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Atoh1 and other related key regulators in the development of auditory sensory epithelium in the mammalian inner ear: function and interplay

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ABSTRACT

Damage or loss of auditory hair cells leads to irreversible sensorineural hearing loss in human, thus regeneration of these cells to reconstruct auditory sensory epithelium holds the promise for the treatment of deafness. Regulatory factors involved in the development of auditory sensory epithelium play crucial roles in hair cell regeneration and hearing restoration. Here, we first focus on the transcription factor Atoh1 which is critical for hair cell development and regeneration, and comprehensively summarize the current understanding of the protein structure, target binding motif, developmental expression pattern, functional role, and upstream and downstream regulatory mechanism of *Atoh1* in the context of controlling the cell fate commitment to hair cells or transdifferentiation from supporting cells. We also discuss cellular context dependency of *Atoh1* in hair cell induction which should be taken into consideration when using *Atoh1* gene therapy for hair cell regeneration. Next, we review the roles of *Gfi1*, *Pou4f3*, and *Barhl1* in hair cell maturation and maintenance, and suggest that manipulation of these genes and their downstream targets will be helpful for the generation of functional hair cells with long-term viability. Finally, we provide an overview of the interplay between Notch, Wnt, Shh, and FGF signaling pathways during auditory sensory epithelium development. By analyzing crosstalk between these pathways, we suggest that combination of Wnt signaling activation with *Hey1* and *Hey2* inhibition will be crucial for hair cell regeneration and hearing restoration. Furthermore, this review highlights the importance of deeper understanding of the cellular context for hair cell development and the interconnection between these key regulators in developing new strategies to treat sensorineural hearing loss.

1. Introduction

The auditory sensory epithelium (the organ of Corti) in the mammalian inner ear is a sophisticated structure which contains a cellular mosaic pattern of sensory hair cells and non-sensory supporting cells. The formation of this highly ordered auditory sensory mosaic is directed by multiple developmental events. After the generation of otocyst from otic placode, individual region in the otocyst become specified to develop as the prosensory cells (Barald and Kelley, 2004; Kelley, 2006). As development continues, these prosensory cells exit cell cycle (Chen and Segil, 1999) and form a postmitotic region in a gradient from apex to base along the length of cochlear duct (Ruben, 1967; Lowenheim et al., 1999; Matei et al., 2005; Lee et al., 2006). Subsequently, hair cells and supporting cells arise from common postmitotic prosensory cells (Fekete et al., 1998; Driver et al., 2013). Specifically, auditory hair cells differentiate in a temporal basal-to-apical gradient (Ruben, 1967; Sher, 1971; Lim and Anniko, 1985; Chen et al., 2002), and simultaneously, inner and outer hair cells appear sequentially from medial to lateral across the width of cochlear duct (Sher, 1971; Chen et al., 2002). With the differentiation of hair cells, supporting cell differentiation also proceeds (Driver et al., 2013; Wan et al., 2013), eventually giving rise to the orderly matrix of hair cells and surrounding supporting cells.

Auditory hair cells are sensitive to aging, noise exposure, infection, and ototoxic drugs. Due to inability of the adult mammalian auditory hair cells to regenerate spontaneously, damage or loss of these cells

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leads to permanent sensorineural hearing loss. In stark contrast, in non-mammalian vertebrates such as birds, auditory function can be fully restored as a result of hair cell replacement through proliferation and/or direct transdifferentiation of surviving supporting cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Stone and Cotanche, 2007). Importantly, neonatal mammals still retain the ability of hair cell regeneration although this capacity is lost at one week of age (Romand et al., 1996; White et al., 2006; Savary et al., 2007; Cox et al., 2014). Therefore, supporting cells are considered to be an ideal target for hair cell regeneration and reconstruction of auditory sensory epithelium. Fundamental mechanistic understanding of the development of mammalian auditory sensory epithelium is thus of importance for the development of new therapeutic strategies to treat hearing disorder. To date, a variety of key regulators involved in auditory sensory epithelium development have been discovered, among which the basic helix-loop-helix (bHLH) transcription factor Atoh1 is the most well-known. *Atoh1* is the first known transcription factor expressed in hair cells. Previous loss-of-function and gain-of-function studies have shown that *Atoh1* is both necessary and sufficient for hair cell development (Bermingham et al., 1999; Woods et al., 2004; Pan et al., 2012; Cai et al., 2013; Chonko et al., 2013; Zheng and Gao, 2000; Kelly et al., 2012; Liu et al., 2012a, 2014). Besides, these key regulators also include genes (*e.g., Gfi1* , *Pou4f3*, and *Barhl1*) and signaling pathways (*e.g.,* Notch, Wnt, Shh, and FGF). These regulatory factors and molecular pathways act cooperatively to regulate the development of auditory sensory epithelium, and their roles are also being elucidated in detail.

Here, we review recent advancements in understanding the roles of key regulators in the development of mammalian auditory sensory epithelium with a focus on *Atoh1*, hair cell maturation- and maintenance-related genes (*Gfi1*, *Pou4f3*, and *Barhl1*) and several important signaling pathways (Notch, Wnt, Shh, and FGF) that interact with each other, and also propose the future perspectives of manipulating them for hair cell regeneration and hearing restoration. This will definitely facilitate our effort in targeting these key regulators to treat human sensorineural hearing loss.

2. *Atoh1* is a crucial gene in auditory sensory epithelium development

2.1. Characterization of Atoh1

bHLH transcription factor *Atoh1*, the mammalian homolog of the Drosophila proneural gene *atonal*, is required for auditory hair cell development. Mouse *Atoh1* (also known as *Math1*) is an intronless gene with a 1.053 Kb coding sequence (CDS) that encodes a protein with 37.9 kDa in size. The bHLH domain of Atoh1 is 56 residues in length located in the middle of the protein which shows 70% homology with atonal, demonstrating that this motif is highly conserved. Additionally, Atoh1 contains relatively high proportion of proline residues, indicating their possible involvement in protein interaction, while the high proportion of serine residues at the C-terminus of Atoh1 implies that function of Atoh1 may be regulated by phosphorylation of these serine residues. In addition to the conserved bHLH domain of Atoh1, the C-terminus rich in serines is also well conserved among vertebrates. Furthermore, the N-terminus of Atoh1 shares high identity only among mammals (Akazawa et al., 1995; Mulvaney and Dabdoub, 2012).

Atoh1 belongs to class II bHLH transcription factors, it binds to a second class I bHLH transcription factor such as E47 to form a heterodimer which then directly binds to E-box motif (CANNTG) such as CAGCTG and CAGGTG to activate transcription of downstream target genes (Helms et al., 2000; Saba et al., 2005; Scheffer et al., 2007; Masuda et al., 2011). A genome-wide study using chromatin immunoprecipitation-sequencing (ChIP-seq) further defined Atoh1 DNA-binding motif termed AtEAM during mouse cerebellar development as RMCAKMTGKY (R is G/A, M is A/C, K is G/T, and Y is C/T) (Klisch et al., 2011), which differs from binding motifs of other related bHLH factors, demonstrating that E-box flanking sequences also determine DNA binding specificity of Atoh1 in addition to E-box motif itself. However, it is not known whether Atoh1 shares common DNA-binding motif in both developing cerebellum and auditory hair cells, therefore, the Atoh1 DNA-binding signature during hair cell development remains to be investigated using a genome-wide approach.

2.2. Developmental expression pattern of Atoh1 in the cochlea

During cochlear development, *Atoh1* was previously thought to be initially expressed in a wide range of prosensory cells in cochlear duct (Bermingham et al., 1999; Woods et al., 2004) through examination of *Atoh1* promoter activity, and thus *Atoh1* was first considered to be a proneural gene like *atonal*. However, this point of view is not supported by other studies directly detecting *Atoh1* mRNA or protein (Lanford et al., 2000; Chen et al., 2002). Consistent with these studies, recent work using $Atoh1^{A1GFP/A1GFP}$ knock-in mouse model also confirmed that *Atoh1* is initially expressed just in groups of postmitotic sensory progenitors that lined up in columns at the mid-basal part of cochlear duct around embryonic day (E) 13.5-E14.5 and is not expressed broadly in the prosensory epithelium (Cai et al., 2013). Thus, *Atoh1* is not likely to be a proneural gene. Interestingly, studies have suggested that the prosensory cells that initially express *Atoh1* in the cochlea will eventually give rise to some of the supporting cells in addition to all the hair cells, and through Notch signaling, *Atoh1*-expressing nascent hair cells will induce the silencing of *Atoh1* in surrounding prosensory cells to allow supporting cell differentiation (Driver et al., 2013; Abdolazimi et al., 2016).

After the onset of *Atoh1* expression near the base of mouse cochlear duct, developmental expression of *Atoh1* proceeds in a wave to the apex during E13.5-E17.5, and *Atoh1* expression domain gradually changes from columns of cells spanning the thickness of the prosensory epithelium to just auditory hair cells (Bermingham et al., 1999; Chen et al., 2002; Woods et al., 2004; Cai et al., 2013). Simultaneously, *Atoh1* expression also progresses from medial to lateral region across the cochlear duct, and eventually exists in one row of inner hair cells and three rows of outer hair cells. During cochlear development, *Atoh1* expression level increases steadily from E13.5 to E17.5 followed by a rapid reduction during the first week after birth, and the silencing of *Atoh1* spreads longitudinally from basal to apical part of cochlear duct (Cai et al., 2013; Driver et al., 2013; Stojanova et al., 2016). By postnatal day (P) 6, *Atoh1* expression level is extremely low (Stojanova et al., 2016) and will be shut down with auditory hair cell maturation.

2.3. Multiple roles of Atoh1 in cochlear development: not just hair cell differentiation

Atoh1 is the first known transcription factor expressed in hair cells, and is required for hair cell formation (Bermingham et al., 1999). The absence of *Atoh1* leads to a complete loss of both cochlear hair cells and vestibular hair cells (Bermingham et al., 1999). Since *Atoh1* has been initially considered as a pro-hair cell gene that is essential for the generation of inner ear hair cells (Bermingham et al., 1999), much attention has been paid to the potential of *Atoh1* in hair cell induction and regeneration. Previous studies have shown that *Atoh1* misexpression can induce the generation of hair cells from non-sensory cells (*e.g.,* Kölliker's organ) in neonatal cochlear epithelium (Zheng and Gao, 2000; Kelly et al., 2012; Liu et al., 2012a, 2014). However, this competency of hair cell induction significantly reduces with age and becomes largely lost at 2 weeks after birth in mice (Kelly et al., 2012; Liu et al., 2012a), indicating that the competency to respond to *Atoh1* will get lost as the cochlea matures. However, some studies have also found that hair cell regeneration by *Atoh1* overexpression occurs in the adult cochlea (Kawamoto et al., 2003; Izumikawa et al., 2005; Atkinson

et al., 2014). Importantly, these new hair cells in the adult cochlea induced by *Atoh1* overexpression may not result in functional improvement (Atkinson et al., 2014), and the efficiency of this method also depends on the severity of degeneration of auditory sensory epithelium (Izumikawa et al., 2008).

Atoh1 was previously thought to be simply a hair cell terminal differentiation factor. Recently, one study has established an *Atoh1* conditional knock-out mouse system to comprehensively investigate the role of *Atoh1* in different stages during auditory hair cell development. They found two critical periods for *Atoh1* function during hair cell development. Firstly, *Atoh1* deletion during E15.5-E17.5 resulted in rapid hair cell death, but removal of *Atoh1* after this period did not influence nascent hair cell number (Cai et al., 2013; Chonko et al., 2013), indicating that continuous *Atoh1* expression in this temporal window is critical for hair cell survival. Secondly, although *Atoh1* deletion shortly after this temporal window did not cause immediate hair cell death, it disrupted normal hair bundle structure required for auditory function and eventually caused delayed hair cell death (Cai et al., 2013), suggesting the existence of a second developmental window of *Atoh1* responsible for hair cell function and long-term survival. Altogether, it can be concluded that *Atoh1* is essential for the differentiation, survival, maturation, auditory function establishment, and long-term viability of hair cells. Identifying *Atoh1* downstream targets in hair cells will interpret these roles of *Atoh1* in hair cell development on the molecular level.

In addition to direct involvement in hair cell development and function, *Atoh1* also indirectly controls auditory sensory mosaic development. Within the critical time window for hair cell survival, loss of *Atoh1* results in severe loss of supporting cells (Woods et al., 2004; Cai et al., 2013) and defect in innervation of the cochlea (Cai et al., 2013), causing disruption of the entire auditory sensory epithelium. Moreover, *Atoh1* overexpression in the cochlea can promote the formation of ectopic sensory mosaics comprised of ectopic hair cells and associated supporting cells, which were also innervated by auditory neuronal fibers (Kawamoto et al., 2003; Woods et al., 2004; Izumikawa et al., 2005; Kelly et al., 2012). Furthermore, *Atoh1* plays a role in cell proliferation regulation in the mammalian cochlea. In response to *Atoh1* overexpression, cell proliferation can be observed in the normally postmitotic cochlear epithelium (Kelly et al., 2012). Previous genome-wide study has shown that *Atoh1* directly regulates genes involved in cell cycle control and proliferation during mouse cerebellar development (Klisch et al., 2011). Therefore, *Atoh1* is also likely to control these genes to promote cell proliferation in cochlear epithelium.

It is now believed that *Atoh1*-dependent supporting cell development and patterning of sensory patch rely on Notch signaling-mediated lateral inhibition. At the onset of hair cell differentiation, the nascent hair cells express Notch ligands Dll1 and Jag2 to induce Notch signaling in neighboring prosensory cells, and finally the auditory sensory mosaic forms. Targeted deletion of *Dll1* and/or *Jag2* results in an increase in hair cell number through a switch in cell fate (Lanford et al., 1999; Kiernan et al., 2005; Brooker et al., 2006). Similarly, the absence of Notch receptor *Notch1* (Kiernan et al., 2005; Li et al., 2015), inhibition of γ-secretase (Takebayashi et al., 2007; Doetzlhofer et al., 2009), and genetic deletion of Notch effectors *Hes1* and *Hes5* (Zheng et al., 2000; Zine et al., 2001) can also lead to upregulation of *Atoh1* and supernumerary hair cell formation at the expense of supporting cell number. Recently, the molecular mechanism by which Notch signaling represses *Atoh1* expression in prosensory cells destined to be supporting cells has been elucidated (Abdolazimi et al., 2016). After the Notch ligands Dll1 and Jag2 produced by nascent hair cells activate Notch signaling in surrounding prosensory cells, the Notch effectors (Hes5 and Hey2) inhibit *Atoh1* expression by directly binding to the *Atoh1* promoter region, thus preventing these adjacent cells from acquiring a hair cell fate and forcing them to become supporting cells instead (Abdolazimi et al., 2016).

Taken together, *Atoh1* plays multiple roles in the generation and patterning of auditory sensory epithelium, not only in hair cell development and function, but also in auditory sensory mosaic development.

2.4. Upstream and downstream regulatory mechanism of Atoh1 during hair cell development

Atoh1 expression is accurately regulated to ensure proper developmental program. In differentiating hair cells of cochlear prosensory region, transcription factors Eya1, Six1 and Sox2 act cooperatively to initiate *Atoh1* expression through direct binding of Six1 and Sox2 to *Atoh1* 3' enhancer (Ahmed et al., 2012). Notably, Sox2 has been previously reported to exert prosensory function by directly promoting *Atoh1* expression (Neves et al., 2012; Kempfle et al., 2016). However, Sox2 also plays an inhibitory role in *Atoh1* activation through an incoherent feed forward loop mechanism: simultaneous with *Atoh1* activation by Sox2, additional factors that negatively regulate *Atoh1* expression such as *Id1–3*, *Hes5*, *Hey1*, *Neurog1* and *NeuroD* are also induced by Sox2 (Neves et al., 2012). Therefore, *Sox2* expression must be downregulated in order to maintain *Atoh1* expression level in developing auditory hair cells, and recently, the downregulation of *Sox2* is found to be mediated by Six1 (Zhang et al., 2017). During initiation of *Atoh1* expression in the cochlea, Sonic Hedgehog (Shh) signaling produced by spiral ganglion acts as an *Atoh1* inhibitor which is required for establishing the temporal basal-to-apical expression pattern of *Atoh1* (Bok et al., 2013). Loss of *Shh* causes an abnormal wave of *Atoh1* expression and hair cell differentiation that progresses in an apical-to-basal gradient along the cochlear duct (Bok et al., 2013). Recently, regulation of *Atoh1* has been shown to be involved in selection of sensory progenitors for hair cell and supporting cell fate. *Atoh1* expression is initially induced in groups of postmitotic sensory progenitors, and then Notch signaling pathway effector Hes/Hey induced by nascent hair cells binds to *Atoh1* 5' promoter rather than its 3' enhancer to repress *Atoh1* transcription in selected progenitors that are to become non-sensory supporting cells, while the remaining *Atoh1*-expressing cells proceed to differentiate into auditory hair cells (Abdolazimi et al., 2016). Thus, the common sensory progenitors give rise to different cell types in auditory sensory epithelium. After nascent hair cell formation, *Atoh1* expression becomes upregulated through a well-known autoregulation mechanism by which Atoh1 can recognize and bind to its 3' enhancer (Helms et al., 2000). Apart from transcriptional regulation, *Atoh1* expression is also regulated by epigenetic modification during hair cell development. Analysis of histone modification of the *Atoh1* locus revealed that dynamic changes of H3K4me3/H3K27me3, H3K9ac and H3K9me3 are strongly associated with *Atoh1* activation and subsequent inactivation after birth during hair cell differentiation and maturation (Stojanova et al., 2016). Importantly, perinatal supporting cells maintain bivalent H3K4me3/H3K27me3 epigenetic status at the *Atoh1* locus which is similar to that of sensory progenitors, indicating latent potential of these supporting cells to activate *Atoh1* expression to become sensory hair cells (Stojanova et al., 2016). In addition, post-translational regulation of Atoh1 protein is also an important way to regulate Atoh1 function. Atoh1 degradation is found to be mediated by Huwe1 dependent ubiquitin proteasome pathway (Cheng et al., 2016). Deletion of *Huwe1* in embryonic or early postnatal supporting cells results in *Atoh1*-mediated extra hair cell formation, and hair cell-specific *Huwe1* deletion causes hair cell death (Cheng et al., 2016), indicating that appropriate degradation of Atoh1 level by Huwe1 is necessary for normal hair cell development and survival.

Identifying Atoh1 downstream targets can help us to better understand molecular mechanism underlying *Atoh1*-mediated hair cell development. The comprehensive Atoh1 targetome has been revealed in mouse developing cerebellum by a combination of genome-wide Atoh1 ChIP-seq, Histone-seq and RNA-seq methods. The identified

direct Atoh1 target genes were shown to be associated with a broad range of functions including cell proliferation, differentiation, survival, migration, cell adhesion and metabolism, and many were involved in various cell signaling pathways (Klisch et al., 2011). This study not only expands our knowledge about roles of Atoh1 in neurogenesis but also provides a framework for identification of direct Atoh1 targets in other Atoh1-expressing cell types especially when these cells are scarce such as inner ear hair cells. Indeed, by combining the cerebellar Atoh1 ChIP-seq data with gene expression profile of developing dorsal neural tube, several genes were identified as dorsal spinal cord interneurons-specific direct Atoh1 targets (Lai et al., 2011). Importantly, one recent study also applied this strategy to the identification of direct Atoh1 target genes during auditory hair cell development. In this study, a total of 233 potential direct Atoh1 target genes in auditory hair cells were identified, most of which have not been previously reported (Cai et al., 2015). This study lays a foundation for understanding the diverse set of molecular functions of Atoh1 during hair cell development. However, this strategy of cross-referencing cerebellar Atoh1 ChIP-seq data with hair cell transcriptome only allows identification of Atoh1 targets common to both cell types. The unique direct Atoh1 target genes in hair cells relative to cerebellum cannot be identified using the current strategy. Given that auditory hair cells are scarce in the cochlea, ChIP-seq experiment based on small numbers of cells will be required to generate a systematic list of hair cell-specific direct Atoh1 target genes. Furthermore, using *in vitro* hair cell differentiation systems to efficiently obtain pluripotent stem cell-derived hair cell-like cells may circumvent the paucity of hair cells. A recent study induced hair cell-like cells through *Atoh1* overexpression during *in vitro* mouse embryonic stem cell (mESC) differentiation, and then RNA-seq analysis of the resultant hair cell-like cells showed that *Atoh1* overexpression significantly influenced pathways such as Notch signaling, neuron migration, glutamate receptor, action potential regulation, and neurotransmitter regulation (Lee et al., 2017). Besides this method, many other *in vitro* hair cell induction methods have been established (*e.g.*, Oshima et al., 2010; Ouji et al., 2013; Costa et al., 2015; Duran Alonso et al., 2018). Therefore, these well established *in vitro* hair cell differentiation systems offer a good platform for hair cell-related molecular studies such as identification of direct Atoh1 target genes during hair cell development.

2.5. *Atoh1*-mediated auditory hair cell genesis is cellular context dependent

Non-mammalian vertebrates have the ability to spontaneously regenerate their damaged or lost auditory hair cells which probably involves *Atoh1* derepression (Cafaro et al., 2007). However, the ability of auditory hair cell spontaneous regeneration is very limited in neonatal mammals and more importantly, the regenerative ability decreases and becomes lost at just one week after birth in mice (*e.g.*, Cox et al., 2014), thus leading to irreversible sensorineural hearing loss.

As discussed above, *Atoh1* is a crucial gene controlling auditory hair cell development and it has gained tremendous attention in gene therapy-based hair cell regeneration and hearing restoration. Indeed, it has been reported that *Atoh1* gene therapy can successfully regenerate new hair cells or its hair bundles, and to some extent improve hearing in adult deaf mammals (Izumikawa et al., 2005; Yang et al., 2012). However, other studies have also shown ineffectiveness of *Atoh1* in hair cell regeneration and hearing improvement (Izumikawa et al., 2008; Atkinson et al., 2014).

In fact, the efficiency of hair cell induction by *Atoh1* is relatively low, since most cochlear non-sensory cells cannot be converted to hair cells after *Atoh1* ectopic expression (Kuo et al., 2015) and this efficiency further decreases with age (Kelly et al., 2012). In addition, the newly generated hair cells are often immature without proper differentiation as they lack typical mature hair cell markers and mechanical response (Liu et al., 2012a, 2014). It is known that besides expression in hair cells, *Atoh1* is also expressed in other tissues including cerebellum, dorsal spinal cord, intestinal, and skin, and is also essential for their development (Mulvaney and Dabdoub, 2012). Thus, cellular context is necessarily an important aspect of *Atoh1*-mediated developmental program (Jahan et al., 2015; Costa et al., 2017). A defined cellular context for hair cell fate commitment, differentiation and maturation could allow *Atoh1* to specifically direct functional hair cell formation rather than generation of related cell types. However, the majority of cochlear non-sensory cells may lack the proper cellular context for hair cell development. Consequently, creating a proper cellular context that recapitulates *in vivo* auditory sensory epithelium development may be important to improve *Atoh1* gene therapy-based hair cell induction and regeneration (Jahan et al., 2015). The transcriptional profile of hair cell progenitors provides one key insight into how the gene transcriptional network varies with cellular context. Recent studies have applied RNA-seq or DNA microarray technique to examine gene expression profile of inner ear hair cells at different developmental time points (Cai et al., 2015; Scheffer et al., 2015). These studies reveal dynamic gene expression changes that accompany hair cell differentiation and provide a basis for understanding cellular context for hair cell fate commitment and differentiation. Building upon these high-throughput sequencing datasets, identification of hair cell development-related factors acting upstream of Atoh1, Atoh1 co-factors, and Atoh1 downstream targets will be a crucial step for further establishing a proper cellular context on which *Atoh1* gene therapy-based hair cell regeneration depends (Jahan et al., 2015). For example, Eya1, Six1 and Sox2 have been identified to be upstream regulators of *Atoh1*, and coexpression of these three factors can efficiently activate hair cell developmental program (Ahmed et al., 2012). Besides, delivery of Atoh1 in combination with its co-factors TCF3, GATA3, ETV4, NMYC or ETS2 to cochlear epithelium dramatically increased the efficiency of hair cell induction as compared with Atoh1 treatment alone (Masuda et al., 2012; Ikeda et al., 2015). This is because Atoh1 and its co-factors can directly activate expression of the downstream target gene *Pou4f3* (Masuda et al., 2012; Ikeda et al., 2015), an essential gene for late hair cell differentiation and survival, which then may further define a proper cellular context for hair cell induction. In a second study, a combination of Atoh1, Pou4f3 and Gfi1 expression was found to efficiently generate hair cell-like cells from mESCs *in vitro* and in the developing avian inner ear *in vivo*, while Atoh1 alone failed to induce a hair cell fate (Costa et al., 2015), suggesting that Atoh1 downstream target genes *Pou4f3* and *Gfi1* can program a precise cellular context for *Atoh1* to drive hair cell differentiation. Importantly, DNA microarray further revealed the molecular context induced by the combined expression of *Atoh1*, *Pou4f3* and *Gfi1* which will definitely enhance our understanding of cellular and molecular context for *Atoh1*-mediated hair cell development. Surprisingly, one recent study found that *Atoh1* overexpression combined with $p27^{kip1}$ deletion or combined activation of *Atoh1* and *GATA3* or *Pou4f3* is even sufficient to convert supporting cells to hair cells in adult mice (Walters et al., 2017), confirming again the requirement of proper cellular context for hair cell fate commitment and differentiation which currently remains poorly understood. Therefore, the current available transcriptome of hair cells or auditory sensory epithelium during development (Cai et al., 2015; Scheffer et al., 2015; Perl et al., 2018) will provide highly valuable information about cellular context on which *Atoh1* function in hair cell development depends, and from which more cellular context-related genes and pathways will be identified and serve as therapeutic targets for *Atoh1* gene therapy-based hair cell regeneration.

Another key insight into cellular context-dependent features of the transcriptional network is the epigenetic modification within hair cell progenitors (supporting cells). It is likely that the epigenetic status of hair cell differentiation-related genes such as factors acting upstream of Atoh1 and Atoh1 downstream targets leads to their repression in

supporting cells during aging (Mulvaney and Dabdoub, 2012), thus causing inability of these supporting cells to respond to *Atoh1*. However, little is known about epigenetic regulation during auditory sensory epithelium development (Stojanova et al., 2016). Accordingly, it is necessary to examine the histone modification and DNA methylation of supporting cells during different developmental stages using genome-wide approach, and then cross-reference these data with datasets such as transcriptome of developing hair cells and direct Atoh1 target genes (Cai et al., 2015; Scheffer et al., 2015) to eventually reveal epigenetic context of supporting cells in genome-wide scale.

Altogether, *Atoh1* is thus conditionally necessary and sufficient for hair cell development and function. As *Atoh1* activity is context dependent, integration of *Atoh1* into a genetic program of hair cell development will efficiently instruct Atoh1 to drive hair cell fate commitment, differentiation and maturation. Consequently, understanding the molecular and cellular context for hair cell development is of fundamental importance for *Atoh1* gene therapy-based hair cell regeneration.

3. Essential genes for auditory hair cell maturation and maintenance

Auditory hair cell maturation and maintenance is an important aspect of hair cell development. Without proper maturation and maintenance after hair cell initial differentiation, the nascent hair cells will eventually degenerate. To date, *Gfi1*, *Pou4f3*, and *Barhl1* are genes required for auditory hair cell maturation and maintenance, the loss of which are associated with severe to profound hearing loss.

The zinc-finger transcription factor *Gfi1* is first expressed around the onset of hair cell genesis in the developing cochlea, and later its expression becomes restricted in auditory hair cells (Wallis et al., 2003). The absence of *Gfi1* results in profound deafness which can be directly attributed to abnormal hair cell development and a complete hair cell loss during just before and soon after birth as a result of programmed cell death (Wallis et al., 2003). Thus, *Gfi1* is essential for proper hair cell differentiation, maturation and survival. The POU-domain transcription factor *Pou4f3*, which shows a similar developmental expression pattern to that of *Gfi1* during cochlear development, is also required for hair cell maturation and survival (Xiang et al., 1998). In *Pou4f3* null mutants, although hair cells are initially produced in the developing cochlea, they are not able to mature and develop stereociliary bundles. Moreover, these immature hair cells become completely lost during the late embryonic and early postnatal development, thus leading to complete hearing loss (Erkman et al., 1996; Xiang et al., 1997, 1998). Recently, *Pou4f3* ectopic expression has been found to convert supporting cells towards the hair cell lineage in adult mouse cochleae, and this can be further promoted by combined expression of *Pou4f3* and *Atoh1* (Walters et al., 2017), indicating the key role of *Pou4f3* in hair cell development and regeneration. It is now supposed that the activation of downstream targets by *Pou4f3* largely contributes to the efficiency of *Atoh1*-mediated hair cell development and survival (Walters et al., 2017). As *Gfi1* and *Nr2f2* expression have been reported to be regulated by *Pou4f3* (Hertzano et al., 2004; Tornari et al., 2014), they may be considered as additional therapeutic targets critical for hair cell regeneration. In addition, *Pou4f3* has also been identified as a direct target gene of Atoh1 during hair cell development (Masuda et al., 2011, 2012; Cai et al., 2015; Ikeda et al., 2015). Therefore, the *Atoh1-Pou4f3*-target genes of *Pou4f3* (*e.g., Gfi1*) is an important molecular pathway controlling not only hair cell fate commitment and differentiation but also hair cell maturation and survival. Indeed, a combination of Gfi1, Pou4f3 and Atoh1 is sufficient to reprogram cells towards the hair cell lineage (Costa et al., 2015; Duran Alonso et al., 2018). Besides, another essential gene for auditory hair cell viability is the BarH class homeobox gene *Barhl1*. In the mouse cochlea, *Barhl1* is specifically expressed in developing hair cells starting at E14.5 when nascent hair cells have been initially generated (Li et al., 2002). Targeted disruption of *Barhl1* leads to age-related deafness resulting from progressive auditory hair cell degeneration during a relatively long period from P6 to at least P300 (Li et al., 2002). As all auditory hair cells appear to be normal both in morphology and hair cell marker expression at early postnatal period and there is a long time course of hair cell degeneration in *Barhl1* null mice, *Barhl1* is likely to be exclusively responsible for hair cell long-term maintenance and maybe its terminal differentiation. Recently, based on the highly efficient *in vitro* hair cell differentiation system and CRISPR/Cas9 approach, potential downstream target genes of Barhl1 in hair cell-like cells were identified using RNA-sequencing and bioinformatics (Zhong et al., 2018), which allows us to better understand the molecular function of *Barhl1* in hair cell development. Interestingly, like *Pou4f3*, *Barhl1* is also a potential direct target of Atoh1 in hair cells, as several Atoh1-binding regions have been identified in the *Barhl1* locus and its expression closely follows that of *Atoh1* in the developing cochlea (Klisch et al., 2011; Pan et al., 2012; Chonko et al., 2013). Therefore, the *Atoh1-Barhl1*-target genes of *Barhl1* represents another molecular pathway controlling hair cell differentiation and long-term survival.

Altogether, after initial auditory hair cell genesis, *Gfi1* and *Pou4f3* are both required for late stage of hair cell differentiation, maturation and short-term survival while *Barhl1* plays an essential role in hair cell long-term maintenance. Given that *Gfi1*, *Pou4f3*, and *Barhl1* lie downstream of Atoh1, manipulation of the molecular pathways *Atoh1-Pou4f3*-target genes of *Pou4f3* (*e.g., Gfi1*) and the *Atoh1-Barhl1*-target genes of *Barhl1* will probably be necessary to generate fully functional auditory hair cells with long-term viability.

4. The interplay between signaling pathways during auditory sensory epithelium development

In recent years, our knowledge about the roles of cell signaling pathways in regulating auditory sensory epithelium development has grown rapidly. These signaling pathways exert different effects at different cochlear developmental stages (reviewed in Atkinson et al., 2015). Despite the progress in understanding the functions of signaling pathways during cochlear development, manipulation of individual pathways to promote hair cell regeneration in the postnatal or mature cochlea yields unsatisfactory results (Liu et al., 2012b; Mizutari et al., 2013; Shi et al., 2013; Tona et al., 2014): when targeting individual Notch or Wnt signaling in the cochlea, only modest hair cell regenerative response or no any effect can be observed. Given that these signaling pathways act cooperatively to regulate cochlear development, it is suggested that targeting several pathways is likely to be a more promising strategy for hair cell regeneration (Atkinson et al., 2015). Therefore, it is crucial to elucidate the complex interplay between signaling pathways during auditory sensory epithelium development.

Notch and Wnt signaling are both well characterized pathways that are involved in prosensory cell proliferation and cell fate commitment during cochlear development. Modulating either of these pathways is a promising approach for hair cell regeneration. With the deepening of research, one type of interaction between Notch and Wnt signaling in hair cell development and regeneration has been recently discovered (Li et al., 2015; Romero-Carvajal et al., 2015). During Notch inhibition-mediated mitotic hair cell generation, Wnt signaling is activated in Lgr5-positive supporting cells and promotes their entry to the cell cycle and differentiation into hair cells, indicating that Notch signaling exerts an inhibitory effect on Wnt signaling activity in hair cell progenitors (Li et al., 2015). Thus, targeting both pathways by interfering with the interplay between Notch and Wnt signaling provides a potential method for mitotic genesis of hair cells. Following this strategy, subsequent studies confirmed that concurrent Notch inhibition and Wnt activation in supporting cells significantly promoted mitotic generation of vestibular hair cells which was more efficient than modulating either signaling alone (Wu et al., 2016), and when

combined with *Atoh1* overexpression, it led to extensive proliferation of supporting cells followed by mitotic hair cell genesis in the neonatal mouse cochlea (Ni et al., 2016a). Importantly, transcriptome analysis further identified multiple genes required for cell proliferation and hair cell development that are either regulated by individual signaling or by the combination of Notch and Wnt signaling (Ni et al., 2016a), which may provide additional therapeutic targets crucial for mitotic hair cell regeneration and allow us to better understand the molecular mechanism behind proliferative hair cell generation. Remarkably, instead of concurrent signaling manipulation, one recent study devised a two-step approach of Wnt activation to stimulate progenitor cell proliferation followed by Notch inhibition to drive hair cell differentiation which simulate the process of hair cell development, and found that mitotic generation of hair cells occurred not only in normal cochleae but also in injured cochleae (Ni et al., 2016b), indicating that sequential manipulation of Wnt and Notch signaling according to hair cell developmental program could hold the promise for auditory hair cell replacement therapy.

It has been previously known that Shh signaling from spiral ganglion is responsible for temporal pattern of auditory hair cell development, with the sensory progenitors exiting from the cell cycle in a wave from apical to basal part of cochlear duct while they subsequently differentiate into hair cells in an opposite basal-to-apical gradient (Bok et al., 2013). Recently, it has been found that Shh signaling interacts with FGF signaling and Notch signaling target genes to control the graded pattern of auditory hair cell differentiation. In cochlear prosensory cells, Shh signaling maintains Notch effectors *Hey1* and *Hey2* expression level, thus negatively regulating the initiation of *Atoh1* expression to prevent premature hair cell differentiation,moreover, this positive regulation of *Hey1* and *Hey2* by Shh signaling is likely to be mediated by FGF signaling (Tateya et al., 2013; Benito-Gonzalez and Doetzlhofer, 2014). Therefore, this characterized molecular pathway Shh signaling-FGF signaling-Notch effectors *Hey1* and *Hey2-Atoh1* is possibly a potential target for hair cell induction. By interrupting the interplay between these signaling pathways, it may be feasible to combine inhibition of Shh signaling and/or its downstream pathways with *Atoh1* overexpression to stimulate auditory hair cell genesis. However, the effectiveness of this strategy has not been examined and further work is needed to investigate the interaction of these signaling pathways in hair cell regeneration after damage.

Maintenance of cochlear supporting cell fate also requires cooperative regulation of signaling pathways. After nascent inner hair cells appear, these hair cells activate FGF signaling as well as Notch signaling in surrounding pillar cells. Both signaling pathways then activate the expression of the Notch effector *Hey2*, thus inhibiting *Atoh1* expression and maintaining pillar cell fate (Doetzlhofer et al., 2009). Importantly, as a result of the redundancy of FGF and Notch signaling function in *Hey2* regulation, inhibition of either signaling alone can still keep pillar cell identity unchanged without converting them to hair cells, only combined inhibition of both FGF and Notch signaling leads to activation of *Atoh1* expression and thus drives pillar cells to transdifferentiate into hair cells (Doetzlhofer et al., 2009). Therefore, the fate of cochlear pillar cells depends on coordination of FGF and Notch signaling activity. The FGF and Notch signaling-*Hey2-Atoh1* thus provides a potential molecular pathway for the generation of auditory hair cells from supporting cells which can be achieved by inhibiting the synergistic activation of *Hey2* expression by both FGF and Notch signaling pathways.

As discussed above, it can be found that crosstalk between Notch, Wnt, Shh, and FGF signaling pathways regulates the development of auditory sensory epithelium. Insight into the interplay between Notch, FGF, and Shh signaling pathways reveals that the regulation of Notch effectors *Hey1* and *Hey2*, which control cell fates in the auditory sensory epithelium through *Atoh1,* are the converging points for these pathways. Therefore, the regulation of *Atoh1* by Hey1 and Hey2 will be the key step in controlling cell fates in the auditory sensory epithelium and is a potential useful target for hair cell induction. By eliminating the inhibitory effect of Hey1 and Hey2 on *Atoh1* expression, hair cell regeneration will likely to be induced more efficiently. Besides, since a balanced ratio of hair cells and non-sensory supporting cells is required for the function of auditory sensory epithelium (Atkinson et al., 2015), mitotic hair cell regeneration without at the expense of supporting cell number is thus of importance. Given the role of Wnt signaling in promoting supporting cell proliferation, therefore, combining Wnt signaling activation with *Hey1* and *Hey2* inhibition will be crucial for hair cell regeneration and hearing restoration.

Although the roles of individual signaling pathways in the development of auditory sensory epithelium have been well studied, our understanding of the interplay between them is rather limited and requires further investigations to characterize the complex cell signaling regulatory network. Moreover, it is undoubted that activation of *Atoh1* expression in supporting cells through manipulating regulatory factors and signaling pathways is a promising strategy to induce hair cell regeneration. However, this strategy only has limited value in clinical application because the adult cochlea loses the competency to respond to manipulation of signaling molecules and factors (Walters and Zuo, 2013; Atkinson et al., 2015). Previous study has shown that various changes occur as the cochlea matures including the structure, signaling molecules, otic stem cell, gene expression, microRNAs and epigenetics (Walters and Zuo, 2013). It is likely that these changes contribute to the loss of regenerative potential in the adult cochlea. Therefore, the cochlear sensitivity to manipulation of signaling molecules and factors is likely to be cellular context dependent. Consequently, more work is needed to fully characterize the changes in gene expression profile and epigenetic state between the neonatal and adult cochleae using genome-wide approaches such as RNA-seq and histone ChIP-seq. This can provide valuable information about age-related decline of regenerative potential during cochlear development and additional targets that are critical for the cochlear responsiveness to manipulation of signaling molecules and factors to activate *Atoh1* expression.

5. Conclusion

Sensorineural deafness caused by loss of auditory hair cells in human is permanent, which has become a serious problem threatening human health. Understanding the molecular mechanism underlying the development of mammalian auditory sensory epithelium is of fundamental importance to treat sensorineural deafness through induction of auditory hair cell regeneration. In recent years, a large number of studies have identified and defined the roles of key regulators such as *Atoh1*, *Gfi1*, *Pou4f3*, *Barhl1* and Notch, Wnt, Shh, and FGF signaling pathways in controlling auditory sensory epithelium development and shed light on the potential of manipulating these key factors in future clinical therapies of hearing loss. However, current approaches of targeting these regulators alone to regenerate auditory hair cells are still unsatisfactory. Here, we review recent studies investigating functions and the interplay of key regulators involved in auditory sensory epithelium development, and suggest the potential strategy that may be more effective in auditory hair cell regeneration. In summary, the functions of *Atoh1* and other related key regulators in the development of auditory sensory epithelium are cellular context dependent, and these regulators also interact with each other to constitute a complex molecular regulatory network during auditory sensory epithelium development (Fig. 1 A, B). Therefore, manipulation of these key regulators in combination and integration of them into a proper cellular context for hair cell development will be a promising strategy to regenerate auditory hair cells. Consequently, deeper understanding of the cellular context for hair cell development and the interconnection between these key regulators will be of great importance for developing new strategies to treat sensorineural hearing loss.

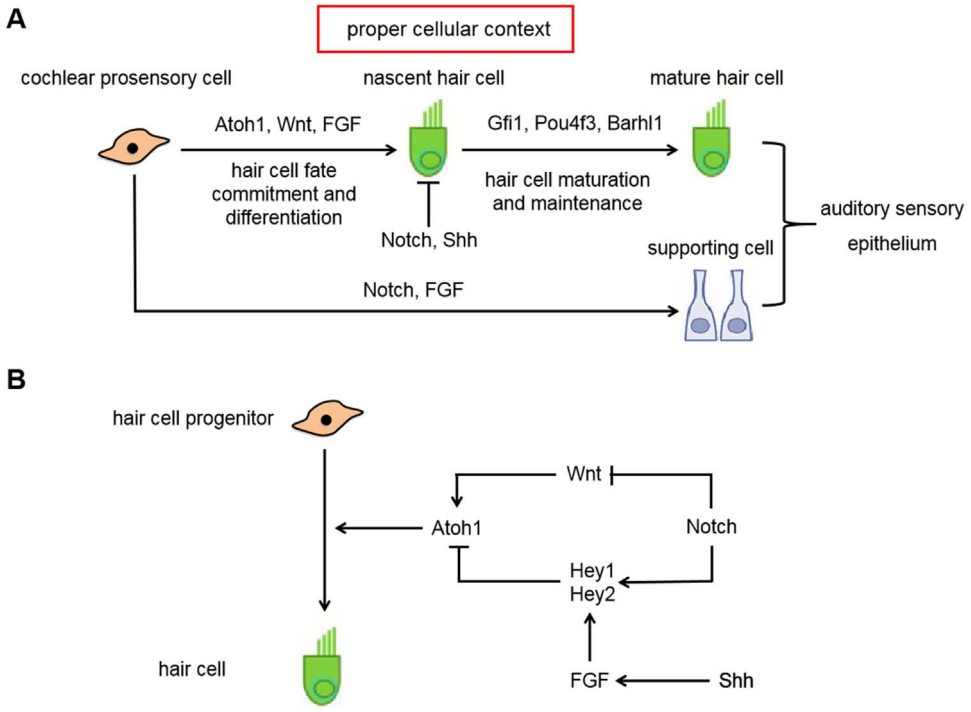

Fig. 1. Schematic depictions of the development of auditory sensory epithelium and its associated key regulators. (A) The roles of key regulators in the development of auditory sensory epithelium. Atoh1, Wnt and FGF signaling are required for auditory hair cell fate commitment and differentiation from prosensory cells, which is negatively regulated by Notch and Shh signaling. After nascent hair cell formation, Atoh1 downstream targets Gfi1, Pou4f3, and Barhl1 play essential roles in the maturation and maintenance of hair cells, eventually generating fully functional auditory hair cells with long-term viability. Simultaneous with the differentiation of hair cells, Notch signaling is activated and further inhibits *Atoh1* expression in surrounding prosensory cells, thus forcing them to acquiring a supporting cell fate, while FGF signaling induces supporting cell subtypes differentiation. The functions of these regulators in controlling auditory sensory epithelium development are cellular context dependent. **(B)** Interplay between Notch, Wnt, Shh, and FGF signaling pathways involved in the development of auditory sensory epithelium. Targeting these pathways by interrupting the interconnection between them is likely to be a promising strategy for auditory hair cell regeneration and hearing restoration.

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Conflicts of interest

The authors declare no conflict of interest.

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