cells in avenue “A”.

No matter the starting point, in forcing cell cycle re-entry in differentiated cells, not only must the correct number of cells be formed by a controlled balance between proliferation and differentiation as in avenue "A" but this re-entry into the cell cycle must produce the normal cytoarchitecture of the OC. Nonetheless, re-entry through inhibition of cell cycle inhibitors (Laine et al. 2007; Liu and Zuo 2008; Minoda et al. 2007; Oesterle et al. 2011; Ono et al. 2009; Rocha-Sanchez et al. 2011; Sulg et al. 2010; Weber et al. 2008; Yu et al. 2010) or over-expression of proto-oncogenes (Loponen et al. 2011; Ozeki et al. 2007) have been successful for at least a short period of time but all newly formed cells eventually undergo apoptosis as there appears to be a more complicated intrinsic regulation than previously thought (Huang et al. 2011). Because of the possibility of cross-talk between bHLH TFs and the intrinsic interplay evident with the Mycs, IDs and differentiation cues such as Atoh1 and Neurod1, the understanding of both the positive (proto-oncogenes such as *N-Myc*) and negative (tumor suppressors such as p21/27Kip1 and pRb) regulation of the cell cycle has never been more important to hair cell restoration. Ultimately, to fully control the cell cycle, it is likely that manipulation of protooncogenes, tumor suppressors and differentiation TFs must be fully utilized, just as the gas and brakes must be in a car; otherwise, the cell cycle will speed out of control or stop dead in its tracks.

bHLH transcription factors that are involved in differentiation in the ear

Mutational analysis in mice has identified three closely related group A bHLH TFs (*Atoh1*, *Neurog1*, *Neurod1*) that are essential for neurosensory cell differentiation (Bermingham et al. 1999; Kim et al. 2001; Ma et al. 2000). Based on data on replacement of the mouse *Atoh1* by the fly *atonal* gene showing that they can fully substitute each other for normal hair cell differentiation (Wang et al. 2002), it was assumed that *atonal* might be close to the ancestral bilaterian gene and thus it has served as the founder for a growing family of *atonal*-like genes. *atonal*-like bHLH genes have been found in diploplastic metazoans (Seipel et al. 2004), which have mechanosensory cells with a high degree of similarity to choanocytes of sponges and choanoflagellates (Burighel et al. 2011; Fritzsch et al. 2007; Jorgensen 1989). In these animals, the sensory cells always have their own axon (Fig. 1) that feeds sensory information into the nerve net for appropriate responses. In contrast, derived bilaterians such as vertebrates or cephalopods have sensory cells without an axon and sensory neurons connecting these sensory cells to the central nervous system (Fritzsch et al. 2010). From this observation, it follows that some bilaterians have evolved molecular means to convert a single neurosensory cell type into two distinct cell types. We have proposed that the multiplication of bHLH genes is tied into this cellular diversification (Fritzsch et al. 2000), although the exact triggering mechanism remains speculative.

It was proposed that the apparently newer bHLH genes of the *atonal* family that play a role in neuronal development, *Neurod1* and *Neurog1*, have evolved later (Simionato et al. 2007), as they were specifically associated with the neuronal precursors. However, more recent data, including multiple genome sequences of basic metazoans, show that the situation in flies is uniquely derived through the loss of *Neurod1* (Simionato et al. 2008). Most importantly, using a non-*atonal*-related bHLH gene as an out-group, a maximum likelihood tree suggests that *Neurog1* is closer to the *atonal* family root, with *Neurod1* being more derived and *atonal/Atoh1* being most derived (Simionato et al. 2008). Obviously, if more data confirm this reformed evolutionary relationship, it appears that the sequence of discovery of these genes in flies may have biased our perception of their evolution and the *atonal* family of genes should be renamed the *neurogenin* family of genes.

No matter the ancestry of these bHLH TFs, the multiplication of these closely related bHLH genes happened in basal bilaterians and resulted in an initial co-expression of these three TFs in the same cell. As indicated in numerous studies on such gene duplication, there is a

limited chance that each gene evolved into a novel regulatory cascade by activating a restricted set of downstream genes through evolutionary changes in their DNA binding sites. Essentially, TFs and their DNA binding sites co-evolve (Yang et al. 2011), much like the better studied co-evolution of hormones and their receptors (Bridgham et al. 2006). Presumably, the bHLH gene multiplication generated a set of redundant signaling molecules that provided the robust basis needed to explore novel functions without compromising the essential basic function (Espinosa-Soto et al. 2011; Wagner 2011). Ultimately, a random mutational walk through gene-space via mutagenesis (Wagner 2011) may have resulted in the specific association of each TF with a specific aspect of cellular evolution: a split of the simple precursor that generated only one neurosensory cell type into two partially overlapping precursor populations was accomplished in vertebrate ancestors. This partial split resulted, through an as yet only partially explored interactive developmental cascade (see below), in the differentiation of two distinct cell types, the sensory hair cell and the sensory neuron of the vertebrate ear (Fritzsch et al. 2006, 2010).

Many metazoans have these three bHLH genes; however, only some species have, most possibly convergently, explored the innovatibility inherent in such gene multiplication (Wagner 2011) and evolved a morphologically dissimilar set of sensory cells connected by neurons to the central nervous system (Budelmann 1992; Burighel et al. 2011). Unfortunately, no expression or experimental data on bHLH genes exist in these specific non-vertebrate animals, making it currently impossible to establish molecular similarities or differences in how these animals arrived at a comparable cellular diversification by evolving the bHLH genes into a developmental module that can generate two distinct cell types instead of one. In particular, cephalopods, with the co-existence of sensory cells with their own axon next to sensory cells that are connected to the central nervous system via sensory neurons (Budelmann 1992), need to be analyzed to understand how bHLH genes are regulated into such distinct outcomes. It is notable that flies have apparently lost one of those three ancestral genes (*Neurod1*) but have also evolved novel multiplications of bHLH genes that seem not be tied into the generation of radically different cell types but rather in the modification of existing types (Cachero et al. 2011). It is conceivable that expression of these bHLH genes in the brain or intestine could have driven their evolution and their implementation in the evolving neurosensory cell types was secondary. However, the basic evolutionary principle of gene duplication followed by cell type multiplication, further outlined below, would remain the same.

Once a sensory cell with an axon had evolved the expression of three bHLH TFs, the precursors for this single cell were multiplied to form an array of sensory cells through an enhanced proliferation of a precursor population, instead of a single cell (Fig. 2a, b). Such an array adopted the universal planar cell polarity signal to adjust the sensory cells within such an epithelium into a distinct and typically opposing, polarity as found in virtually all mechanosensory epithelia (Fig. 2b). Overlapping with this multiplication, a segregation of bHLH gene expression must have occurred and the increased populations were reorganized into two partially segregated populations in terms of bHLH TF expression. Discrete differences in overlap of diffusible factors combined with the evolutionary changes in bHLH gene activation patterns likely evolved as a consequence of the increased size and limitations in diffusible gradients as well as the multiplication of these diffusible factors; for example, the Fgf, Wnt, Bmp and HH families (Groves and Fekete 2012). As a consequence, some cells at one end of the larger array of uncommitted cells could have developed as sensory neurons without mechanotransduction abilities, whereas cells at the other end of the array could have developed as hair cells without an axon but with mechanotransduction capabilities (Fig. 2c). These different developmental trajectories evolved as a consequence of the gene multiplication, mutations in their respective promoter regions and consequently, a slight difference in response to diffusible factors. Once such a segregated developmental

pathway had evolved, the pairing of neuronal and hair cell development was fixed permanently, forming a novel pathway to ensure that specific hair cells and associated sensory neurons had to evolve to guarantee an epithelial- and polarity-specific presentation stimuli in the central nervous system (Fig. 2d). In the second part of this review, we will provide developmental data that are consistent with such an evolutionary scenario and show that many neurons derive from one part of a sensory epithelium or from areas between sensory epithelia (Fig. 3) (Fritzsch et al. 2002).

A critical step in this scenario is the multiplication of sensory cell precursors into a larger array that allowed differential expression of bHLH genes as a consequence of relaxed expression specification within that array through differential signal strength of diffusible factors (Groves and Fekete 2012). Both overlapping of multiple bHLH genes as well as multiplication of a single precursor are prerequisites for the innovation to generate a novel cell type: the sensory neuron without mechanotransduction through relaxed, altered cell fate specification.

In summary, in basal metazoans an ancestral bHLH TF that was originally involved in regulating the development of the highly conserved mechanosensory cell morphology (Burighel et al. 2011; Fritzsch et al. 2007) was multiplied (Fritzsch et al. 2010; Simionato et al. 2008). This multiplication provided the genetic robustness needed for the innovative split of the ancestral sensory cell with its own axon into two cells: a hair cell that specializes in mechanotransduction and a sensory neuron that connects the hair cell to the central nervous system (Fig. 1). Below, we will highlight the complexity of the TF interaction in mice during development and speculate how it could have been tied into the evolutionary split of a simple precursor population into two partially overlapping populations that generate the two discrete cell types, as well as the two subtypes of the hair cells.

bHLH transcription factors regulate both type- and subtype-specific development of inner ear neurosensory cells

In principle, cell fate decision making in any developing system (including the ear) follows three steps (Lander 2011; Peter and Davidson 2011):

1) Diffusible signals establish, through gradients, the position of specific cell types;

2) Regulation of various factors “prime” cells to respond appropriately to other topologically more specific TFs with cell type-specific differentiation;

3) Consolidation of cell fate through cell–cell interactions ensures a coordinated assembly of multiple cell types in a functionally relevant pattern.

This general principle applies to the OC development and uses the following known factors for each level of molecular regulation:

1) Bmps, Fgfs, Shh and Wnts are diffusible factors that define the otic placode and later sensory epithelia (Bok et al. 2007; Chang et al. 2008; Groves and Fekete 2012; Huh et al. 2012; Ohyama et al. 2011; Pauley et al. 2003; Pirvola et al. 2000; Riccomagno et al. 2002; Riccomagno et al. 2005).

2) *Eya1, Six1, Foxg1, Gata3, Jag1, Pax2, Sox2* and other genes act as cell fate primers to solidify the transient pattern generated by the diffusible factors (Ahmed et al. 2012a; Bouchard et al. 2010; Fritzsch et al. 2006, 2011; Kiernan et al. 2006; Pauley et al. 2006; Zou et al. 2008).

3) At least three proneural bHLH TFs (Atoh1, Neurod1, Neurog1) define and differentiate sensory neurons and hair cells (Bermingham et al. 1999; Kim et al.

2001; Ma et al. 1998, 2000). In addition, neurogenic TFs regulate the initial patterning of supporting cells and the overall pattern of the OC development, through both the Delta/Notch system and through feedback loops of diffusible factors, such as Fgf8 expressed in IHCs (Basch et al. 2011; Doetzlhofer et al. 2009; Fritzsch et al. 2011; Jacques et al. 2007; Pirvola et al. 2000).

Although original loss-of-function studies show a simple one gene–one cell type developmental scheme, the results of various more detailed knockout studies and expression analysis show that the three proneural bHLH TFs regulate neurosensory development through extensive intra- and intercellular interactions (Fig. 4). For example, *Neurog1* drives the expression of *Neurod1* in sensory neurons (Ma et al. 1998) and deletion of either gene leads to neuronal loss. Deletion of *Neurog1* also results in hair cell loss and premature expression of *Atoh1* (Ma et al. 2000; Matei et al. 2005). This suggests that *Neurog1* inhibits *Atoh1*, which in turn cross-regulates expression of *Neurog1* (Jahan et al. 2012; Matei et al. 2005; Raft et al. 2007). In addition, *Atoh1* expression is now known to be necessary for *Neurod1* expression in developing hair cells (Jahan et al. 2012; Pan et al. 2012). Thus, possibly through disinhibition of *Atoh1* expression, absence of *Neurog1* increases expression of *Neurod1* in hair cells (Matei et al. 2005). *Neurod1* also suppresses both *Neurog1* (Jahan et al. 2010b) and *Atoh1* expression (Jahan et al. 2010b). These data indicate an unprecedented level of complexity of the cross-regulation of these three bHLH TFs with a developmentally incomplete segregation of expression and interaction (Fig. 4). We hypothesize that this incomplete developmental segregation is possibly a consequence of the incomplete evolutionary segregation of their initially identical developmental signaling of both downstream and upstream expression regulation. In essence, while at least some hair cells will differentiate in the absence of either *Neurog1* or *Neurod1*, the presence of both *Neurog1* and *Neurod1* is necessary to balance the proportion of neurons to hair cells.

Beyond the cell decision-making process to differentiate sensory neurons, hair cells and supporting cells, the OC development also requires the specification of morphologically and physiologically distinct subtypes of hair cells (IHCs and OHCs), sensory neurons (type I and II spiral ganglion neurons) and supporting cells (seven subtypes) at specific positions for proper function. It is unclear how these different cell subtypes form in their specific positions and what unique mixture of TFs drives their differentiation. As progress in hair cell regeneration is inching toward hair cell restoration, the as yet unspecified pathways and molecular cues to make different subtypes of hair cells in specific locations will become crucial for the reconstitution of a functional OC (Yang et al. 2012). To achieve reconstruction, we will need to understand how to generate IHCs at the correct position as generation of OHCs or vestibular hair cells instead might not provide the proper mechanoelectric sound transduction needed for hearing. Below is a summary of our recent studies on the cell fate decision-making in the ear.

Formation of neurons and sensory hair cells results from interplay of bHLH TFs

Most revealing for a more detailed understanding of the intracellular cross-regulation of these bHLH factors are experiments that remove *Neurod1. Neurod1* loss results in degeneration of most of the inner ear sensory neurons (Jahan et al. 2010a; Kim et al. 2001). Since some of the other bHLH TFs in the developing ear have the capacity to rescue some neurons (Kruger et al. 2006) but apparently not to suppress *Atoh1*, the de-repressed *Atoh1* expression converts some neurons into hair cells through the maintenance of the initial expression of *Atoh1* (Figs. 5, 6). Selective overexpression of *Atoh1* in the developing sensory neurons is now needed to show how widespread this effect of transformation into hair cells can be in sensory neurons and how much the *Neurog1* mediated expression of *Neurod1* in neurons is not only needed in normal development but can counteract an induced *Atoh1* expression.

Equally interesting is our data on a targeted misexpression of *Neurog1* under the *Atoh1* promoter control. Based on the fact that absence of *Neurod1* alters hair cell subtype differentiation as it results in a higher and earlier expression of *Atoh1* (Jahan et al. 2010b), one would expect that co-expression of one allele of *Atoh1* and one expressing *Neurog1* would alter the hair cell phenotype due to additional disabling of the *Atoh1* signaling. Indeed, that is what we recently reported in a $Atoh1^{kiNeurog1}$ knockin mouse model (Figs. 7, 8) (Jahan et al. 2012). However, while *Atoh1* upregulation in sensory neurons, after eliminating the repressor *Neurod1*, leads to hair cell differentiation, no differentiation of hair cells as neurons can be induced with homozygotic misexpression of *Neurog1* under the *Atoh1* promoter control (Jahan et al. 2012).

In line with these differential effects of *Atoh1* levels of expression in conjunction with co-expression of other bHLH genes, in particular *Neurod1*, are recent data on a conceptually novel mouse mutant, a “self-terminating” *Atoh1* mouse (Pan et al. 2012). In this mouse, the *Atoh1-cre* transgene (Matei et al. 2005), which uses the previously isolated *Atoh1* enhancer (Helms et al. 2000) to drive *cre*, is only activated after the floxed *Atoh1* gene (Maricich et al. 2009) is transcribed into mRNA and translated into protein. The Atoh1-induced *cre* subsequently “self-terminates” continued transcription of *Atoh1* mRNA by recombining the two floxed *Atoh1* alleles. These mice show only a transient expression of *Atoh1* mRNA that is never as profound as the expression in control littermates. Most interesting is the fate of the hair cell precursors. Despite only a transient expression of *Atoh1*, they initiate near normal differentiation, express Myo7a but fail to express *Neurod1*. The initial differentiation is followed by hair cell death with a variable time constant. Some hair cell precursors die within days whereas others remain for weeks. In particular, the IHCs die early whereas the first row of OHCs seems to have the longest viability. This effect indicates that continued but low level of *Atoh1* expression (just enough to be detected using a LacZ reporter; Matei et al. 2005) is important for hair cell maintenance. Indeed, in *Neurod1* mutant mice, *Atoh1* expression remains elevated relative to control postnatal littermates, indicating that *Neurod1* may function not only as a transcriptional activator but also as a repressor. If true, it indicates that overexpression of *Atoh1* could ameliorate noise or chemically induced hair cell loss. A recent paper indicates that this might indeed be the case (He et al., in revision).

Inner versus outer hair cell identity may be defined by level and duration of Atoh1 expression

An optimized regenerative therapy that leads to the restoration of a functional OC will need the proper distribution and functional characteristics of IHCs and OHCs. As far as neurosensory subtype specification is concerned, many genes are differentially expressed in various subtypes of hair cells and supporting cells in the developed OC (Belyantseva et al. 2000; Montcouquiol and Kelley 2003; Zheng and Gao 1997). Consistent with these expression differences are differential defects in mutants (Deol 1981; Holley et al. 2010; Huh et al. 2012) or after chemical or noise ablations for IHCs and OHCs (Abrashkin et al. 2006). Best known is the differential distribution of *Fgf8* in IHCs (Jacques et al. 2007; Pirvola et al. 2000) and *Fgfr3* in pillar and Deiter's cells (Huh et al. 2012; Puligilla et al. 2007). Absence of *Fgfr3* leads to alteration of pillar cell development including disorganization of type II afferent growth to OHCs (Puligilla et al. 2007), absence of *Fgf20* results in loss of the outermost row of OHCs (Huh et al. 2012) and loss of *Eya1/Six1* results in loss of all OHCs (Ahmed et al. 2012a). However, how *Fgf8* is upregulated only in IHCs (Jahan et al. 2010b; Pirvola et al. 2000) and how *Fgf20* and *Eya1/Six1* manage to regulate formation of only some hair cells remain unclear. Despite the differential distribution of several markers in IHCs and OHCs (Cotanche and Kaiser 2010), no molecular steps are currently known that convert the general hair cell development, driven by *Atoh1*, toward differentiation into the two subtypes of hair cells found in all mammalian sensory epithelia.

In fact, we do not yet understand at a molecular level how vestibular and OC hair cells are distinctly specified. However, studies in mutant mice suggest that segregation of sensory epithelia facilitates epithelia-specific hair cells differentially. This suggestion derives from data that show that lack of epithelia segregation results in overlapping distribution of vestibular and OC hair cells adjacent to each other (Nichols et al. 2008).

The only insight into this basic problem are recent data that suggest alterations in the interactions of bHLH TFs in developing neurosensory cells may play a role in cell subtype decision in the ear. Eliminating the bHLH gene *Neurod1* results in transformation of OHCs into IHC-like cells that express *Fgf8* and develop the thick stereocilia characteristic of IHCs (Jahan et al. 2010b). This transformation seems to come about through eliminating the *Neurod1* mediated suppression of *Atoh1* expression in the apex (Jahan et al. 2010b), suggesting that timing of *Atoh1* expression, in combination with the level of its expression, plays a yet to be fully characterized role in the differentiation of IHCs and OHCs. Assessing this further requires the in vivo manipulation beyond the simple haploinsufficiency of *Atoh1* (no phenotype has been reported for those mice) and the complete null of *Atoh1*. While *Atoh1* null mice have no hair cells, much like after chemically induced ablation (Bermingham et al. 1999; Chen et al. 2002; Fritzsch et al. 2005), they are early lethal precluding any further study. Conditional mutant lines that eliminate *Atoh1* prior to hair cell differentiation using, for example, *Pax2-cre*, can survive but have no OC remaining after birth and almost no sensory neurons (Pan et al. 2011). Our “self-terminating” *Atoh1-cre:Atoh1* conditional deletion mice have a progressive and near complete loss of all IHCs shortly after birth but retain some OHCs in the apex until postnatal day 38. Interestingly, this mouse model retains many of the afferent and efferent innervations to the OC, mimicking a very severe early onset human presbycusis.

In these “self-terminating” mice, the IHCs are lost first (Pan et al. 2012). This is the opposite effect seen after *Neurod1* loss, which mediates disinhibition of *Atoh1* expression, which leads to transformation of OHCs into IHC-like cells (Jahan et al. 2010b). Combined, these two sets of data imply that it is not only the topology of *Atoh1* expression that is essential for the initiation of hair cell differentiation in the sensory epithelia (and not in ganglia as in *Neurod1* null mice) but also that both level and duration of expression play a major part in hair cell subtype specification in the ear. Our preliminary data obtained by combining the *Neurog1* knockin allele (Jahan et al. 2012) with the self-terminating mouse model (Pan et al. 2012) directly test the hypothesis presented here. Consistent with our presumption, our data show an uncoupling of hair cell subtypes from their topology: cells in the position of inner or outer hair cells can differentiate either as inner or outer hair cells. Further analysis of these mice can provide the groundwork to align quantitative understanding of ear development with data in other systems where effects of intensity and duration of expression on cell fate decision are well known (Niwa et al. 2000; Pelet et al. 2011; Sansom et al. 2009), including quantitative effects of other bHLH genes (Conway et al. 2010).

In summary, studies in mouse ear development show a complex intra- and intercellular cross-regulation of the three *atonal* family bHLH TFs that indicates an incomplete segregation during development. We propose that this complex interplay reflects the evolutionary history of multiplying *atonal* family members and diversifying them to differentiate into two (hair cells and sensory neuron) instead of the single ancestral neurosensory cell. Combined, these three interacting *atonal* family members (and an undisclosed set of other bHLH TFs) are needed to generate the right number of the two neurosensory cell types. Intra- and intercellular cross-regulation between these TFs also affect the level and duration of expression of each factor and thereby determine not only cell type-specific differentiation but also help define the neurosensory subtypes. The formation of neurosensory cell types are largely a matter of temporal regulation (neurons first,

followed later by hair cells in mice) possibly of clonally related cells (Fritzsch et al. 2006), while the subtype specification depends on both the time and intensity of expression of these TFs, one of the least understood aspects of gene expression regulation (Crocker et al. 2008).

Remarkably, 600 million years after the vertebrate ancestors evolved the interactive cascade of three *atonal* family members to guide the development of two cell types in the ear and the lateral line system, there is still overlapping expression and cross-regulation within cells suggestive of incomplete segregation of their promoter-mediated expression. Both spatial and temporal expression changes enhance the degree of segregation of sensory neuron precursors from hair cell precursors but do not eliminate them. Whether this remaining overlap is under positive selection or simply reflects a historic anachronism that will eventually disappear over evolutionary time remains to be explored.

What regulates *Atoh1, Neurog1* and *Neurod1* expression in the ear and how much does their downstream gene repertoire overlap?

From the above insight into the evolutionary and developmental dynamics of inner ear bHLH gene expression and their effects on various neurosensory cell types in the ear, it should be clear that the regulation of these TFs has to be tightly controlled in space, time and intensity. While the above-outlined data, combined with previous analysis of promoter regions of these genes (Ahmed et al. 2012a, b; Gowan et al. 2001; Helms et al. 2000; Quinones et al. 2010), suggest an intricate intracellular and extracellular cross-regulation, it is unclear what is regulating the highly patterned initial expression to specific cells of the developing prosensory epithelia but diffusible factors such as Fgfs and Bmps are likely candidates (Fritzsch et al. 2006). It is clear that *Neurog1* is the first bHLH gene to be expressed at embryonic day (E) 8.75 in the mouse (Ma et al. 1998) followed by *Neurod1*, which is evidently regulated by *Neurog1* (Jahan et al. 2012; Ma et al. 1998). The last of these TFs to be expressed is *Atoh1*, which makes its appearance at E10.5 as revealed by RT-qPCR (Matei et al. 2005). Apparently, expression of *Neurod1* in hair cells is even further delayed as it is dependent on *Atoh1* for its expression, while it inhibits *Atoh1* in a negative feedback loop of unknown complexity (Jahan et al. 2010b; Matei et al. 2005).

Several candidate genes can be proposed as upstream regulators of these three *atonal* family members based on data generated in insects (Garcia-Bellido and de Celis 2009) but none of them have been tested in vertebrates and thus remain speculative at the moment. Multiple TFs have been identified in ear development that are needed for hair cell and neuronal development (Ahmed et al. 2012a, b; Bouchard et al. 2010; Duncan et al. 2011) but their interaction remains to be elucidated. The various TFs needed for neurosensory cell precursors to respond to expression of a given bHLH TF with proper differentiation should not be confused with those factors regulating the expression of bHLH TFs. Clearly, the Delta/Notch system is an essential stabilizer of the differentiation pattern of the OC (Adam et al. 1998; Brooker et al. 2006; Daudet et al. 2007; Pan et al. 2010; Zine et al. 2001). However, the entire Delta/Notch signaling cascade can be blocked without affecting initial proneural gene upregulation in specific positions (Basch et al. 2011). Indeed, the first publication describing the effect of eliminating a proneural gene in the ear already showed that the Delta/Notch expression is dependent on the expression of *Neurog1* and is not inducing it (Ma et al. 1998). Without knowing exactly which TFs combine to regulate topographically restricted proneural gene expression in the ear, we will not be able to design ways to regulate their expression in vivo through selective activation of their promoter regions to restore an OC. Some data suggest that multiple TFs with a complex interaction in *Atoh1* promoter regions are needed for that expression (Ahmed et al. 2012a), without detailing the additional TFs already characterized in the *Atoh1* promoter region required to

drive *Atoh1* in a topologically restricted fashion such as *Gata3* (Duncan et al. 2011) or *Pax2* (Bouchard et al. 2010).

The known aspects of promoter regions of these three bHLH genes are composed of multiple TF binding sites, including E-boxes for self-binding and binding of other related bHLH TFs (Ahmed et al. 2012a). Atoh1 binds to an E-box consensus binding site in its enhancer and autor-egulates its expression (Helms et al. 2000). In addition, the *Atoh1* enhancer also contains a second E-box that is highly conserved. But the binding factor has not been identified and its role in regulating *Atoh1* expression is unknown. The promoters of *Neurod1* and *Neurog1* have not been fully characterized; however, it has been suggested that the activity of remote regulatory sequences may direct the spatio-temporally specific expression of these genes (Gowan et al. 2001). Further studies to identify and characterize these regulatory elements and the binding factors will also provide the molecular mechanism for the intracellular cross-regulation of these bHLH genes.

In the absence of identified genes that could directly mediate the bHLH gene activation in vivo through binding to their promoters, the only alternative for promoter regulation-driven gene activation would be the direct activation of relevant downstream genes. However, studies in the nervous system have shown that each of the three relevant bHLH TFs has several hundred downstream target genes (Dalgard et al. 2011; Klisch et al. 2011; Seo et al. 2007). Roughly half of them are present in the ear during different development stages (Sajan et al. 2007, 2011) (Fig. 9). Studies in the ear may identify even more ear-specific targets for these bHLH genes. Which ones are the most important to elicit a cascading effect remains unclear. Obviously, understanding the interaction of TFs to regulate the space, intensity and duration of *Atoh1* expression might be the more logical way to go, despite its emerging complexity (Ahmed et al. 2012a).

How can these insights translate into treatment to help against hearing loss?

There are still several hurdles to overcome for an effective translation of these molecular insights into treatment. If we take the approach that the main problem we need to understand is the topologically restricted upregulation of a few TFs that induce hair cells directly through transdifferentiation (Yoo et al. 2011), or allow transfection with *Atoh1* to effectively differentiate new hair cells, or allow hair cells generated in vitro to differentiate appropriately, we can then begin to work on genetically engineered mouse models that allow us to test certain premises. Alternatively, we can use the molecular knowledge at hand and try, for example, to transform the basilar papilla of frogs (Fritzsch and Wake 1988) into a mammalian type of organ using modern genetic techniques now possible in these animals (Abu-Daya et al. 2012), thus demonstrating that we indeed understand all the relevant genes.

When confronted with a complete loss of an OC and replacement by a flat epithelium (Izumikawa et al. 2008; Pan et al. 2011), there is no clear path to success in the foreseeable future. However, such cases of complete loss of all hair cells are mostly limited to treatment with ototoxic drugs in humans, as in experimental animals. In fact, most hearing loss, in particular age-related hearing loss, does not lead to a sudden and near complete loss of all hair cells. While several mouse lines show signs of presbycusis, the molecular basis of these effects is typically as unknown as the genetic predisposition is for presbycusis in humans. Like in these mouse lines, the common phenotype of hair cell loss in humans with presbycusis is probably the convergence of a multitude of minor genetic defects that require several genome-wide association studies to narrow them down. The unclear etiology notwithstanding, generating mouse lines that are genetically modified to show progressive hair cell loss with specific loss of one subtype of hair cells could help provide mouse models

for human presbycusis. Several mouse models (see below) could allow insights toward curing the progressive hearing loss through restoration of the lost hair cells while maintaining the remaining hair cells. Such data could provide a beginning to reveal manipulations to ensure proper hair cell subtype development in cases with a more complete loss of hair cells.

Furthermore, in contrast to the past animal models with a chemically induced complete loss of all hair cells, genetically engineered mouse models could demonstrate whether the remaining hair cells can function as a template to help organize the differentiation of hair cells into two subtypes in the right position and thus restore hearing. Such a mouse model should, therefore, have only a partial loss, preferentially only of one subtype of hair cells, with retention of others that may or may not be differentiated fully as hair cells. Likewise, these mice should also show a progressive and complete loss of all hair cells over time but should retain most of the sensory neurons to assess the function of a restored OC. The complete loss over time is essential to verify the effectiveness of reconstitution of hair cells and their retention. Existing mouse models with a knockout of genes relevant for hair cell maintenance such as *Pou4f3* (previously *Brn3c*) or *Barhl1* (Chellappa et al. 2008; Xiang et al. 2003) are too complex to be useful here, as they require not only the restoration of hair cells but in addition the full functional replacement of the knockout genes, as otherwise the regenerated hair cells will degenerate again. Essentially, this model would need to have a progressive hair cell loss driven by the loss of the same gene that is typically used to restore hair cells, *Atoh1*.

Unfortunately, *Atoh1* null mice are early lethal and have also a complete loss of all hair cells much like chemically induced ablations (Bermingham et al. 1999; Chen et al. 2002; Fritzsch et al. 2005). Conditional mutant lines that eliminate *Atoh1* prior to hair cell differentiation using, for example, *Pax2-cre*, can survive but have no OC left and almost no sensory neurons (Pan et al. 2011). Using an available *Atoh1-cre* line (Matei et al. 2005), we genetically engineered a “self-terminating” *Atoh1* expression (Pan et al. 2012). This mouse model has a progressive and near complete loss of all IHCs shortly after birth, retains only a few OHCs in the apex at postnatal day 38 but retains most of the afferent and efferent innervation (Pan et al. 2012). Most importantly, these mice lose all endogenous *Atoh1* in remaining hair cells due to the self-termination mechanism whereby the *Atoh1* protein activates the *Atoh1* enhancer-mediated *cre* expression that recombines the floxed *Atoh1* gene (Pan et al. 2012).

These new mice are an ideal genetically engineered model for attempts to induce novel hair cell differentiation, rescuing otherwise slowly dying hair cells, using established protocols for adenoviral transfection (Izumikawa et al. 2005, 2008; Staecker et al. 2011). Using adenovirus-mediated *Atoh1* transfection, one can directly test in these mice if the remaining undifferentiated Myo7a-positive “hair cells” initiate differentiation into functional hair cells that remain viable over a longer period. This mouse model can also help to understand how duration and intensity of a virally mediated *Atoh1* expression relates to hair cell subtype differentiation independent of their position. Therefore, combining the genetically engineered mouse with the adenoviral transfection approach, one could critically test the hypothesis that the level and duration of *Atoh1* determine not only overall viability but also the subtype of hair cells. Such a test would also validate whether existing hair cells can serve as a template to expand the residual OC to fill in hair cells between patches of remaining hair cells or to fill in gaps in remaining rows of existing hair cells. If such effects occur, it could lead to a “replenishment therapy” prior to the complete loss of the OC, an approach that could benefit particularly patients with slow progressing age-related hearing loss.

In summary, understanding how non-mammals can replace hair cells at a molecular level does not by itself guarantee transferability to induce similar processes in humans. In contrast, recently molecularly engineered mice are an ideal model for attempts to induce novel hair cell differentiation of slowly dying hair cells using established protocols for adenoviral transfection (Izumikawa et al. 2005, 2008) with a high level of translatability to humans. Using these mice (Pan et al. 2012), one could establish how the transient expression of a limited amount of *Atoh1* translates at a molecular level to a variable hair cell loss over time. This will also test whether existing hair cells can serve as a template to expand the residual OC to fill in gaps between patches of remaining hair cells.

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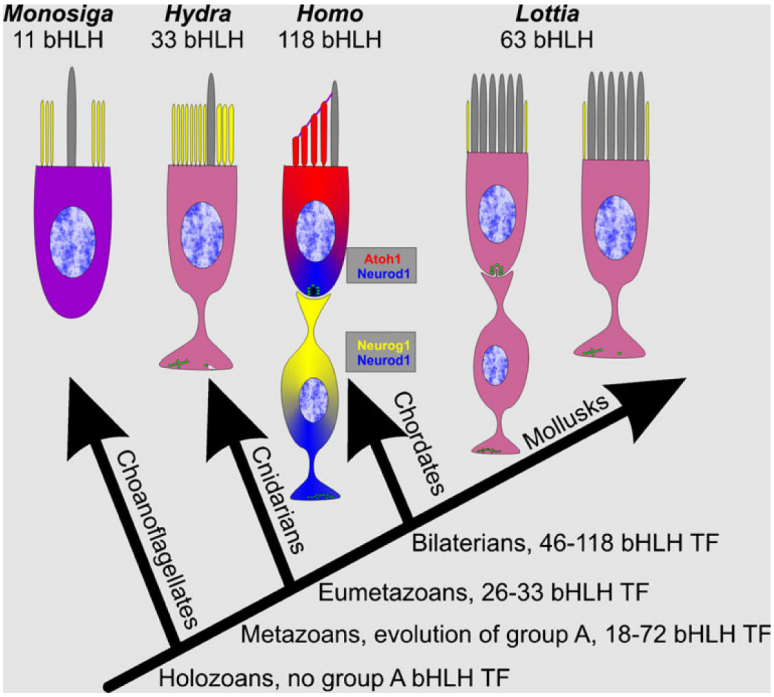

Fig. 1.

Available data relevant for the evolution of human hair cells and sensory neurons are shown. Note that Monosiga has 11 bHLH TFs, none of which are orthologous to Metazoans. Eumetazoans have sensory cells with axons and display asymmetric distribution of microvilli (*yellow*) and kinocilium (*gray*). In mammals, the three bHLH TFs are partially overlapping to drive neuronal (*Neurog1, Neurod1*) and hair cell (*Atoh1, Neurod1*) development. A superficially similar arrangement is found in some mollusks but which bHLH genes are expressed in these cells is unknown. Compiled after Budelmann (1992), Degnan et al. (2009), Fritzsch et al. (2007) and Galliot and Quiquand (2011)

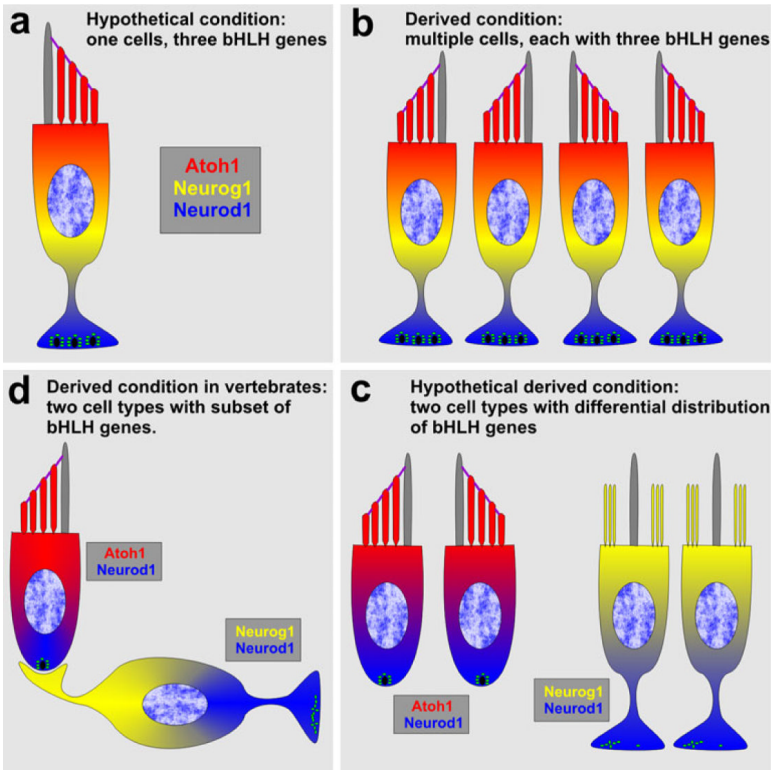

Fig. 2.

A hypothetical evolutionary transformation of a single sensory cell with an axon expressing three bHLH TFs (**a**) into two connected hair cell/sensory neuron (**d**). Hypothetical intermediate steps could be the formation of a multicellular array (**b**) that allowed differential expression of the three bHLH TFs to induce formation of two distinct cell types (**c**). Once these two cell types were formed, additional changes led to the two cell types recognized in vertebrate ears (**d**)

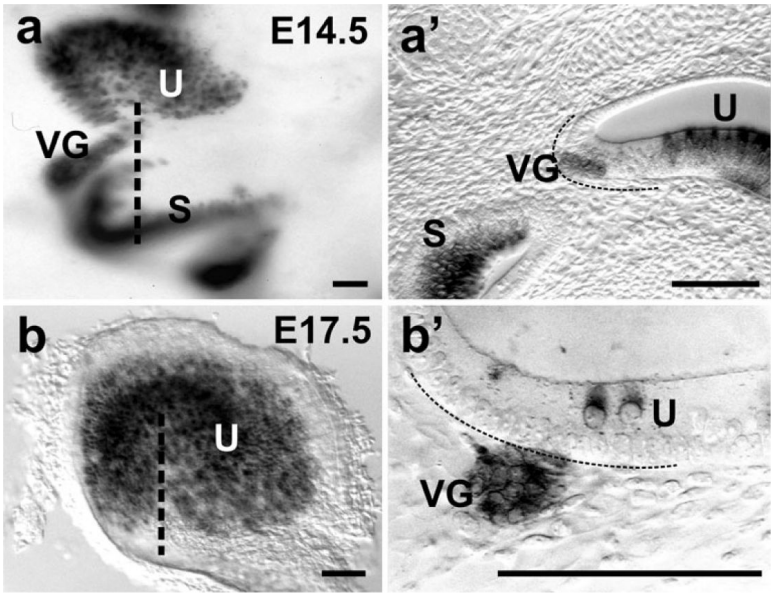

Fig. 3.

This image shows the delamination of Bdnf–LacZ positive neurons from two sensory epithelia, the utricle (U) and saccule (S). Note that the area of delamination of neurons is devoid of hair cell labeling at *E14.5* in the whole-mounted ear (**a**). Thick sections at the plane indicated in (**a**) by a *dotted line* show that vestibular ganglion neurons (*VG*) are at the neuronal edge of the utricle and separated by a region of proliferating precursors that are not yet differentiating as either neurons or hair cells (**a'**). At *E17.5*, only a few neurons remain immediately underneath the utricle and are clearly distinct from the differentiated hair cells (**b'**). *S* saccule, *U* utricle, *VG* vestibular ganglion. *Bar* 100 μm

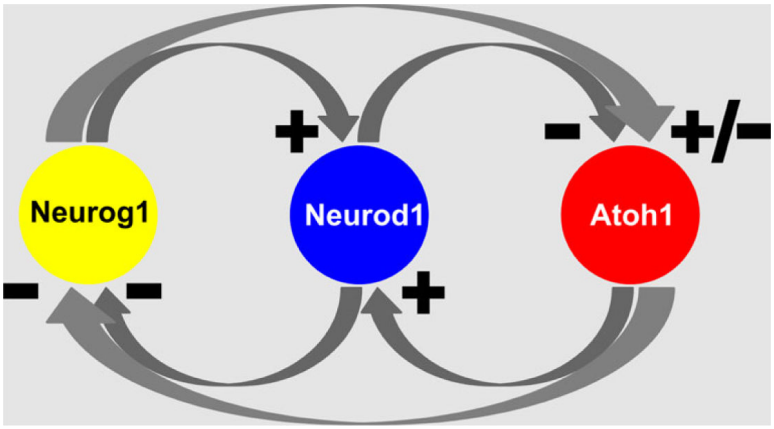

Fig. 4.

Known intra- and intercellular interactions of *Atoh1* bHLH TF family members are depicted. *Neurog1* and *Atoh1* are necessary for *Neurod1* expression in neuronal and hair cell precursors, respectively. *Neurod1*, in contrast, inhibits *Neurog1* and *Atoh1* in neurons and hair cells, respectively. Possible intercellular interactions cross-regulate *Atoh1/Neurog1* expression. *Atoh1* appears to inhibit *Neurog1*, whereas absence of *Neurog1* results in disinhibition of *Atoh1*. *Neurog1* can reduce hair cell development in some parts of the ear but also results in extra hair cells in other parts but it is unclear how this relates to cross-regulation of *Atoh1*

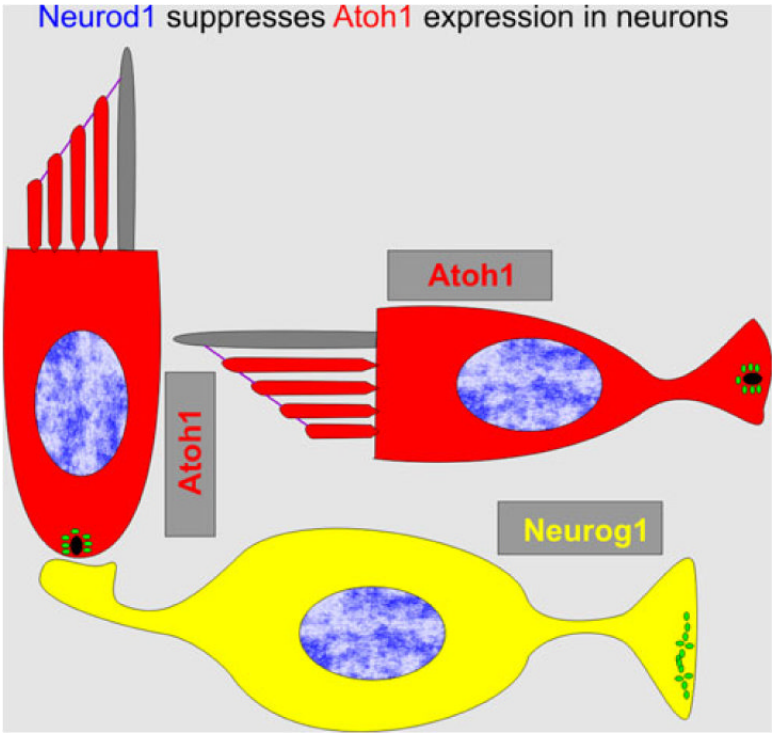

Fig. 5.

Deletion of *Neurod1* results in loss of many sensory neurons. Among the surviving neurons some develop as quasi-hair cells through expression of *Atoh1* and Myo7a, two well-accepted markers for hair cells in the ear. Occasionally, these hair cells develop short processes that may contain the synaptic contacts, as indicated. For details, see (Jahan et al. 2010b; Sebe-Pedros et al. 2011)

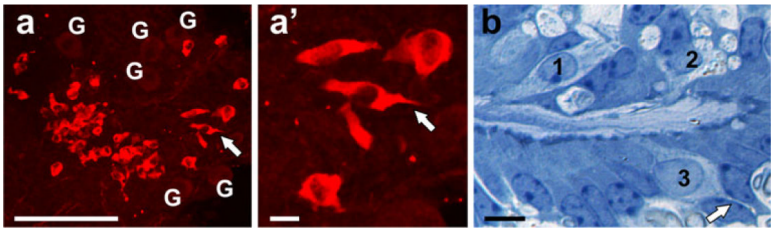

Fig. 6.

Deletion of *Neurod1* results in transformation of cells, most likely sensory neurons into Myo7a positive "hair cells" (**a**). These cells are typically irregular or spindle-shaped and some of them display a more or less extended projection on the opposite part of the stereocilia/kinocilia, indicating a distinct polarity of the apex and the base of hair cells (*arrows* in **a** and **a'**). Most of these Myo7a-positive cells are found along ventricles formed inside the ganglia, into which they extend with their apical specializations. While some of these cells display multiple vesiculated structures near their base (*1*, *2* in **b**), others display a basal extension somewhat similar to the axon emanating from a neuron (*3*, *arrow* in **b**). G ganglion neurons. *Bars* (**a**) 100 μm, (**a'**, **b**) 10 μm

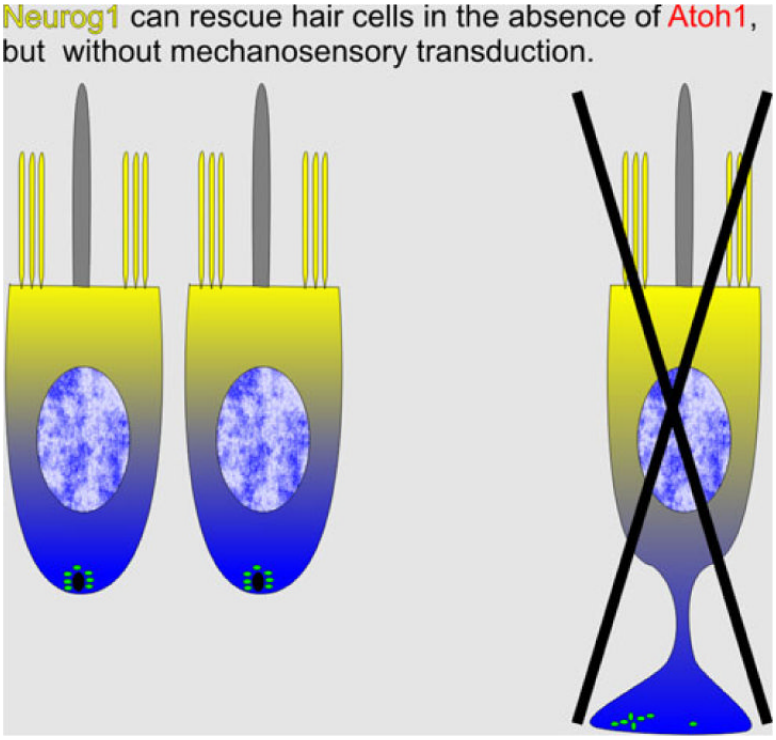

Fig. 7.

Expression of *Neurog1* under *Atoh1* promoter control results in partial differentiation of hair cell precursors. However, these cells rapidly degenerate without ever developing stereocilia. Instead, these cells develop central kinocilia surrounded by many microvilli. Despite a profound expression of *Neurog1* and *Neurod1* in these cells, none develop as neurons. This suggests that hair cell precursors are committed to differentiate as hair cells prior to *Atoh1* upregulation and misexpression of a different bHLH gene cannot change that fate. This contrasts sharply with the fate change of sensory neurons in *Neurod1* null mice shown in Fig. 5

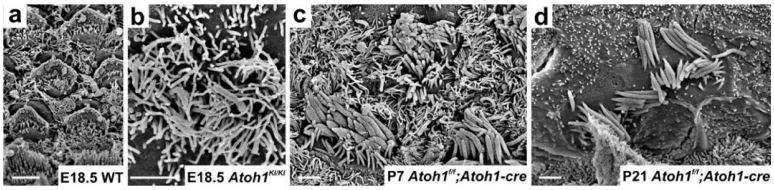

Fig. 8.

Scanning electron microscopy images show abnormal OC cells with long microvilli surrounding a central kinocilium in the E18.5 mouse mutant where *Atoh1* is replaced with *Neurog1* (**b**). In the *Atoh1* conditional knockout mouse mutant using *Atoh1-cre* to "self-terminate" after its limited transient expression, some apical hair cells develop stereocilia at an early postnatal stage (**c**). However, the stereocilia development is incomplete and transient, leading to further loss of stereocilia (**d**) and eventually loss of hair cells. This suggests that *Atoh1* is necessary for both the initiation and the maintenance of the mechanotransduction development. Although the replacement of *Atoh1* with *Neurog1* can partially rescue the viability of the OC cells, *Neurog1* can neither functionally support hair cell development nor transdifferentiate them into neurons. *Bars* (**a**) 10 μm, (**b**–**d**) 1 μm. The images are modified from Jahan et al. (2012), Pan et al. (2012)

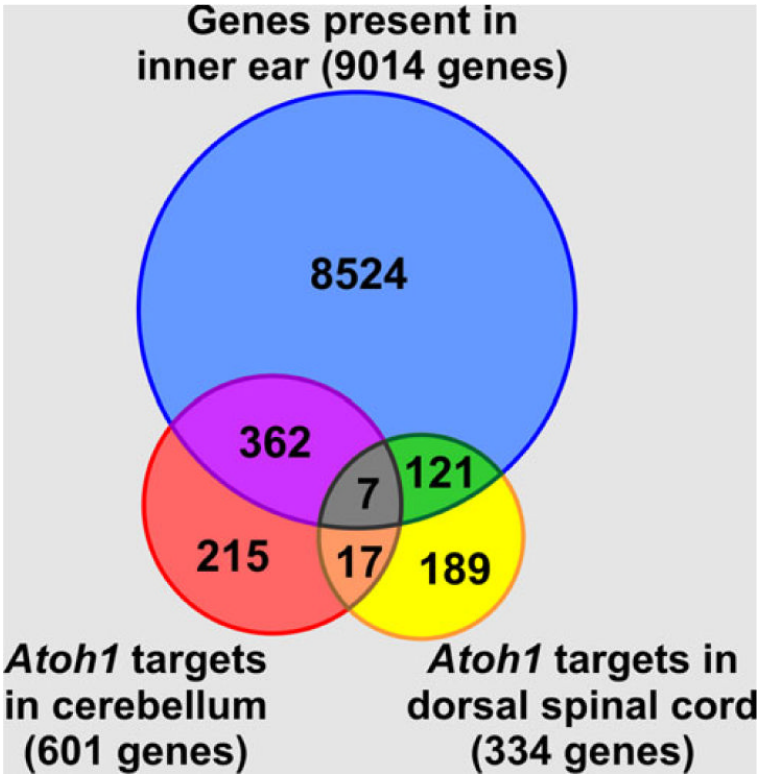

Fig. 9.

Atoh1 downstream targets identified in the cerebellum and the dorsal spinal cord were compared with the list of genes that are expressed in the inner ear; 61 % of the *Atoh1* target genes in the cerebellum and 38 % in the dorsal spinal cord are also expressed and potentially regulated by *Atoh1* in the inner ear