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Insights into inner ear-specific gene regulation: epigenetics and non-coding RNAs in inner ear development and regeneration

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Abstract

The vertebrate inner ear houses highly specialized sensory organs, tuned to detect and encode sound, head motion and gravity. Gene expression programs under the control of transcription factors orchestrate the formation and specialization of the non-sensory inner ear labyrinth and its sensory constituents. More recently, epigenetic factors and non-coding RNAs emerged as an additional layer of gene regulation, both in inner ear development and disease. In this review, we provide an overview on how epigenetic modifications and non-coding RNAs, in particular microRNAs (miRNAs), influence gene expression and summarize recent discoveries that highlight their critical role in the proper formation of the inner ear labyrinth and its sensory organs. In contrast to non-mammalian vertebrates, adult mammals lack the ability to regenerate inner ear mechano-sensory hair cells. Finally, we discuss recent insights into how epigenetic factors and miRNAs may facilitate, or in the case of mammals, restrict sensory hair cell regeneration.

Keywords

microRNA; DNA methylation; histone modification; inner ear development; hair cell regeneration; deafness

1. Introduction

The development of the inner ear is a dynamic process, eventually leading to a complex tissue enabling hearing and balance, represented by the auditory and vestibular systems, respectively. In the auditory system, the sensory epithelium embedded within the cochlea contains the organ of Corti responsible for hearing. The vestibular system contains five organs, including the three semicircular canals with cristae sensory epithelia that detect

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angular acceleration by fluid motion and the saccule and the utricle, which contain the macula sensory epithelium that detects linear acceleration due to gravity.

The inner ear sensory apparatus and its innervating sensory neurons originate from the otic placode, a thickening of surface ectoderm adjacent to the hindbrain. Otic induction and its regionalization occur in a series of steps that requires the reiterative interplay of inductive signals and the action of transcription factors [1]. Once formed, the otic vesicle grows in size and undergoes extensive morphological changes, forming the auditory and vestibular apparatus and the endolymphatic duct. The first cell lineage specified within the developing otic placode is the neuronal lineage. As early as the otic cup stage, neuroblasts begin to delaminate from a broad anteriorly located neurogenic domain and differentiate into sensory neurons of the auditory-vestibular ganglion. The auditory and vestibular hair cells, sensory receptors of the inner ear, and their surrounding supporting cells are derived from their respective vestibular and auditory pro-sensory patches within the developing otocyst [2].

Non-mammalian vertebrates such as birds, fish and amphibians have the capacity to regenerate hair cells in response to injury. In fish and amphibians, hair cells are added to the inner ear and the lateral line sensory organs throughout the lifetime of the animal as part of ongoing growth [3]. The newly generated hair cells originate from neighboring non-sensory supporting cells. Such ongoing hair cell production is dramatically up-regulated in response to injury [4, 5]. In the avian inner ear, a steady turnover of hair cells is observed within vestibular sensory organs but not within the auditory sensory organ [6, 7]. However, upon damage, auditory hair cells are readily regenerated from supporting cells through both mitotic [8, 9] and non-mitotic mechanisms [10]. The mammalian inner ear has a very limited ability to replace lost hair cells. Once matured, the auditory sensory epithelium lacks the ability to regenerate lost hair cells [11] and the vestibular sensory epithelia mount only a limited regenerative response to trauma [12, 13] (see chapter by Burns and Stone in this issue).

During early development, transcriptional pathways have been elucidated that govern the differentiation of the otocyst towards sensory or non-sensory regions [reviewed in [14]. Temporal and spatial triggers of otic development and maturation have been characterized, including the molecular controls that guide otic induction, patterning, morphogenesis and neurosensory development [15] (see chapter by Whitfield and Alsina in this issue). Knowledge about transcriptional pathways has laid the groundwork for establishing early and late developmental pathways of the inner ear. Moreover, pathogenic mutations in several transcription factors are associated with deafness [16, 17].

In this review we will discuss a new layer of gene regulation involving mechanisms of noncoding RNAs, chromatin remodeling, histone modifications, DNA methylation and research progress in these areas in the auditory and vestibular systems. It has become clear in recent years that the epigenetic layer of regulation plays an essential role in the development and maintenance of the inner ear, and has potential in regenerative capacities, with a great deal yet to be explored.

2. Gene regulation by epigenetic pathways and non-coding RNAs

2.1 Epigenetic gene regulation

Epigenetic regulation, above the DNA sequence, is controlled by a combination of dynamic nucleosome occupancy, post-translational modifications (PMTs) of histones, DNA methylation, and nuclear regulatory non-coding RNAs (Fig. 1) [18–20]. This level of regulation plays an essential role in development from a single totipotent oocyte through defined subsets of multipotent progenitor cells, eventually yielding the entire variety of cell types comprising the highly complex adult organism. Global epigenomic maps are essential to our understanding of gene regulation, or misregulation, as well as critical for controlling cell fate commitment and maintenance [21–23].

Chromatin allows for DNA to be tightly packed within eukaryotic nuclei. Nucleosomes constitute the fundamental subunit of chromatin. Nucleosome cores are comprised of 145–147 base pairs (bps) of DNA, wrapped around an octamer of two copies of histone proteins H2A, H2B, H3 and H4 [24].

Open chromatin structure is an identifier of regulatory elements. Areas of chromatin with lower nucleosome occupancy have markedly increased accessibility to the nuclease DNaseI and are referred to as DNase Hypersensitive sites (DHS). Using a DNaseI hypersensitivity assay coupled with high-throughput sequencing, DNaseI-seq, DHS sites were found to be associated with most of the defined *cis*-regulatory elements such as promoters, insulators, silencers, enhancers and locus control region [25].

The ENCODE (The Encyclopedia Of DNA Elements) Project analysis clearly demonstrates the overlap between transcription factor binding sites (TFBS) and a less dense nucleosome structure. Furthermore, DHS and transcription factor binding appear in a developmental stage or cell lineage dependent context [26, 27]. Genome-wide DHS maps will reveal high resolution, ~150–200 bp, open regulatory segments that allow more accurate predictions of TFBS.

Histone modifications are present as enrichment patterns at promoters and enhancers. The core histones comprising the nucleosome undergo various modifications at numerous positions across their N-terminal tails that project out of the mostly globular octamer. Common PTMs of histone proteins include acetylation, methylation, phosphorylation and ubiquitination. The modification pattern of histones is highly dynamic and is regulated by the opposing action of enzyme families. For instance, histone acetyltransferases catalyze the transfer of an acetyl group to the ε-amino group of lysine side chains, whereas histone deacetylases (HDACs) remove the acetyl group [28].

The overall chromatin architecture is directly linked to the composition of histone PMTs at a certain genomic location [29]. Different chemical modifications imparted on the histones have different effects over the degree of association between the negatively charged DNA and the nucleosome. They can tighten the DNA loop around each nucleosome and/or compress nucleosomes together, or it can loosen each nucleosome’s hold upon the DNA and separate nucleosomes from each other. In addition, histone modifications serve as docking

sites for regulatory proteins and chromatin remodelers that may alter the placement or position of nucleosomes in an ATP-dependent manner [30]. As a result, histone PTM compositions can be analyzed for its effect on gene expression resulting from its underlying influence on chromatin architecture.

Histone PTMs allow exploration of the basic epigenetic landscape dynamics. For example, histone 3 lysine 4 tri-methylation (H3K4me3) is positively correlated to the transcription start sites of actively transcribed genes [31]. Histone 3 lysine 27 tri-methylation (H3K27me3) is a repressive modification that spans over inactive genes; however, it is typically restricted to repressed key lineage developmental genes, not all inactive genes. Co-localization of H3K4me3 and H3K27me3 is known to mark genes found in a poised expression state, mainly in pluripotent stem cells or multipotent progenitor cells. This bivalent state is likely enriched in cell populations earlier in development to confer the ability to respond to signaling cues and readily activate gene expression (by loss of H3K27me3) or long-term repression (by loss of H3K4me3) as cells commit to their terminal fates [32, 33]. ChIP-Seq combines chromatin immunoprecipitation (ChIP) and high-throughput sequencing [34] and is used to establish genome-wide histone modification profiles [35].

DNA methylation is a key regulator of promoter and enhancer activity. DNA methylation, carried out by DNA methyltransferases (DNMTs) involves the covalent addition of a methyl group at the 5′ carbon of the cytosine ring, resulting in 5-methylcytosine [36]. In general, DNA methylation of promoters and enhancers negatively correlates to their activity and the expression of genes [37] through the disruption of transcription factor binding. For example, there is a gain of DNA methylation in pluripotency-related enhancers and gene promoters as differentiation progresses, and differential methylation in lineage specific enhancers between various lineages of blood and skin stem cells [38]. Altered patterns of promoter and enhancer DNA methylation are frequent in tumorigenic and aging cells. Connexin 26 is the most prevalent gene associated with profound congenital deafness [39]. Aberrant methylation in its promoter region has been found in breast [40] and lung cancer [41]. Aging rats have hypermethylation of the connexin 26 promotor region associated with a reduction in connexin 26 expression [42]. Recent advances and manipulations of next-generation sequencing platforms have presented a great advance in mapping the cell’s methylome in a genome-wide fashion, in particular MethylCseq [43]. This method provides genome-wide, nucleotide resolution of DNA methylation.

2.2 Gene regulation by non-coding RNAs

The central dogma of molecular biology that genetic information begins with DNA and ends with protein was challenged with the discovery of the wide range of non-protein coding RNA transcription and RNA-mediated genetic and epigenetic events in higher organisms [44]. The vast majority of the genome, approximately 97%, is non-coding. While less is known about all the regulatory and other elements of this larger portion, a significant role is attributed to RNA. Non-coding genome contains multiple forms of RNAs, including long non-coding (lncRNA) [45], piwi-interacting RNAs (piRNAs), circular RNAs (circRNAs), small nucleolar RNAs (sno-RNAs) and microRNAs (miRNAs). Small or short non-coding

RNA (ncRNA), which are under 200 nucleotides (nt) in length, include miRNAs (Fig. 1). The primary miRNA (pri-miRNA) transcripts are processed in the nucleus by a microprocessor complex that includes Drosha, an RNase III-like enzyme, and DGCR8 [46, 47]. A precursor miRNA (pre-miRNA) hairpin product 70 nt long is generated, which is exported by the receptor Exportin 5 to the cytoplasm. There, a second RNase III enzyme, Dicer, converts the RNA into mature miRNA duplexes. These duplexes are separated to produce a mature 22-nt miRNA, which is incorporated into the RNA-induced silencing complex (RISC). Finally, the complex targets mRNAs in order to repress them by degradation or translational repression.

In mammals, a single miRNA can bind any of hundreds of mRNA targets by complementation of the seed region of the miRNA to the target's 3′ untranslated region (UTR) binding site. This leads to a reduction in gene expression by translational suppression and mRNA destabilization [48]. The second major class of ncRNA, greater than 200 nt, is lncRNA [45]. They can either be cis-acting and function in local gene silencing (e.g., Xist RNA, a key regulator of X chromosome inactivation [49]); or trans-acting and target multiple genes throughout the genome, such as lncRNA-p21 [45]. lncRNAs mechanisms of action are likely unique and can be difficult to identify, as they do not have structural characteristics and their biogenesis and processing may differ.

In the inner ear field, the history of research in non-coding RNAs goes back to 2005, when the triad miRNA-96, 182 and 183 were detected in zebrafish hair cells of sensory epithelia by *in situ* hybridization [50]. Microarray analysis of mouse inner ears revealed expression of miRNAs and in particular, miRNA-96, 182 and 183 in mammalian hair cells [51]. Since then profiling using microarray and next generation sequencing platforms uncovered hundreds of miRNAs in the auditory and vestibular portions of the inner ear, including 74 differentially expressed miRNAs [52, 53]. MiRNAs specific to age-related hearing loss were identified in a microarray screen, and differential regulation of specific miRNAs led to the suggestion that pro-apoptotic miRNAs and those promoting proliferation and differentiation are both involved in age-related degeneration of the organ of Corti [54].

Much less is known about lncRNAs in the developing and mature inner ear. The *Meg3* RNA has been proposed to function as a lncRNA and based on expression studies, to play a role in pattern specification, differentiation and maintenance of inner ear cells [55]. *Rubie* is a lncRNA positioned upstream of the Bmp4 gene, and its disruption by an intronic endogenous retrovirus is predicted to be the cause of the *Epistatic circler* (*Ecl)* mouse vestibular phenotype [56].

3. Role of epigenetic factors and miRNAs in inner ear development

3.1 Epigenetics and otic induction, patterning and morphogenesis

Cranial placodes, including the otic placode, arise from a common precursor field, the preplacodal region, adjacent to the neural plate. Mutual repression between the homeodomain transcription factors OTX2 and GBX2 segregate placode progenitors of different fate, with GBX2 being required for otic placode specification [57]. A recent study identified DNMT3A, an enzyme required for de-novo DNA methylation as an essential

upstream regulator of otic GBX2 expression. De-novo DNA methylation, which creates new patterns of DNA methylation, occurs predominantly during early development and gametogenesis [58]. In the early chick embryo, *Dnmt3a* is expressed in the pre-placodal region and later in the otic placode itself. Knockdown of *Dnmt3a* selectively reduces the expression of early otic marker and specifier genes *Gbx2* and *Pax2,* followed by a loss of late otic marker genes and a severe reduction in otic vesicle size [59]. How DNMT3A regulates *Gbx2* expression has yet to be established. DNA methylation of cis-regulatory elements undergoes dynamic changes during development and cell differentiation [60, 61]. The authors propose a model by which DNMT3A activates *Gbx2* gene expression by selectively methylating a repressor binding site in the *Gbx2* promotor.

Following otic placode formation, the otic placode invaginates to form the otic cup, which pinches closed dorsally to form the otic vesicle (otocyst) [62]. A study by Uribe and colleagues revealed a critical role for histone demethylase KDM4B (alternative name JMJD2B) in otic invagination [63]. KDM4B is a histone demethylase that preferentially catalyzes demethylation of tri and di-methylated lysine residue 9 on histone 3 (H3K9me2/3). H3K9me3 and H3K9me2 are considered repressive epigenetic marks and are correlated with transcriptional silencing of a gene locus [64]. In the developing chick embryo KDM4B is expressed in pre-otic ectoderm and later becomes restricted to the border of the otocyst. In the absence of KDM4B, cytoskeletal rearrangements and cell polarization, required for otic invagination, fail to occur. The authors provide compelling evidence that the observed defects are due to a failure of *Dlx3* induction. *Dlx3*, a member of the vertebrate Distal-less related (*Dlx*) homeodomain genes, is initially expressed throughout the otic placode and during otic invagination becomes restricted to the rim of the invaginating otic pit [65]. The authors show that KDM4B directly binds to the *Dlx3* regulatory region and is required for demethylation of H3K9me3 at the promoter of *Dlx3.* Knock down of *Dlx3* causes similar otic invagination defects, and forced expression of *Dlx3* is sufficient to rescue otic defects due to the loss of KDM4B [63].

Otic morphogenesis requires a highly coordinated and complex pattern of growth. The histone deacetylase HDAC1 has recently emerged as an important regulator of cell proliferation and cell survival. In mice, targeted deletion of *Hdac1* dramatically impairs proliferation and is associated with elevated levels of the cyclin-dependent kinase (CDK) inhibitor expression [66]. In zebrafish, HDAC1 is highly expressed in the developing otocyst and posterior lateral line primordium. *Hdac1* knockdown results in a smaller otic vesicle, fused otoliths, malformed or absent semicircular canals, and fewer sensory hair cells [67]. These defects can at least in part be attributed to defects in otic cell proliferation and cell survival, which in *Hdac1* morphants is severely reduced. In addition, knockdown of *Hdac1* reduced otic expression of fibroblast growth factor (FGF) ligands FGF3 and 8 [67]. FGF3 and FGF8 play a critical role in otic patterning and hair cell fate specification in zebrafish [68, 69], and disrupted FGF signaling likely contributes to the observed inner ear defects in *Hdac1* morphants. Whether HDAC1 plays a similar role in murine inner ear morphogenesis has yet to be established.

Haploinsufficiency in *Chd7*, a gene that encodes for a chromatin modifier protein, causes CHARGE Syndrome, a multiple congenital anomaly disorder, which among other effects is

characterized by inner ear defects [70]. CHD7 is a member of the chromodomain helicase DNA-binding (CHD) family of ATP-dependent chromatin remodeling enzymes. ATP-dependent chromatin remodeling complexes facilitated by ATP hydrolysis shift, exchange or remove adjacent nucleosomes, enabling binding of transcription factors to DNA (reviewed in [71]). In both humans and mice, *Chd7* haplo-insufficiency results in severe semicircular canal malformation, and accompanying vestibular defects [72–75]. These defects are in part due to an abnormal pattern of cell proliferation. In addition, analysis of heterozygous *Chd7* mutant and conditional *Chd7* knockout mice revealed that CHD7 regulates the expression of vestibular regulatory genes in a gene dosage dependent manner [76]. Interestingly, inhibition of retinoic acid (RA) signaling can rescue semicircular canal defects in heterozygous *Chd7* mutant mice, suggesting that hyperactive RA signaling contributes to inner ear malformations in *Chd7* mutants [77]. The exact mechanism through which CHD7 regulates vestibular–specific genes has yet to be determined. CHD7 occupancy is cell-type specific, and most CHD7 sites show features of gene enhancer elements. It has been proposed that CHD7 provides access to transcriptional activators and co-factors by locally remodeling chromatin structure [78, 79]. In neuronal stem cells CHD7 directly interacts with HMG-box transcription factor SOX2 and cooperatively regulates the expression of key developmental regulators including the Notch ligand Jagged1 (JAG1) [80]. JAG1 is an essential regulator of inner ear neuro-sensory development and is required for semicircular canal formation [81]. *Jag1* expression is severely reduced in E10.5 heterozygous *Chd7* mutant otocysts, suggesting that *Jag1* deficiency may in part contribute to the vestibular defects observed in *Cdh7* mutants [80]. Future studies are warranted to address whether SOX2 and CHD7 directly interact in otic progenitor cells and cooperate in regulating otic gene expression.

3.2 MiRNAs in otic patterning and morphogenesis

Most of our understanding of how miRNAs might control inner ear development stem from studies of *Dicer1* mutant animals. Dicer1 function is essential for the processing of pre-miRNAs into functional mature miRNAs. In addition, Dicer1 is required for processing of double stranded RNA into small-interfering RNAs (siRNAs) [82].

In zebrafish, *Dicer1* mutant larvae have a severely malformed inner ear and lack otoconia [83]. Similarly, in mice, early otic deletion of the *Dicer1* gene (*Pax2-Cre* $Dicer1^{flox/flox}$ or $Foxg1^{Cre/+}$ $Dicer1^{flox/flox}$) results in gross inner ear malformations. The overall size of the inner ear is reduced, cochlear outgrowth is severely stunted and the development of semicircular canals is arrested at the canal plate stage [84, 85]. FGF10 dependent signaling plays a critical role in otic morphogenesis. Loss of FGF ligand FGF10 leads to severe inner ear malformations including partial failure of semicircular canal formation and cochlear outgrowth defects [86, 87]. *Fgf10* expression is severely diminished in *Dicer1* mutants, suggesting that partial loss FGF10 function contributes to *Dicer1* mutant phenotype [84]. The contribution of individual miRNAs to inner ear morphogenesis has yet to be examined.

A potential key regulator of inner ear morphogenesis is *miR-200b*, which is selectively expressed in cochlear and vestibular epithelial cells [88]. *MiR-200b* belongs to the *miR-200* family, which is comprised of *miR- 200a, miR- 200b, miR- 200c, miR- 141* and *miR- 429* [89]. Epithelial-to-mesenchymal transition (EMT) and its reversal (MET) are critical for

tissue remodeling during development (reviewed in [90]). Recent studies revealed that EMT and MET are controlled by a double negative feedback loop between members of miR-200 family and the transcription factors ZEB1 and ZEB2. *miR-200* family members enforce an epithelial fate and oppose EMT by transcriptionally silencing *Zeb1* and *Zeb2* genes [91, 92]. The pro-mesenchymal fate transcription factors ZEB1 and ZEB2 in turn repress the transcription of epithelial genes, including *miR-200* genes [93]. In the developing inner ear, epithelial remodeling is required for the formation of semicircular canals [94]. Twirler mice, which have a noncoding point mutation in the first intron of the *Zeb1* gene, have severe vestibular and auditory defects [95]. Transcriptome analysis revealed that in Twirler mice, ZEB1 target genes are de-repressed, suggesting that ZEB1 function is compromised in Twirler mice and that the resulting disruption of epithelial and mesenchymal cell identities underlies the inner ear malformations observed in the Twirler mice [88]. It has yet to be determined whether and to what extend *miR-200b* expression is de-regulated in Twirler mice.

3.3 Epigenetic factors in neurogenesis and hair cell formation

Central to otic neurogenesis are the basic helix-loop-helix (bHLH) transcription factors Neurogenin1 (NEUROG1) and NEUROD1 [96]. NEUROG1 is required for neuronal fate specification [97, 98], whereas NEUROD1, which functions downstream of NEUROG1 is required for the progression of the otic neurogenic program [99].

A recent study found that subunits of SWI/SNF-like chromatin remodeling complex cooperate with EYA1/SIX1 in the transcriptional activation of *Neurog1* and *Neurod1* in the developing inner ear [100]. Haplo-insufficiency for EYA1 leads to branchio-oto-renal syndrome, which is characterized by combinations of craniofacial defects and hearing loss with or without kidney anomalies [101, 102]. Animal models revealed that the transcriptional cofactor EYA1 and the homeobox transcription factor SIX1 are required for formation of the cochlea and vestibulocochlear ganglion [103, 104]. EYA1 and SIX1 are members of Eyes absent (*Eya*) and Sine oculis (*So/Six*) gene families. EYA proteins have a potent transactivation domain but lack a DNA binding domain and require the presence of transcription factors to act as potent transcriptional activator (reviewed in [105]). Employing gain and loss-of-function strategies, the authors uncovered that EYA1/SIX1 co-activator complex controls otic neural induction and neural differentiation by positively regulating the expression of *Neurog1* and *Neurod1*. Interestingly, the neural inductive activity of EYA1/SIX1 depended on the presence and enzymatic function of core components of SWI/SNF like BAF complex. Mammalian SWI/SNFlike BAF complexes are ATP-dependent chromatin-remodeling complexes that are made up of a core ATPase subunit BRG1 (official name SMARCA4) or BRM (SMARCA2), and BRG1 Associated Factors (BAFs) (reviewed in [106]). The authors found that EYA1 and SIX1 directly interact with SWI/SNF subunits BRG1 and BAF170, and BRG1 ATPase activity is required for normal otic neurogenesis as well as ectopic neurogenesis induced by *Eya1/Six1* overexpression, indicating a central role for SWI/SNF-like chromatin remodeling complex in otic neurogenesis [100]. A parallel pathway of *Neurog1* regulation involves the ATP-dependent chromatin-remodeler CHD7. In *Chd7*-deficient otic tissue the cochlear vestibule ganglion is missing or severely reduced. TBX1, a T-box transcription factor selectively expressed in the non-neurogenic domain of

the developing otocyst, is known to limit the extent of the neurogenic domain within the otic vesicle [107]. Analysis of *Chd7*-deficient otic tissue revealed that the expression domain of *Tbx1* is expanded into the neurosensory domain, and *Neurog1* expression is initially induced but fails to be maintained [75]. Future studies are needed to clarify whether CHD7 occupies *Tbx1* and *Neurog1* cis regulatory sequences.

The bHLH transcription factor ATOH1 plays a central role in mechano-sensory hair cell development. ATOH1 is both necessary and sufficient for hair cell formation and differentiation [108–110]. ATOH1 expressing hair cells and their surrounding supporting cells derive from sensory progenitor cells commonly referred to as pro-sensory cells. Pro-sensory cells and later differentiating supporting cells are inhibited from inducing *Atoh1* expression through a lateral inhibition mechanism mediated by the Notch signaling pathway [111–113]. A recent study by Stojanova and colleagues examined whether epigenetic mechanisms control *Atoh1* expression in the developing murine cochlea. Developmentally regulated genes often exhibit both repressive (H3K27me3) and active (H3K4me3) epigenetic marks within their promoter and enhancer sequences, referred to as bivalent state [33]. The authors found that these bivalent epigenetic marks are frequent within the *Atoh1* locus of pro-sensory cells and perinatal supporting cells, indicating that *Atoh1* is poised for expression in these cell types. In differentiating hair cells, these bivalent marks were infrequent and H3K9 acetylation, a mark, associated with actively transcribed genes, was prevalent [114]. Inhibition of histone acetyltransferase activity reduced H3K9 acetylation at the *Atoh1* locus and prevented *Atoh1* induction, suggesting a central role for histone acetyltransferases in *Atoh1* induction. In turn, shut down of *Atoh1* expression in maturating hair cells required H3K9 deacetylation and was accompanied by an increased frequency of H3K9 tri-methylation (H3K9me3) [115], a mark associated with gene silencing [116].

3.4 MiRNAs in neurogenesis and hair cell formation

Early otic *Dicer1* conditional knockouts (*Foxg1*$^{Cre/+}$ *Dicer1*$^{flox/flox}$ *or Pax2-Cre Dicer1*$^{flox/flox}$) have, in addition to defects in otic morphology, severe neuro-sensory defects. In these mutants, both vestibular and auditory sensory epithelia are severely reduced in size, innervating neurons are largely missing and the organization and apical specialization of remaining hair cells is severely disrupted [84, 117] [84, 85]. Innervating sensory neurons depend on trophic support from hair cells and supporting cells. Defects in neuronal survival may be a consequence of the stunted sensory development in early otic *Dicer1* conditional knockouts; alternatively it may be due to defects in neuronal differentiation as previously reported for *Dicer1* mutant cortical neurons [118]. Early otic *Dicer1* conditional knockouts were not viable so hearing could not be tested. The targeted removal of functional mature miRNAs from hair cells using *Pou4f3-Cre* mice crossed with *Dicer1*$^{flox/flox}$ led to a mouse that survived until approximately one month of age, enabling hearing to be tested [52]. These mice were profoundly deaf, as evaluated by auditory brainstem response. The *Dicer1* mutant hair cells began degenerating soon after birth, with surviving cells having an abnormal morphology, suggesting a critical role for miRNAs in hair cell differentiation/maturation.

Re-examination of the cochlear phenotype of early otic *Dicer1* mouse mutants by Huyghe and colleagues revealed previously undetected defects in pro-sensory cell proliferation and hair cell fate specification. *Dicer1* deficient pro-sensory cells were delayed in their cell cycle withdrawal and showed a bias to differentiate as hair cells. In addition, defects in planar cell polarity (PCP) as well as defects in formation or maintenance of kinocilia were noted. These defects are likely due to de-regulation of Wnt signaling. Among the genes de-repressed in the absence of Dicer1 were secreted frizzled-related protein (Sfrp)4 and Sfrp5, which encode for known modulators of both canonical and non-canonical Wnt signaling [119]. Exposure of cochlear tissue to SFRP4 and SFRP5 protein caused cell fate specification and PCP defects that largely resembled the defects observed in *Dicer1* mutants. *Sfrp4* and *Sfrp5* expression in the developing cochlea is likely repressed by *miR-124.* The authors found *that miR-124* is selectively expressed in the differentiating auditory sensory epithelium, *miR-124* negatively regulates *Sfrp4* and *Sfrp5* expression, and similar to Sfrp4/5 overexpression, *miR-124* inhibition biases pro-sensory cells towards a hair cell fate [117].

The failure of *Dicer1* deficient auditory pro-sensory cells to exit the cell cycle at the appropriate time may, at least in part, be due to the loss of *let-7* miRNA function. A recent study by Golden and colleagues revealed that a double negative feedback loop between *let-7* miRNAs and the RNA binding protein (RBP) LIN28B times and coordinates cell cycle exit and differentiation of pro-sensory cells in the murine cochlea. *Let-7* (lethal-7), originally identified as a regulator of developmental timing (heterochrony) in *C. elegans* [120], was among the first miRNAs to be discovered [121, 122]. In human and mice, members of the *let-7* family (*let-7a, b, c, d, e, f, g, i, mir-98*) function as tumor suppressors and are highly associated with a terminal differentiated state. *Let-7* miRNAs target and transcriptionally silence pro-growth genes including *Lin28a* and *Lin28b.* In turn, LIN28 RBPs bind *let-7* primary and precursor transcript and interfere with their processing by Drosha and Dicer1 (reviewed in [123]). In the developing inner ear, *Lin28b* is selectively expressed in pro-sensory cells, whereas *let-7* miRNAs are expressed in terminally differentiated cells including hair cells and supporting cells [124]. Using inducible *LIN28B* and *let-7g* transgenic mouse lines the authors found that higher than normal levels of *let-7* miRNAs resulted in premature pro-sensory cell cycle exit, whereas lower than normal *let-7* levels, due to LIN28B overexpression delayed pro-sensory cell cycle exit [124]. LIN28B overexpression led to an increase in cochlear *N-Myc (Mycn)* and *cyclin D1 (Ccnd1)* expression. *N-Myc* and *cyclin D1* are known let-7 target genes [125, 126] and loss of either gene has been shown to disrupt cell proliferation in the developing inner ear [127, 128], suggesting that the effects of LIN28B and *let-7* on proliferation are at least in part due to deregulation of *N-Myc* and *cyclin D1* expression. Over-expression of *let-7* miRNAs accelerates development and induces precocious differentiation in many tissues including the retina [129]. However, even though lower than normal *let-7* levels due to LIN28B overexpression delayed auditory hair cell differentiation, *let-7g* overexpression failed to induce precocious differentiation [124]. These findings suggest that in the developing cochlea, *let-7* mainly functions as an inhibitor of progenitor-specific gene expression.

Sensory cells including hair cells express three homologous miRNAs, *miR-96, miR-182*, and *miR-183* [51]. These evolutionary conserved miRNAs are expressed from a single genetic locus and are commonly referred to as *miR-183* cluster or family [130, 131].

Overexpression of *miR-183* family members in sensory progenitors biases them towards a hair cell fate. In zebrafish overexpression of *miR-96* or *miR-182* results in ectopic sensory patches and increases the number of hair cells within the inner ear vestibular sensory organs, whereas knockdown of *miR-96*, *-182*, and/or-*183* generates fewer hair cells [132]. A similar bias towards a hair cell fate was observed when *miR-183* family members were overexpressed in the developing chicken auditory organ. However, in contrast to zebrafish, overexpression of *miR-183* cluster members failed to induce ectopic sensory patches [133]. Mutations in the seed region of *miR-96* are associated with hearing loss in humans and mice [134, 135]. The mouse mutant diminuendo (*Dmdo*) has a single base change in the seed region of the *miR-96* gene [135]. In homozygous and heterozygous *Dmdo* mice, hair cells form relatively normal but have misshapen stereocilia and their biophysical maturation and specialization is arrested [135, 136]. At one month of age, *Dmdo* homozygous mice have virtually no hair cells and *Dmdo* heterozygous mice show severe outer hair cell loss [135]. The observed defects could be due loss of *miR-96* dependent target regulation and/or gain of novel targets. Qualitative similar hair cell defects are observed in hair cell-specific *Dicer1* mutants, suggesting the former to be the case.

4. Non-coding RNAs and epigenetics in human sensory disease

4.1 miRNAs in deafness

Mutations in miRNAs in humans have been found in three families [134, 137]. Of the *miR-183*, *miR-182*, and *miR-96* triad that is specifically expressed in inner and outer hair cells, only *miR-96* is associated with human disease. While attempts have been made to discover if there is a larger number of miRNAs contributing directly to human deafness, this is unlikely to be the case [138].

4.2 Epigenetic factors associated with deafness

Mutations in epigenetic factors recently emerged as potential causes for deafness in humans. Germline mutations in histone methyltransferases have been identified in human syndromes associated with sensorineural hearing loss. WHSC1 (Wolf-Hirschhorn Syndrome candidate 1, also known as NSD2 and MMSET) is a histone methyltransferase that preferentially catalyzes H3K36 methylation, an epigenetic modification highly associated with transcriptional activation [139]. Haploinsufficiency in *Whsc1* is linked to Wolf-Hirschhorn syndrome (WHS), a complex genetic disorder that causes severe growth retardation, craniofacial defects, seizures, and in some cases hearing impairment [140]. *Whsc1* mutant mice are developmentally delayed and manifest similar craniofacial defects and external ear and eye anomalies seen in humans with WHS [141, 142]. The authors found that cochlear hair cells form in *Whsc1* heterozygous and homozygous mutants, but their shape, arrangement, morphological specialization and innervation are disrupted, suggesting that WHSC1 dysfunction, might contribute to sensorineural hearing loss in individuals with WHS [142]. How WHSC1 controls cochlear hair cell arrangement, specialization and innervation are currently unknown, but components of the Wnt/ PCP machinery are among the likely targets of WHSC1 mediated regulation.

5. Role of epigenetic factors and miRNAs in hair cell regeneration

5.1 Supporting cell proliferation in birds and fish

Histone modifying enzymes and miRNAs recently emerged as critical regulators of supporting cell proliferation in fish and birds.

In the mature vestibular epithelia of birds, supporting cells divide at a low rate and continually and spontaneously replace lost hair cells. Treatment of hair cell-damaged chicken utricle with broad spectrum HDAC inhibitors or class I selective HDAC inhibitors resulted in a decrease in supporting cell proliferation, but did not affect the differentiation of replacement hair cells [143]. In developing zebrafish, class I HDACs, HDAC1 and HDAC3 are selectively expressed in lateral line primordium and neuromast [144, 145]. Knockdown of HDAC3 or broad inhibition of HDAC activity severely impaired cell proliferation within the lateral line primordium [144] [146]. Similarly, in hair cell-damaged zebrafish larvae, inhibition of HDAC activity with broad spectrum HDAC inhibitors reduced supporting cell proliferation and subsequent hair cell regeneration. In mice, CDK inhibitors p27/Kip1 (CDKN1B) and p21/Cip1 (CDKN1A) are required for maintaining auditory supporting cells and hair cells in a postmitotic state [147–149]. The authors found that in the hair cell-damaged zebrafish larvae HDAC inhibition increased p21/Cip1 and p27/Kip1 expression, suggesting that HDACs facilitate supporting cell proliferation through restricting p27/Kip1 and p21/Cip1 expression [145].

Lysine-specific demethylase 1 (LSD1, official name KDM1A) is a FAD-dependent enzyme that catalyzes the demethylation of mono- and di-methylated lysine 4 and 9 on histone 3 (H3K4me1/2 and H3K9me1/2) repressing and activating transcription, respectively (reviewed in [150]). Two recent studies revealed an important role for LSD1 in regulating sensory progenitor cell proliferation. In the developing zebrafish larvae, pharmacological inhibition of LSD1 using trans-2-phenylcyclopropylamine (2-PCPA) disrupted cell proliferation and induced apoptosis in neuromast cells, causing a reduction in the numbers of sensory hair cells and supporting cells produced [67]. Similarly, in the hair cell-damaged zebrafish, 2-PCPA treatment significantly decreased supporting cell proliferation and subsequent production of hair cells. Based on gene expression data the authors propose that LSD1 promotes cell proliferation by inhibiting the expression of p21/Cip1 and p27/kip1 as well as by promoting the expression Wnt/β-cateninsignaling components [151].

Supporting cells within the auditory sensory epithelia (basilar papilla) of birds are quiescent, but re-enter the cell cycle within hours following hair cell damage [152]. Enrichment analysis of gene sets differentially expressed in the quiescent and proliferating basilar papilla uncovered *miR-181a* as a potential upstream regulator of supporting cell proliferation. The authors found that in the intact chicken basilar papilla *miR-181a* overexpression was sufficient to stimulate supporting cell proliferation and the production of new hair cells [153]. In a follow up study the authors found that knock-down of endogenous *miR-181a* in basilar papilla inhibited the regenerative proliferation of supporting cells in response to hair cell damage [154]. *miR-181a* has been shown to interfere with p27/Kip1 transcription and translation in tumor cell lines [155, 156], suggesting that *miR-181a* might trigger cell cycle

re-entry of supporting cells in a p27/Kip1 dependent manner. Whether *miR-181a* is capable of stimulating supporting cell proliferation in mammalian species is currently unknown.

5.2 Targets for regenerative strategies in mammals

Prior to the onset of hearing, mammalian supporting cells have a latent ability to spontaneously regenerate hair cells. Isolated auditory supporting cells placed in culture re-enter the cell cycle and generate hair cells through both mitotic and non-mitotic mechanisms [157, 158]. In response to hair cell damage, auditory supporting cells spontaneously regenerate hair cells *in vivo* [159, 160]. The limited regenerative response of immature auditory supporting cells can be further enhanced by either inhibiting the Notch signaling pathway [161, 162], or by over-activating Wnt/ β-catenin signaling [163–165].

The polycomb group protein BMI1 is required to sustain Wnt/β-catenin signaling in neonatal supporting cells. BMI1 is part of the multi-protein polycomb repressive complex 1 (PRC1), which is needed for ubiquitination of histone H2A lysine 119. This epigenetic mark is associated with gene repression (reviewed in [166]). In cochlear explants, obtained from neonatal *Bmi1* mutant mice, supporting cells failed to re-enter the cell cycle in response to hair cell damage and BMI1 deficiency reduced the sphere-forming ability of unfractionated cochlear epithelial cells and Wnt-responsive supporting cells. The authors found that in the *Bmi1*-deficient cochlear tissue, Wnt antagonist DKK1 was increased and Wnt/β-catenin signaling was attenuated. Over-activation of Wnt signaling using GSK-3β inhibitor partially reversed the proliferation deficit of *Bmi1* mutant supporting cells, suggesting that BMI1 facilitates supporting cell proliferation by sustaining high levels of canonical Wnt signaling [167].

DNA methylation may restrict hair cell lineage potential. DNMT1 activity is required to maintain patterns of DNA methylation in daughter cells after DNA replication [168]. The nucleotide analog 5-azacytidine (5-aza) incorporates into DNA and interferes with DNMT function, leading to a decrease in global DNA methylation [169]. A recent study used a prosensory-like cell line (MUCS), initially derived from adult mouse utricle, to determine the role of DNA methylation in hair cell fate induction and differentiation. MUCS cells have mesenchymal characteristics and the authors found that treatment of MUCS cells with 5-aza increased expression of epithelial genes and hair cell genes on both transcript and protein levels, which facilitated the formation of hair cell-like cells [170].

The newt, a urodele amphibian, possesses a remarkable ability for tissue and cell regeneration independently of its age. Regeneration in adult newts is mediated primarily by dedifferentiation and re-differentiation (trans-differentiation) of terminally differentiated cells [171]. Gene expression profiling of newt inner ear and lens tissue revealed that the early phase of lens and hair cell regeneration is accompanied by a sharp drop in *let-7* miRNA expression, suggesting that high *let-7* miRNA levels may be refractory to trans-differentiation of terminal differentiated cells [172]. A recent study indicates that indeed, high *let-7* miRNA levels impair hair cell regeneration in the early postnatal murine cochlea. In early postnatal cochlear explants, pharmacological inhibition of Notch signaling allows for the production of new hair cells through supporting cell-to-hair cell conversion (trans-differentiation) [173]. Using a similar experimental approach, the authors found that *let-7g*

overexpression significantly decreased the production of new hair cells in response to Notch inhibition, whereas overexpression of *let-7* antagonist LIN28B enhanced hair cell production in response to Notch inhibition [124]. Expression of mature *let-7* miRNAs rise within the auditory sensory epithelium during postnatal maturation, and it is intriguing to speculate that such rise in *let-7* miRNA expression may be functionally linked to the decline in regenerative capacity of supporting cells.

Another miRNA recently implicated in auditory hair cell regeneration is *miR-210*. *miR-210*, an evolutionary conserved miRNA, is critical for the cells response to hypoxia, with roles in mitochondrial metabolism, angiogenesis, DNA repair, and cell survival [174]. *miR-210* was identified in a screen for miRNAs that are differentially expressed during hair cell differentiation using the inner ear cell line (UB/OC-1). *MiR-210* knockdown in UB/OC-1 cells induces premature hair cell marker expression, suggesting that *miR-210* functions in maintenance of a progenitor state. Overexpression of *miR-210* in early postnatal cochlear explants resulted in spontaneous formation of new hair cells, which based on Cre-loxP mediated lineage tracing, originated from supporting cells. A subset of in silico predicted *miR-210* targets were experimentally validated. However, none of the validated *miR-210* targets have yet been associated with sensory cell development [175]. Futures studies are needed to resolved how *miR-210* facilitates supporting cell-mediated hair cell regeneration.

6. Conclusions

The past decade has seen a remarkable increase in our understanding of non-coding RNAs and chromatin modifying enzymes that has changed how we view gene expression and regulation in every organism and tissue studied. Advances in genomic technologies have enabled a comprehensive look at the key mechanisms of epigenetics. Investigations of the auditory and vestibular systems have lagged behind partly due to the lack of a robust cell culture system for the inner ear, the prior need for a large number of cells for genomic experiments, and the requirement for a model organisms rather than direct examination of the human inner ear. Despite these hindrances, we predict that the next decade will lead to dramatic discoveries, following the progress made to date and summarized in this review. Most promising, epigenomic dynamics uncovered during development or hair cell regeneration may help identify key regulators, which will in turn aid in prioritizing genes for future studies.

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Abbreviations

bps	base pairs
DHS	DNase Hypersensitive sites

TFBS	transcription factor binding sites
PTMs	post-translational modifications
HDACs	histone deacetylases
H3K4me3	histone 3 lysine 4 tri-methylation
H3K27me3	histone 3 lysine 27 tri-methylation
ChIP	chromatin immunoprecipitation
DNMTs	DNA methyltransferases
lncRNA	long non-coding RNA
piRNA	piwi-interacting RNA
circRNA	circular RNA
sno-RNA	small nucleolar RNA
miRNA	microRNAs
pri	primary
pre	precursor
ncRNA	non-coding RNA
nt	nucleotides
RISC	RNA-induced silencing complex
UTR	untranslated region
CDK	cyclin-dependent kinase
FGF	fibroblast growth factor
CHD	chromodomain helicase DNA-binding
RA	retinoic acid
siRNAs	small-interfering RNAs
EMT	epithelial-to-mesenchymal transition
MET	mesenchymal-to-epithelial transition
bHLH	basic helix-loop-helix
H3K9me3	H3K9 trimethylation
PCP	planar cell polarity
Sfrp	secreted frizzled-related protein

RBP RNA binding protein

WHS Wolf-Hirschhorn syndrome

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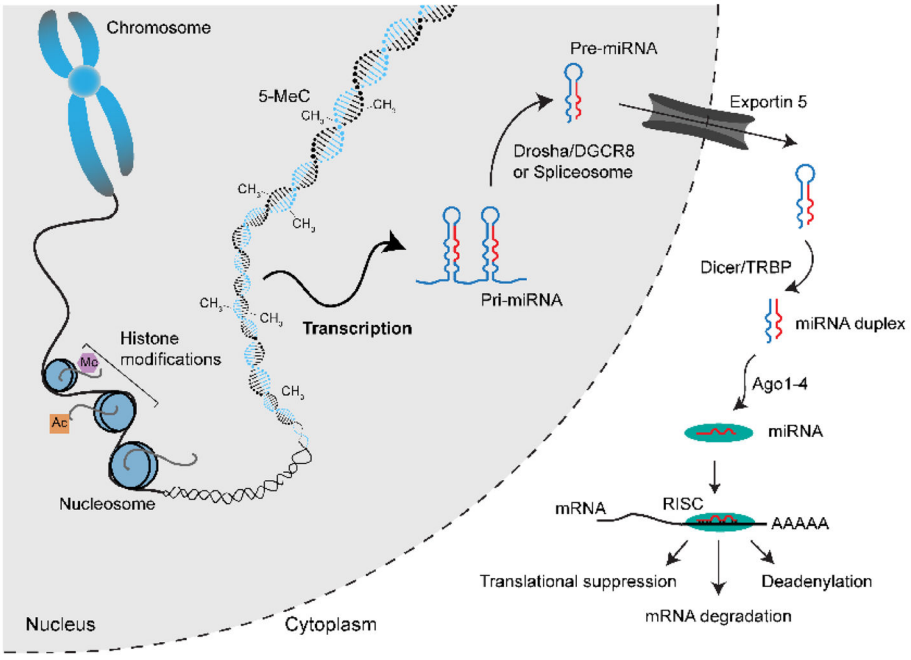

Fig. 1.

Schematic diagram of miRNA biogenesis and epigenetic mechanisms in the mammalian cell. Common histone PMTs such as histone methylation (me) and acetylation (ac) are indicated. DNA methylation is shown on the DNA double helix, as 5-MeC and CH_3. Gene transcription produces a pri-miRNA that is processed by Drosha to form a pre-miRNA. Exportin-5 facilitates its export to the cytoplasm, where Dicer cleaves the pre-miRNA into short double-stranded RNA. The RISC complex is activated and its catalytic component argonaute (Ago) leads to degradation of mRNA, translational suppression and deadenylation.