Neurog1, Neurod1, and Atoh1 are essential for spiral ganglia, cochlear nuclei, and cochlear hair cell development

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Abstract

We review the molecular basis of three related basic helix–loop–helix (bHLH) genes (Neurog1, Neurod1, and Atoh1) and upstream regulators Eya1/Six1, Sox2, Pax2, Gata3, Fgfr2b, Foxg1, and Lmx1a/b during the development of spiral ganglia, cochlear nuclei, and cochlear hair cells. Neuronal development requires early expression of Neurog1, followed by its downstream target Neurod1, which downregulates Atoh1 expression. In contrast, hair cells and cochlear nuclei critically depend on Atoh1 and require Neurod1 and Neurog1 expression for various aspects of development. Several experiments show a partial uncoupling of Atoh1/Neurod1 (spiral ganglia and cochlea) and Atoh1/Neurog1/Neurod1 (cochlear nuclei). In this review, we integrate the cellular and molecular mechanisms that regulate the development of auditory system and provide novel insights into the restoration of hearing loss, beyond the limited generation of lost sensory neurons and hair cells.

Keywords

bHLH genes, cochlea development, neuronal differentiation, cochlear nuclei projections

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Introduction

Without a doubt, loss of hair cells, in combination with deprivation of sensory neurons and cochlear nuclei, results in severe aging-related hearing loss. Various approaches to hearing restoration focus mostly on hair cell regeneration, often without a full appreciation of the apparent interaction of hair cells with sensory neurons and cochlear nuclei. For instance, the loss of hair cells also reduces most, but not all, spiral ganglion neurons extending centrally to smaller cochlear nuclei extending centrally to smaller cochlear nuclei.

Three basic helix-loop-helix (bHLH) genes were shown to be crucial for hair cell, sensory neuron, and cochlear nuclei development:

1. Neurog1 plays a crucial role in sensory neuron development, affects hair cells, and has a limited impact on cochlear nuclei

2. Neurod1 plays a role in neuronal differentiation, cochlear nuclei development, and hair cell development

3. Atoh1 is essential for cochlear hair cells and cochlear nuclei development and has a limited effect on sensory neurons

Sensory neurons exit the cell cycle from the base to apex between embryonic day 10 (E10) and E12 in mice, followed by cochlear hair cells from the apex to base between embryonic day 10 (E10) and E12 in mice, followed by cochlear hair cells from the apex to base between embryonic day 10 (E10) and E14. Spiral ganglion neurons project to cochlear hair cells (from base to apex; E13–E16; Figure 1) and nearly simultaneously send central processes to cochlear nuclei (from base to apex; E12–E16). Neurons and hair cells have been suggested to have a clonal relationship because of similarities in bHLH gene expression. This relationship may play a role in neuronal pathfinding for at least the periphery; however, central targeting is less understood but may involve Neurod1.

Spiral ganglion neurons depend upon Neurog1 and Neurod1. In contrast to Neurog1 null mice, which showed a complete loss of neurons, Neurod1 null mice showed residual spiral ganglion neurons extending centrally to smaller cochlear nuclei. Unlike Neurog1, which is possibly transiently expressed in cochlear nuclei, Neurod1 was found massively expressed, overlapping with Atoh1, Ptf1, and Lmx1atemp. Peripherally, it was established that cochlear hair cells critically depend on Atoh1 (Math1). Furthermore, the length of the cochlea depends on Neurog1 and Neurod1. Neurog1 is upstream of Neurod1, and both are upstream of Atoh1. Neurog1 and Neurod1 truncate Atoh1 expression, suggesting that these genes interact in many areas of neuronal development. Also, a loss or reduction of cochlear hair cells occurs following the absence of Gata3, Pax2, Eya1/Six1, Foxg1, and Lmx1a, and many of these genes and others also affect the sensory neurons innervating them.

We will provide a comprehensive review of the interplay of the three bHLH genes (Neurog1, Neurod1, and Atoh1) in the context of spiral ganglia, cochlear nuclei, and cochlear hair cell development. In addition, we will examine the role of other transcription factors (Eya1/Six1, Sox2, Pax2, Gata3, Foxg1, and Lmx1a) known to be involved in their development.

Spiral Ganglion Neurons

Crosstalk of Neurog1, Neurod1, and Atoh1 determines inner ear sensory neuron fate

Both Neurog1 and Neurod1 play important roles in sensory neuron development and differentiation. All inner ear sensory neurons were lost in Neurog1 null mice. Similarly, many sensory neurons were lost in Neurod1 null mice; however, not all neurons were lost. More recent work in Neurod1 null mice showed that of those neurons that survived, there was an intermingled vestibular and auditory sensory neuron projection to cochlear hair cells and showed a reduced and aberrant central projection to cochlear nuclei.

What is unknown is whether there is a direct role of Atoh1 in sensory neuron development or whether it is indirect. Hair cells depend on neuronal innervation for long-term maintenance. Logically, one would assume that the absence of hair cells will eventually cause degeneration of many neurons because of a lack of neurotrophic support. Atoh1 null mouse embryos, which lack hair cells, showed reduced Bdnf-lacZ staining and reduced hair cell innervation in the basal turn of the cochlea (Figure 2). The apex, which retained Bdnf-lacZ staining in undifferentiated cells of these mice, showed a denser spacing of spiral ganglion neurons, suggesting that Bdnf expression may not depend on Atoh1 in the apex. Conditional deletion of Atoh1 resulted in residual innervation correlated to residual hair cell formation, demonstrating that near-normal residual cochlear hair cells receive innervation from a surprisingly large number of neurons.

Pou4f3 (Brn33c) null mice, which develop only immature hair cells and have limited expression of neurotrophins, show little effect on innervation patterns beyond the lack of innervation to outer hair cells (OHCs) birth. The absence of inner hair cells (IHCs), through the loss of Atoh1 or in Bronx-waltzer mutants, results in spiral ganglion projections to OHCs and disorganized central projections. Interestingly, replacing an allele of Atoh1 with Neurog1 in Atoh1Neurog1 mice showed a different pattern of spiral ganglia projections to reach out the organ of Corti, consistent with a reduction in the number of neurons and hair cells.
Figure 1. The auditory system revealed in development. Organization of the cochlear hair cells, the spiral ganglia, and the innervation of the cochlear nuclei (A). Details show the differential innervation of spiral ganglion neurons to the inner hair cells (IHCs) (yellow, expresses both Ntf3 and brain-derived neurotrophic factor (BDNF)) and outer hair cells (OHCs) (red, expresses BDNF). Note that only Ntf3 (green) is expressed in cochlear nucleus neurons (B). After the apex-to-base cell cycle exit (E12.5–14.5), a base-to-apex differentiation of hair cells by Atoh1 follows (E14.5–18.5) (C). In addition, differences in hair cells and supporting cells and the size and thickness of the organ of Corti are depicted (C). DCN, dorsal cochlear nucleus; E, embryonic day; VCN, ventral cochlear nucleus. This figure was adapted with permission from Booth KT et al. under the terms of the Creative Commons 4.0 Attribution License (CC BY 4.0) (A and C) and from Rubel and Fritzsch (B).
Spiral ganglion neurons depend primarily on Neurog1 for the development. BDNF-LacZ of control mice (A) is compared with Atoh1<sup>+/−</sup>; BDNF<sup>+/−</sup> (B) and Neurog1<sup>+/−</sup>; BDNF<sup>+/−</sup> (C). There is an absence of some, but not all, hair cells in Atoh1 null mice (A, B) and loss of sensory neurons and gain and loss of different hair cells in Neurog1 null mice (C). Atoh1<sup>+/−</sup> at embryonic day 14.5 (E14.5) shows near-complete hair cell development near the apex (D). In E14.5 Lmx1a<sup>−/−</sup> mutants, there is a delayed expression of Atoh1<sup>+/−</sup> (E). By postnatal day 7 (P7), the hair cells develop, but there is a fusion of the organ of Corti (OC) with the saccule (SM) (F). Detailed comparisons show normal inner ear afferents in controls (G, G'), reduced afferents in Atoh1-cre; Atoh1<sup>+/−</sup> "self-termination" (H), an expansion of afferents to outer hair cells in the absence of inner hair cells in Bronx waltzer (bv/bv) (I) and Atoh1-cre; Atoh1<sup>+/−</sup>Neurog1 (J) mutants and altered innervation and cell type formation in Neurod1 conditional deletions (K) (arrows). AC, anterior canal crista; DR, ductus reuniens; Ggl, ganglion; HC, horizontal canal crista; P, postnatal day; PC, posterior canal crista; S, saccule; U, utricle. This figure was adapted with permission from Jahan et al.<sup>50</sup> (A–C), from Matei et al.<sup>28</sup> (D–F), and from Copyright Clearance Center: Springer Nature, Cell and Tissue Research, Nichols et al.<sup>49</sup>, Copyright © 2008, Springer-Verlag (G–K).
an uncoupling of innervation and hair cell differentiation. The inactivation of both bHLH transcription factors in double Atoh1/Neurod1 null mutants uncouples fiber growth and expansion of remaining neurons\(^1\) that could be useful for hair cell restoration\(^5,6,9,66,69\). More recent data using Rosa\(^{2\alpha-2\beta}\) or Rainbow mice showed clones of spiral ganglion neurons and hair cells in the organ of Corti, suggesting that they arose from a typical progenitor cell\(^9\). Initially, the meaning of the transient expression of apparently cochlear-derived neurons was unclear.

In contrast to the loss of spiral ganglion neurons in mice lacking Neurog1\(^6,28\), overexpression of Neurog1 in immortalized multipotent otic progenitors (a cellular system for spiral ganglion neuron differentiation) drives proliferation via increased Cdk2. It promotes neuronal differentiation through the expression of Neurod1\(^6,68\). These findings suggest that Neurog1 can promote proliferation or neuronal differentiation and possibly impact hair cells without affecting cochlear nuclei\(^6,69\). It appears that a set of data support the transformation of astrocytes into neurons in Neurod1\(^70\) and Neurog2\(^71\). The induction of neuronal proliferation and otic progenitor cell transplantation is a potential strategy to replace lost spiral ganglion neurons.

Recent work on the characterization of neuronal and hair cell progenitors revealed insights into early gene expression during neuronal development\(^9,72\). Markers for spiral ganglion neurons, Isl1\(^9,24\) and Gata3\(^9,25,76\), were detected in developing neurons, although Neurod1 was seen in only the youngest neurons\(^7\).

In summary, the known deletion of spiral ganglion neurons in Neurod1 and Neurog1 null mice\(^6,28\) suggests these as potential genes for the induction of new neurons with or without inducing hair cells\(^9,74\) and is consistent with predictions of various cell types that require independent inducers\(^9,70\). Understanding how the expansion of neuronal projections in the absence of hair cells could be helpful to restore lost innervation\(^5,6,9,75-78\), in particular, understanding how to reinnervate the flat epithelia after long-term hearing loss, will be beneficial\(^7\).

**Deletion of Sox2 and other genes affect spiral ganglion neuron development**

Initially, deletion of Sox2 was thought to eliminate all sensory neurons\(^80,81\); however, a transient development of vestibular neurons was recently shown\(^81\). A delayed loss of Sox2 in Isl1-cre; Sox2\(^9\) mice showed a transient development of spiral ganglion neurons with abnormal innervation to disorganized hair cells in the base but no hair cells or sensory neurons in the apex\(^9\). That the later-forming neurons in the apex never developed suggests that Sox2 is essential for late neuronal development. Any similarities between different Sox2 deletions (Lcc, Ysh, Isl1-cre; Foxg1-cre) remain to be investigated. Eya1/Six1 induces Sox2 expression to promote pro-neurons-sensory-lineage specification. Ablation of the ATPase-subunit Brg1 or both Eya1/Six1 results in loss of Sox2 expression and lack of neurosensory identity, leading to abnormal apoptosis within the otic ectoderm. Brg1 binds to two of three distal 3’ Sox2 enhancers occupied by Six1, and Brg1 binding to these regions depends on Eya1/Six1 activity\(^82\). Recent work provides insight into SOX2 and NEUROD1 protein expression dynamics during neuronal differentiation. Quantification of the fluorescence intensity of nuclear proteins in immortalized multipotent otic progenitors showed expression dynamics of SOX2 and NEUROD1 from a progenitor into differentiated neurons. During neuronal differentiation, SOX2 levels decreased while NEUROD1 levels increased\(^83\). Evaluation of Neurog1 was excluded because of its dual roles in both proliferation and neuronal differentiation\(^84\). The increase of Neurod1 expression is in line with what is known for Neurod1 in collaboration with Sox2\(^85,86\). Understanding the expression dynamics of crucial transcription factors helps design replacement strategies for lost sensory neurons\(^87\).

The deletion of Pax2 resulted in a near absence of spiral ganglion neurons\(^88\), comparable to the significant loss of spiral ganglion neurons in Isl1-cre; Sox2\(^9\) mice\(^89\). Many additional genes derail the development of the inner ear and its innervation\(^63,66,90\). For example, disorganized projections to the cochlea are shown with Sox10 deletion in Schwann cells\(^9\). In addition, partial loss of hair cells reorganizes the remaining afferent and efferents\(^9,85,91\). These data provide a baseline of various deficits that require further examination, including the disorganized innervation in conditional deletions of Gata3\(^9,92,93\). Other genes, such as those involved in Wnt signaling, affect afferent innervation to OHCs\(^85\), but more work is needed to fine-tune the different effects. Finally, Lmx1a loss results in a delayed upregulation of Atoh1 combined with a transformation of basal turn hair cells into a mix of cochlear and vestibular hair cells\(^9,10,11\). In summary, Sox2 is essential for sensory neuron development\(^9\), in combination with other downstream neuronal inducers (Neurog1 and Neurod1) known to interact with Atoh1\(^9,27\).

**Cochlear Nuclei**

**Neurod1 and Atoh1 are expressed in the cochlear nuclei**

Beyond a transient and limited expression of Neurog1 expression in vestibular nuclei\(^71,91,92\), the other bHLH genes, Atoh1 and Neurod1, are expressed in cochlear nuclei\(^70,93,94\). Atoh1 is expressed in developing cochlear nuclei, and the dorsal cochlear nucleus specifically requires Neurod1\(^22,23\). Atoh1 is expressed dorsally in the central nervous system and its deletion disrupted spiral cord, brainstem, and cerebellum development\(^6,94\). Rhombomere-specific deletion of Atoh1 demonstrates that the cochlear nucleus forms from cells in rhombomeres 3–5\(^5,95,97\). Atoh1 expression is negatively regulated by Neurod1 in the cerebellum\(^41,94\), the cochlear hair cells and neurons\(^8,95\), and the intestine\(^99\) but has not yet been shown for the cochlear nucleus. An additional bHLH gene, bHLHb5\(^97\), is also necessary to properly form the dorsal cochlear nucleus. Both bHLHb5 and another gene, Ptf1a, are strongly expressed in the dorsal cochlear nucleus\(^90,100\); however, details on central projections for losing either of those two genes have not yet been provided\(^84,101\). Loss of Atoh1 or Ptf1a resulted in a loss of excitatory or inhibitory
cochlear nuclei neurons, respectively, suggesting that both genes are important for regulating cell fate determination\(^8,10,39\). Recent molecular work on Atoh1 and Ptf1a lineage contributions to cochlear nuclei development show conserved and divergent origins across species\(^43,102\).

Neurod1 deletion is shown to affect the central targeting of inner ear neurons massively. Not only are auditory neuron projections aberrant, but there is also an overlap of cochlear and vestibular projections\(^46\). Furthermore, the central projections are disorganized to the inferior colliculi\(^36\), expanding previous work on defects generated with Hoxb2 mutants\(^103\). In contrast, Atoh1 null mutants, which lack cochlear nuclei, show near-normal central projections\(^104\), suggesting that neither Atoh1 nor the cochlear nuclei themselves have a notable role in afferent pathfinding centrally. The conditional deletion of Atoh1 in the ear, but retaining Atoh1 expression in cochlear nuclei, shows near-normal segregation of central projections\(^12\), expanding the critical independence of Atoh1 in neuronal pathfinding. Not surprisingly, then, Atoh1/Neurod1 double null mice had little additional disorganized projection of cochlear afferents beyond that of Neurod1 alone\(^27\) (Figure 3). Atoh1/Neurod1 forms a complex interaction in the cerebellum\(^9,28,105\), which is useful for Neurod1 to convert astrocytes and Schwann cells into neurons\(^20,106,107\). Details are needed to determine whether deviations of central projections (Figure 1) would occur in older stages after cochlear nuclei are formed\(^9\) and dependence of cochlear nuclei on neuronal input declines\(^12\). Recent data suggest plastic reinnervation of cochlear nuclei\(^108\), but it remains unclear whether this plasticity is permanent.

These data implicate several different bHLH genes (Atoh1, Neurod1, Ptf1a, and bHLHb5) in cochlear nuclei development. The interactions of these genes in cochlear nuclei development and innervation remain to be fully characterized.

**Sox2 and Lmx1a/b are expressed in cochlear nuclei**

Sox2 is essential for proneuronal regulation throughout the entire brain\(^109,110\) and is broadly expressed in cochlear nuclei, but its role has not been detailed by selective Sox2 deletion in cochlear nuclei. Lmx1a/b double null mutants lack cochlear nuclei and choroid plexus and have a hindbrain reminiscent of a spinal cord\(^11\). In these mice, central projections of spiral ganglion neurons are lost, and vestibular fibers project bilaterally to the dorsal hindbrain and interdigitate with contralateral vestibular fibers\(^11\). The presence of these bilateral projections correlated with the expression of other genes, such as Wnt3a and Tbr2. The suggested Wnt3a attraction expands on previous data showing that loss of the Wnt receptor, Fzd3\(^11\), or downstream Wnt signaling component, Prickle1\(^106\), affects central projections. Recent work suggests that another gene, Npr2, affects central projections, showing the gain and loss of afferents to different cochlear nuclei\(^12,15\).

In summary, the expression of Lmx1a/b for the proper formation of the hindbrain is essential and the deletion of Lmx1a/b causes aberrational projections. In contrast to the detailed description of Lmx1a/b loss, there is limited information on the role of Sox2 and other genes (Npr2, Prickle1, Fzd3, and Wnt3a) on central projections.

**Cochlear Hair Cells**

**Neurog1, Neurod1, and Atoh1 interaction in developing hair cells**

Without a doubt, the development of all hair cells depends upon Atoh1 expression\(^44\). Atoh1 expression initiates in the cochlea at the upper-middle turn around E13.5 and progresses bilaterally toward the base and apex. Atoh1 expression shows a delayed upregulation in the apex compared with the base\(^44,58\), combined with very late apical hair cell differentiation at E18.5\(^12,13\). Interestingly, inner pillar cells were positive for Atoh1, suggesting that Atoh1 expression does not always result in a hair cell fate\(^8,114\). In contrast to differentiation of hair cells starting near the base and progressing toward the apex, hair cells exit the cell cycle first in the apex, at E12.5, and progress toward the base\(^28,29,115\). Furthermore, cell exit progresses radially from IHCs to OHCs\(^10,116,117\), as was shown initially using green fluorescent protein (GFP) labeling\(^18\). Loss of Neurog1 results in hair cells exiting the cell cycle two days earlier than controls\(^26\). Furthermore, there is a premature Atoh1 upregulation in an atypical apex-to-base progression in hair cells following Neurog1 loss\(^19,23\). Likewise, in Neurod1 null mice, early upregulation of Atoh1 from apex to base resulted in the formation of IHC-like cells in the region of OHCs, suggesting a transformation of OHCs into IHCs because of increased Atoh1 expression\(^6,23\). The cellular processes driving remodeling of the prosensory domain during cochlear development indicate that combinations of cellular growth contribute to base-to-apex cochlear extension, allowing different interpretations of OHC progression\(^11,18,117,119,120\). Despite its prominent role in hair cell differentiation, Atoh1 (Figure 4) does not seem to have a role in cochlear length determination\(^7\). In contrast, Neurog1 deletion resulted in a 50% reduction in cochlear length, a reduction in the size of vestibular epithelia\(^28\), and ectopic hair cells in the utricle\(^11\). Likewise, loss of Neurod1 (Figure 4) shortened the cochlea by about 50%\(^16,23\). Atoh1/Neurod1 double knockout added minimally to the cochlear length reduction in Neurod1 loss alone\(^22\). Although this suggests a possible interaction of bHLH genes, the reduction in length may be influenced simply by the loss of Shh normally generated by spiral ganglion neurons\(^22\), which would be absent or reduced in number in Neurog1 or Neurod1 null mice. The reduction of the organ of Corti is affected by several deletions of Shh\(^12\), Gata3\(^9\), Foxg1\(^8,124\), and Lmx1a\(^7,49\) in addition to Neurog1 and Neurod1.

Conditional deletion of Atoh1 using Pax2-cre showed that most hair cells were lost during late embryonic development; however, some undifferentiated cells express Myo7a in postnatal stages and are targeted by neurons. A “self-terminating” system (Atoh1-cre; Atoh1\(^\text{fl}^\text{t}\)), in which a transient expression of Atoh1 results in some initial hair cell development, demonstrated progressive loss of IHCs and most OHCs shortly after birth\(^11\). However, some Myo7a-positive OHCs remained in adults in these mice. This suggests that most hair cells depend upon continued Atoh1 expression for at least some time. Various
other conditional deletions of Atoh1 established that continued Atoh1 expression is essential for hair cell survival and maturation\cite{10,12}. Interestingly, generating a transgenic mouse in which Neurog1 replaces Atoh1 (Atoh1\textsuperscript{Neurog1\textsuperscript{1/1}}) showed that, although Neurog1 cannot fully rescue the Atoh1 null hair cell loss phenotype, it does form additional patches of undifferentiated “hair cells” rather than a flat epithelium\cite{63}. In addition, heterozygote mice expressing one copy of each gene...
Figure 4. Expressed of Atoh1 is needed for cochlear hair cells for development. Loss of Atoh1 has a limited effect of cochlea extension (A, B) compared with the shortened cochlea in Neurog1 (C) and Neurod1 (D) null mice. Detailed images compare control hair cells (E) within Bronx waltzer (bv/bv) (F) and “self-terminating” Atoh1+ (G) mice. They demonstrate near-complete loss of inner hair cells in Atoh1+Neurog1 mice (H, H'), demonstrating incomplete development of different sets of hair cells. Expression of Atoh1 in situ hybridization (ISH) depends on the normal expression pattern in control end organs (I). Ectopic “hair cells” after Neurod1 deletion are shown with ISH for Atoh1, Fgf8, and Nhhl1 (I', J, K). Hair cells within vestibular epithelia (L) as well as ectopic hair cells (L–L”, arrow in L”) are positive for Myo7a. Myo7a labeling also shows ectopic hair cells innervated by tubulin-labeled vestibular neurons (VN) (M). AC, anterior canal crista; eHC, ectopic hair cells; HC, horizontal canal crista; P, passage; PC, posterior canal crista; S, saccule; U, utricle. Bar indicates 100 µm (A–L', M) and 10 µm (L”). This figure was modified after Fritzsch et al.58 (A, B) and was adapted with permission from Matei et al.28 (C), from Jahan et al.54 under the terms of the Creative Commons Attribution License (D, I–M), and from Booth KT et al.64 under the terms of the Creative Commons 4.0 Attribution License (CC BY 4.0) (E–H’).
(Atoh1 and Neurog1) showed some disorganization of hair cell distribution (Figure 2 and Figure 4) not observed in Atoh1 heterozygotes, suggesting cross-interaction between Atoh1 and Neurog1. Using an ingenious system to overexpress Atoh1, in which the Atoh1 coding sequence is under the control of a tetracycline response element (TRE), generated viable ectopic “hair cells” in early postnatal mice in line with an upper induction of proliferation.

Loss of Neurod1 resulted in the formation of Atoh1-positive “hair cell”-like cells within intraganglionic vesicles (Figure 4) in the vestibular ganglion, suggesting a potential conversion of vestibular sensory neurons into hair cells. The ectopic hair cells are forming in addition to the sacculus and utricle and are positive for several genes—such as Atoh1, Fgf8, and Nhlh1—that generally are expressed outside the hair cells (Figure 4). This finding indicates the normal suppression of Atoh1 by Neurod1 in these neurons and implies that Neurod1 might suppress hair cell fate in sensory neurons. Similar Neurod1–Atoh1 interactions were reported in the cerebellum and the intestine and were used to transform astrocyes to neurons. In the absence of both Atoh1 and Neurod1 in double null mutants, these “ectopic hair cells” are not formed, suggesting that Neurod1 and Atoh1 interact upregulate neurons into ectopic hair cells after the loss of Neurod1.

In summary, using progenitor cells for spiral ganglia and hair cell replacement seems to be a possible way forward for hearing restoration, in addition to various other approaches. Unfortunately, generation of new hair cells in later stages beyond the earliest stages has not yet been achieved. Understanding how to generate new hair cells at later stages is needed for older animals and humans with aging-related hearing loss. Fully understanding the various mutations and putting them into the context of different cell fates require identifying the steps necessary to initiate specific distributions of sensory hair cells. What remains is understanding the various interactions of Atoh1, Neurod1, and Atoh1 for the complete formation of all hair cells.

**Sox2 interacts with other genes for hair cell expression**

Sox2 is also essential for hair cell formation, likely through the activation of Atoh1 expression. Interestingly, two independent approaches using delayed deletion of Sox2 showed different results. In one, a delayed loss of Sox2 using Sox2-Cre-ER demonstrated effects in the apex only. In the other study, conditional deletion of Sox2 using Iset1-Cre resulted in the loss of hair cells in the apex and a delayed loss in the base, showing unusual basal turn hair cells/supporting cells and inner pillar cells, suggesting a role for the timing of Sox2 expression. As expected, the timing of Sox2 expression was later demonstrated to be essential for sensory development. Furthermore, a complete deletion of Sox2 in the ear using Foxg1-Cre showed the overall cochlear reduction and no hair cell development. These combined studies provide an essential role of Sox2, although the interaction of Sox2 with Atoh1 is not fully understood.

Other genes are also crucial for inner ear and hair cell development. For example, Eya1/Six1 is essential for early ear development and is needed to form the cochlea and induces Sox2 expression, as described earlier. Another gene, Pax2, is necessary for organ-of-Corti formation and cooperates with Sox2 to activate Atoh1 expression. Conditional deletion of Gata3 using Pax2-Cre showed deletion of many hair cells and a complete loss of all hair cells with an earlier deletion of Gata3 using Foxg1-Cre. In these latter mice, levels of Atoh1 expression were significantly reduced, and genes downstream of Atoh1 were not detected following this early deletion of Gata3. Mice mutant for another gene, Lmx1a, showed a delayed expression of Atoh1 followed by transforming some organ-of-Corti hair cells into differentiated hair cells. Foxg1 null mice show a reduced cochlear length and a disorganized apex of multiple rows of hair cells with disoriented polarities. A somewhat similar phenotype is reported for n-Myc null mutants accompanied with apical cell fate changes.

The partial deletion of some, but not other, hair cells is an exciting perspective that needs to be explored. Inactivation of Fgfr1 in the inner ear by Foxg1-Cre-mediated deletion leads to an 85% reduction in the number of auditory hair cells. Likewise, Sox2 omission shows a partial loss of hair cells in the Yellow submarine (Ysh) mutation. Using Pax2-Cre to conditionally delete Dicer resulted in incomplete hair cell loss compared with the total hair cell loss with Foxg1-Cre conditional deletion, comparable to the equivalent conditional deletions of Gata3. Finally, Bronx-waltzer mice, which are mutant for the gene Srrm4 (Figure 4), lose IHCs and vestibular hair cells but retain OHCs. OHCS, meanwhile, express Srrm3 independent of the Srrm4 gene downstream of REST.

These data show that cochlear hair cells are affected by single gene deletions and complex interactions of several genes, including compound analysis of partial deletions, primarily unexplored in detail. While Atoh1 alone is the dominant gene, interactions with other genes need to be worked out.

**Summary and conclusion**

Inner ear sensory neurons, cochlear nuclei, and cochlear hair cells all require bHLH genes for their proper development. Atoh1 is essential for cochlear hair cell and cochlear nuclei development. Neurog1 and Neurod1 are vital for sensory neuron development and differentiation. All three genes play crucial roles in a feedback network to regulate specific cell fate appropriately and in coordination with other genes. Some of these additional genes interact with the bHLH genes in these contexts, such as Lmx1a/b, requiring more detailed investigation.
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