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MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness

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Abstract Sensorineural hearing loss is the most common sensory disorder in humans and derives, in most cases, from inner-ear defects or degeneration of the cochlear sensory neuroepithelial hair cells. Genetic factors make a significant contribution to hearing impairment. While mutations in 51 genes have been associated with hereditary sensorineural nonsyndromic hearing loss (NSHL) in humans, the responsible mutations in many other chromosomal loci linked with NSHL have not been identified yet. Recently, mutations in a noncoding microRNA (miRNA) gene, *MIR96*, which is expressed specifically in the inner-ear hair cells, were linked with progressive hearing loss in humans and mice. Furthermore, additional miRNAs were found to have essential roles in the development and survival of inner-ear hair cells. Epigenetic mechanisms, in particular, DNA methylation and histone modifications, have also been implicated in human deafness, suggesting that several layers of noncoding genes that have never been studied systematically in the inner-ear sensory epithelia are required for normal hearing. This review aims to summarize the current knowledge about the roles of miRNAs and epigenetic regulatory mechanisms in the development, survival, and function of the inner ear, specifically in the sensory epithelia, tectorial membrane, and innervation, and their contribution to hearing.

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Sensorineural hearing loss and the mammalian inner ear

Sensorineural hearing loss, or deafness in its most severe form, is the most common disability in humans, affecting at least 1 in 500 newborns and over half of individuals older than 80 years. In regions of high consanguinity or poor health care, the incidence of hearing loss is even higher. It is estimated that about 278 million people worldwide have moderate to profound hearing loss in both ears, and over 50 million are living in the US and European countries, with dire consequences on public health and quality of life of the individual [statistics from the World Health Organization (WHO), http://www.who.int/mediacentre/factsheets/fs300/en/index.html, and the US National Institute on Deafness and Other Communication Disorders (NIDCD), http://www.nidcd.nih.gov/health/statistics/hearing.asp].

The mammalian inner ear is one of the most complex tiny organs in the body and is composed of two sensory organs: the cochlea, responsible for hearing, and the vestibule, responsible for balance (Fig. 1, *left*). While cochlear defects induce hearing loss, vestibular defects lead to disorientation, expressed as vertigo or dizziness in humans and circling behavior (movement in circles) in mice (reviewed in Friedman et al. 2007) and zebrafish (reviewed in Whitfield 2002). The sensory epithelia of the cochlea and the vestibule contain supporting cells and mechanosensory receptor cells, called hair cells due to the hairlike stereocilia on the apical surface of these polarized epithelial cells. The hair and supporting cells are arranged in a precise cellular pattern required for proper mechanotransduction to occur. In the cochlea, the organ of Corti is the sensory epithelium, composed of hair cells and supporting cells derived from common progenitor cells. The hair cells

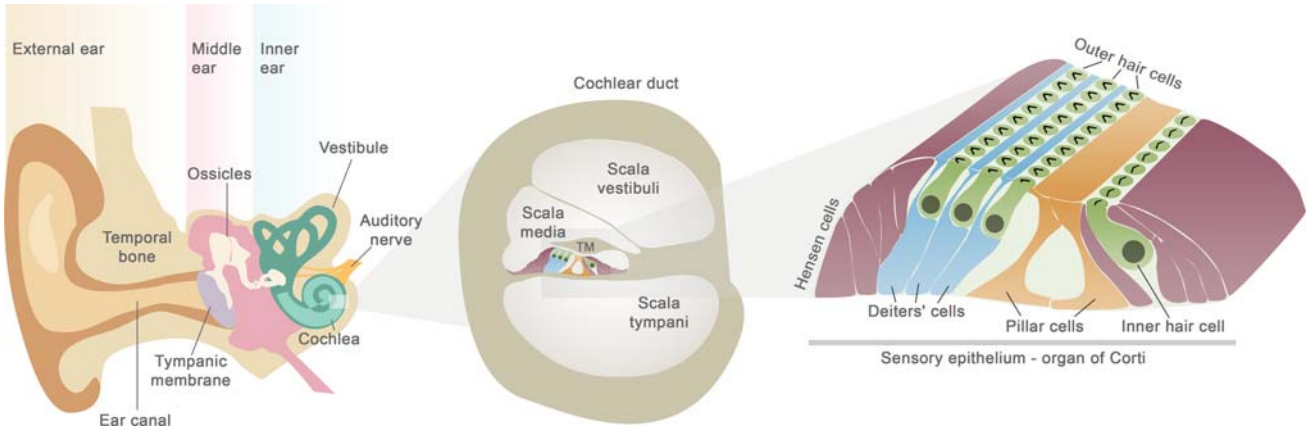

Fig. 1 Schematic illustration of the human ear. *Left* The ear is composed of three parts: the external, middle, and inner ear. The inner ear is composed of two organs, the snail-like cochlea and the vestibule. *Middle* Cross section of the cochlear duct of the snail-shaped cochlea. The cochlear sensory epithelium is illustrated, with the tectorial membrane (TM) above. *Right* Enlarged illustration of the hair and supporting cells in the cochlear sensory epithelium, the organ of Corti. In the right panel: green, hair cells; other colors, supporting cells

are arranged in four rows along the snail-shaped cochlea: three rows of outer hair cells and a single row of inner hair cells, surrounded by supporting cells that include pillar cells, Hensen cells, and Deiter cells (reviewed in Raphael and Altschuler 2003) (Fig. 1, *right*). The stereocilia of the outer hair cells are embedded in the overlying tectorial membrane, which is composed of collagen fibrils and glycoproteins (reviewed in Richardson et al. 2008). The tympanic membrane between the outer ear and the middle ear and the tiny bones of the middle ear convert auditory signals to cochlear fluid waves. The specialized stereocilia of the cochlear hair cells move in response to the fluid movement in the inner-ear canals, which causes ion channels on the stereocilia plasma membrane to open. Thus, the cochlear hair cells convert auditory-derived mechanical stimuli to electrical signals (reviewed in Raphael and Altschuler 2003). The vestibule is composed of three semicircular canals and five patches of sensory epithelia (utricular and saccular maculae and three cristae), which also include several types of sensory hair and supporting cells. Although the cochlear hair cells have a different organization, a slightly different shape, and some differences in gene expression and regulation (Hertzano et al. 2007) compared to the vestibular hair cells, the mRNA and miRNA populations of the cochlear and vestibular sensory epithelia are very similar, with only minor differences (Friedman et al. 2009a; Friedman and Avraham, unpublished).

In most cases, hearing loss in humans derives from abnormal development or degeneration of the cochlear hair cells, and it is estimated that 60-70% of the cases are hereditary (Nance 2003). More than 110 chromosomal loci have been linked with nonsyndromic hearing loss (NSHL) in humans, but thus far the responsible genes have been identified in only a subset of these loci (see Hereditary Hearing Loss homepage, http://webh01.ua.ac.be/hhh/) (Ahmed et al. 2008; Du et al. 2008; Friedman et al. 2009b; Ruel et al. 2008; Uchida et al. 2009; Yang et al. 2009). In addition, many syndromes include sensorineural hearing loss. Until very recently, only mutations in protein-coding and mitochondrial genes were evaluated in NSHL loci, leading to the discovery of mutations in genes encoding 49 different proteins and 2 mitochondrial genes that are responsible for NSHL. Recently, one of the NSHL loci, DFNA50, was found to carry a mutation in a noncoding microRNA (miRNA) gene, and this mutation not only segregated with the hearing loss phenotype, but it is the first miRNA mutation found for a Mendelian disease (Mencia et al. 2009).

The normal development of the inner ear requires coordinated and strictly regulated expression of genes and regulatory factors (Fritzsch et al. 2006; Kelley 2006). After the inner ear develops, the complex sensory transduction of the inner ear is dependent on a tremendous amount of synchronized processes and mechanisms. In addition to the many genes and proteins required for the normal function of the sensory hair cell, many factors are required for the survival of hair cells, some of which are internal to the hair cells [e.g., Usher-related genes (Reiners et al. 2006)], while others are external to the hair cells and affect the hair cell environment [e.g., the connexin genes *Gjb2* and *Gjb6* that are expressed in supporting cells (Nickel and Forge 2008), or collagen genes and *Tecta* that comprise the tectorial membrane (Richardson et al. 2008)]. For example, some of the sensory epithelium supporting cells are responsible for controlling the ionic homeostasis of the endolymph, the solution that fills the lumen (scala media) to which the sensory epithelial cells face. The exact composition of the

endolymph is crucial for the function and survival of the hair cells (reviewed in Kikuchi et al. 2000). External trauma, such as exposure to high-volume acoustics, or ototoxic drugs, such as aminoglycoside antibiotics and the antineoplastic drug cisplatin, can induce apoptosis of the cochlear hair cells (reviewed in Cheng et al. 2005). In such cases, the supporting cells have a limited curative role. While the supporting cell population in the mammalian vestibule has a limited ability to transdifferentiate to new hair cells and thus may compensate for lost hair cells in this system, the mammalian cochlea has no ability to regenerate hair cells after birth (Raphael et al. 2007).

The role of epigenetics in deafness is just beginning to be deciphered; but given its broad function in development and cellular differentiation, it is predicted to provide a significant contribution to this sensory disease. While deafness-linked mutations or chromosomal loci have been identified in many families with NSHL, the reason for most cases of hearing loss in human is still unknown, particularly for the most common form of hearing loss that is age-related, presbycusis. The failure to identify the responsible genes for these cases may suggest that these are complex genetic conditions, derived from an accumulation of several mutations in several genes. Alternatively, some of these cases may derive from aberrant epigenetic regulation and therefore the underlying reason cannot be identified simply by examining the DNA sequence. Moreover, there are cases of human deafness where the severity of the phenotype cannot be explained by the mutation alone, suggesting that epigenetic mechanisms may be involved even when the underlying mutation is known.

The mouse serves as what is probably the most beneficial model to study the mammalian inner ear (Brown et al. 2008). Thirty-five of 51 of the genes that are responsible for hearing loss in humans have mouse models, and half of them were identified as responsible for deafness in mice prior to or concurrently with their identification in humans. More than 130 additional protein-coding genes that have not been linked with hearing loss in humans thus far have been identified as crucial for hearing loss in mice. Mouse models with mutations in these genes enabled the study of their roles in the mammalian inner ear, including a few models with double mutations (i.e., mutations in two different genes that together induce deafness, while each mutation alone does not). Moreover, recently the mouse genome assembly was finished, revealing that most of the mouse protein-coding genes that have no human orthologs belong to the reproductive system, validating yet again that the mouse serves as an excellent model animal to study other organs and systems (Church et al. 2009).

This review focuses on human mutations and mouse models that link noncoding miRNA genes, DNA methylation, and histone modifications (illustrated in Fig. 2) with sensorineural hearing loss.

MicroRNAs and epigenetic mechanisms in the mammalian inner ear

miRNAs and epigenetics, including cytosine methylation and mutagenesis of methylated cytosines (5′-methylcytosine deamination), histone modifications, imprinting, RNA editing, and crosstalk between these different regulatory mechanisms, have been identified as having crucial roles in the regulation of development, survival, and function of many mammalian organs and tissues (for examples, see Cedar and Bergman 2009; Probst et al. 2009). Recently, a few examples and clues that link some of these mechanisms with inner-ear development or survival have been reported.

miRNAs

Biogenesis of miRNAs

miRNAs comprise one of the most prominent classes of noncoding RNAs (ncRNAs) and serve as a major form of regulation in plant and animal cells. Thus far, as of March 2009, 706 human, 547 mouse, and 336 zebrafish miRNAs have been identified (experimentally proven or homologous to experimentally proven miRNAs in other species), and their sequences are listed in the miRBase database [http://www.mirbase.org/; current release: 13.0 (Griffiths-Jones et al. 2008)]. The biogenesis and function of miRNAs in plants and animals are slightly different, and the description here is for animals. miRNA genes (known as Mir or Mirn genes) appear as single genes or in clusters of several Mir genes that are cotranscribed as a single transcription unit. While some of the Mir genes are found outside of protein-coding genes, many other Mir genes are included in introns of protein-coding genes and are transcribed with the host protein-coding gene, under the regulation of the same promoter. The Mir gene is transcribed to a long RNA molecule (pri-miRNAs) that may contain a single or several stem loops. The type III ribonucleases Drosha and Dicer1 release the mature miRNA(s) from the pri-miRNA. Drosha, together with DGCR8 as a cofactor, form the microprocessor complex that isolates the stem-loop structures to obtain the shorter (60-124 nucleotides) hairpin pre-miRNA(s). Each pre-miRNA contains one to two miRNAs (reviewed in Bartel 2004; Carthew and Sontheimer 2009). In some cases, the pri-miRNA may be coprocessed by intron splicing to produce products that are videntical to the Drosha-released pre-miRNAs, and this mechanism may serve as an alternative

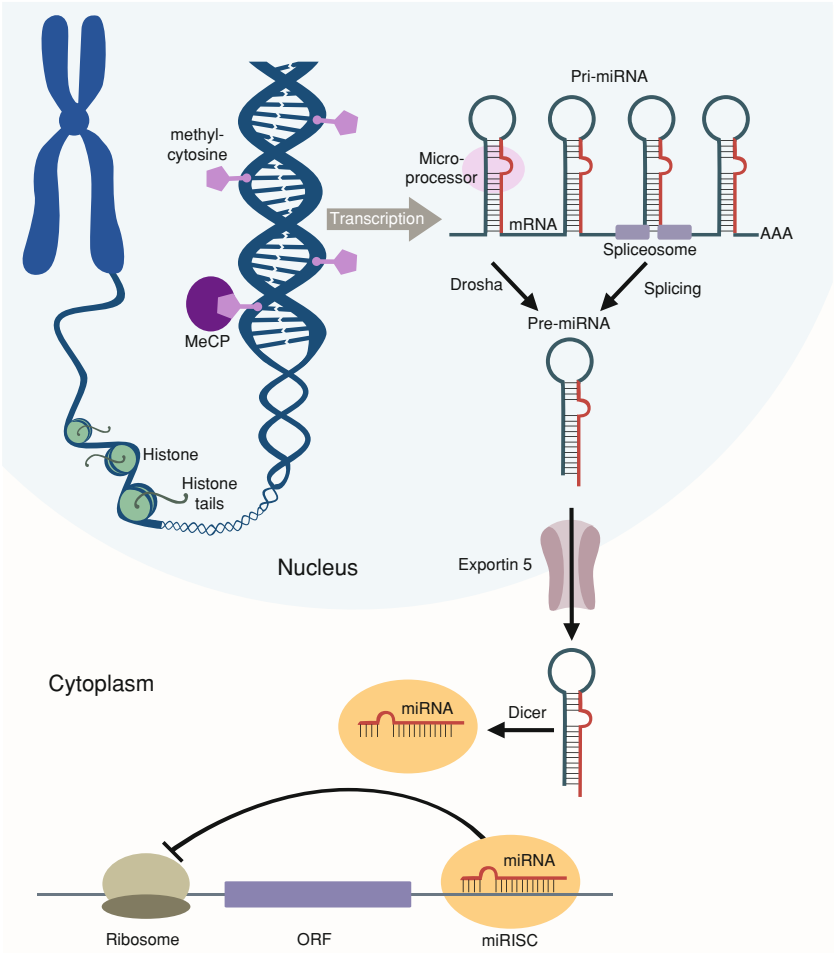

Fig. 2 Illustration of the main components of the three mechanisms described in this review. The DNA is wrapped around a complex of histone proteins to create the nuclesosome. Post-translational modifications of the core histones determine chromatin density and thus the genes' accessibility for transcription factors and enzymes. Methylation of DNA cytosines is also shown. Methylated cytosines can bind MeCP proteins that may induce histone modifications and chromtin remodeling. An "open" chromatin environment enables transcription of genes, including miR genes. miR genes are transctibed to long pri-miRNAs that are processed by the Drosha-including microprocessor complex or by splicing to stem-loop pre-miRNAs. Pre-miRNAs are exported from the nucleus to the cytoplasm, where Dicer catalyzes the release of mature miRNAs from the precursors. The mature miRNA, incorporated in the miRISC complex, can bind to partially complementary sites at 3′UTRs of target mRNAs and reduce their translation

Drosha-independent way to release pre-miRNAs (Ruby et al. 2007). Then, pre-miRNAs are exported from the nucleus to the cytoplasm, and Dicer1 excises the mature 16-29-nucleotide-long miRNA (average length = 22 nucleotides) from the pre-miRNA (illustrated in Fig. 2). Thus, Dicer1 is essential for the production of mature and functional miRNAs (reviewed in Bartel 2004; Carthew and Sontheimer 2009). Dicer1 is incorporated in a complex of proteins, and therefore the released mature miRNA is bound to this riboprotein complex, termed miRISC (miRNA-induced silencing complex). miRISC includes additional proteins that are required for miRNA binding and miRNA-induced regulation of gene transcription and mRNA translation, such as an argonaute (Ago) protein [Ago2/Eif2c2 is the common argonaute in the miRISC complex in mammals, but any of the other three argonautes may supersede it (Su et al. 2009)], TARBP2 (previously known as TRBP) (Chendrimada et al. 2005), GW182, and additional proteins (reviewed in Carthew and Sontheimer 2009).

Functions of miRNAs

miRNAs, incorporated in miRISC, regulate expression of genes that contain *cis* complementary sequence(s), most often in their 3′ untranslated region (3′UTR). The most established function of miRNAs is translational suppression of mRNAs with imperfect complementary sequences and cleavage of mRNAs with a perfect match (reviewed in Bartel 2004). It has been suggested that at least 60% of the vertebrate protein-coding mRNAs are direct targets of

miRNAs (Friedman et al. 2009c; Miranda et al. 2006) and that miRNAs affect, directly or indirectly, virtually all cellular and organismal processes.

In mammals, most of the target mRNAs for miRNAs contain imperfect complementary sequences in their 3′UTRs, and binding of a miRNA to this site suppresses the mRNA translation. When the target mRNA contains several binding sites for the same miRNA, its translation is more suppressed. However, sometimes miRNAs have the reverse effect and upregulate the translation of their target mRNAs. This may depend on the cell cycle stage, since two recent studies proposed that while miRNAs inhibit the translation of their target mRNAs in proliferating human cell lines, they enhance the translation of the same targets in quiescent cells (Vasudevan et al. 2007, 2008). Although in most cases miRNAs bind to complementary (or partial complementary) sequences at the 3′UTRs of their target mRNAs, miRNA binding sites were found also in other locations, such as 5′UTRs. The miRNA effect may depend on the location of its binding site. While translation repression is mediated by the binding of miRNAs to the mRNA 3′UTRs, a case in which miRNA binds to its target 5′UTR and upregulates its translation was recently reported (Orom et al. 2008).

miRNAs are associated with many developmental processes and diseases and have been found to be crucial for normal development in all animals tested (Zhang et al. 2007). They are differentially expressed in different tissues (Landgraf et al. 2007), and certain miRNAs are known as having a role in the development of specific organs and tissues (reviewed in Stefani and Slack 2008; Zhang et al. 2007). In mammals, miRNAs are also essential for early embryogenesis and viability, and *Dicer1* knockout mice arrest miRNA maturation and die at an early embryonic stage, before embryonic day 9.5 (E9.5) (Bernstein et al. 2003). In the zebrafish, Dicer and the production of miRNAs are required at later stages and, thus, *Dicer* knockout in whole zebrafish embryos enables the evaluation of miRNA importance for brain formation, somatogenesis, heart development, and general organogenesis (Giraldez et al. 2005).

Expression of miR-183 family miRNAs in animals

The study of miRNAs in the inner ear began only in 2005, when an atlas of in situ hybridization results in zebrafish embryos, demonstrating the expression of miRNAs, was published (Wienholds et al. 2005). Some of the miRNAs were found to be expressed in the inner ear of the zebrafish embryo. Three miRNAs (miR-182, miR-96, and miR-183) that share similar sequences are coexpressed in zebrafish embryos. Since their genes are clustered within 4 kb in the mouse and human genomes and are most probably transcribed together as a polycistronic gene, these three miRNAs are commonly referred to as the miR-183 cluster or family. Their expression in zebrafish embryos is most prominent in the hair cells of the inner ear and the lateral line neuromasts, and in the sensory cells of the eye and the nose, but they are also expressed in cranial ganglia and epiphysis. The zebrafish lateral line neuromasts contain sensory hair cells that are similar to inner-ear hair cells (Nicolson 2005). These miRNAs are also expressed in inner-ear hair cells and eye retina in chick embryos (Darnell et al. 2006).

The expression of the miR-183 family miRNAs in zebrafish embryos attracted the attention of inner-ear and eye researchers, who used in situ hybridization to detect the expression of these three miRNAs in the mouse inner ear and eye. Indeed, this triad of miRNAs was found to be expressed specifically in the inner-ear hair cells, in both the cochlea and the vestibule (Weston et al. 2006), and in the photoreceptors, bipolar cells, and amacrine cells in the eye retina (Ryan et al. 2006; Xu et al. 2007) of postnatal mice. A recent study (Sacheli et al. 2009) described the expression of these miRNAs along mouse inner-ear development, which begins around E9.5 when the otic vesicle (also termed otocyst) is invaginated from the otic placode. Along inner-ear development, miR-182 and miR-183 expression patterns are similar, while miR-96 is not always coexpressed with them. After birth (P0-P2), the expression of these miRNAs is reduced, and their cochlear expression is limited to the hair cells and the spiral ganglion. As the postnatal cochlea matures, the expression of these miRNAs gradually disappears from cochlear hair cells and moves to other cochlear tissues, the inner sulcus and the spiral limbus. Overall, the dynamic expression of these miRNAs in the developing mouse inner ear, with the higher expression level in differentiating hair cell, suggests that these miRNAs are associated with hair cell differentiation and maturation.

Further study revealed that the miR-183 family miRNAs are expressed in the hair cells of the inner ear and neuromasts of additional vertebrates and have orthologous miRNAs that are specifically expressed in neurosensory cells in invertebrate deuterostomes and in ciliated sensory cells of the fruit fly *Drosophila melanogaster*. However, the miR-183 orthologous miRNA in the nematode *Caenorhabditis elegans* may also be expressed in nonciliated glial or supporting cells in sensory organs (Pierce et al. 2008). Thus, miR-183 family miRNAs are considered to be specifically expressed in neurosensory organs, and in most cases in ciliated or stereociliated neurosensory cells. Although it is widely believed that miR-183 family miRNAs are expressed in postnatal mammals only in the inner ear and the retina, a recent report detected these miRNAs in several additional organs from adult mice, in particular, the submandibular gland (Jin et al. 2009).

Additional miRNAs expressed in the mouse inner ear

miRNA microarrays have been used to detect additional miRNAs in the whole mouse inner ear. Weston et al. (2006) used microarrays to profile the expression of pre-miRNAs or mature miRNAs in postnatal whole mouse inner ear at several ages from newborn to adult. The expression level of several miRNAs changed along inner-ear development. In a subsequent report, our group (Friedman et al. 2009a) used microarrays to compare the expression profiles of mature miRNAs in separated cochleae and vestibules from newborn mice. Because cochlear and vestibular sensory epithelia are similar but not identical, we hoped to identify miRNAs that may be responsible for some of these differences. However, most miRNAs exhibited similar expression levels in whole cochleae and whole vestibules, and the few that were expressed differentially showed only relatively small differences. The cochlea and vestibule are complex organs that include many different tissues and cell types, and the expression level detected for each miRNA was actually an average of its different expression levels in the different cell populations. This was emphasized by the detailed in situ hybridization expression analysis of several miRNAs (Friedman et al. 2009a). The differences in miRNA expression in whole inner ears along postnatal inner-ear development, reported by Weston et al. (2006), were also small, most probably for the same reasons. Overall, these microarray experiments demonstrated that more then 100 different miRNAs are expressed in the mouse inner ear.

Weston et al. (2006) proposed that if the miRNA population in embryonic inner ears is compared to the postnatal population, the differences may be greater. Although the newborn mouse inner ear is still not functional and continues to develop, most of inner-ear development does occur before birth during embryogenesis. To address this question, we selected six miRNAs [miR-15a, miR-18a, miR-30b, miR-99a, miR-199a-3p (also known as miR-199a), and miR-182] with different expression levels in the newborn whole cochleae and vestibules, according to microarray results, and followed their expression levels along inner-ear development from embryonic day 16.5 (E16.5) to postnatal day 30 (P30) by the more sensitive and accurate qRT-PCR assay (Friedman et al. 2009a). At P30, the inner ear is fully developed and functional. We found significant differences in the levels of each miRNA along development. The level of some miRNAs was highest in embryonic (miR-18a) or newborn (miR-99a, miR-199a-3p, and miR-182, a member of the miR-183 cluster described above) developing inner ears, while others peaked in the fully developed P30 inner ears (miR-15a and miR-30b). Differences were also found between cochlear and vestibular levels at the same age for some miRNAs at selected time points. Thus, the relatively low sensitivity and high background of microarrays may mask differences in miRNA levels that may be detected by qRT-PCR. The differences in temporal expression patterns suggest that each of these miRNAs has a different role in the inner ear. miRNAs that are highly expressed before birth may play a role in early inner-ear development; miRNAs that peak in the newborn inner ear (such as miR-182) may regulate the final differentiation of inner-ear tissues; while miRNAs with a higher expression level in P30 inner ears may play a role in cell maintenance and function. However, most miRNAs may play a role during inner-ear development because the miRNA populations in P100 inner ears (Weston et al. 2006) and P30 separated cochleae and vestibules (Friedman and Avraham, unpublished) are much smaller than the miRNA populations at P0.

Quantitative assays to determine miRNA levels in whole organs are not sufficient to understand the role of each miRNA. In complex organs like the cochlea and the vestibule it is very important to know the exact cells that express each miRNA. We showed, by in situ hybridization of the mouse newborn inner ear, that miRNAs with similar average expression levels (in whole cochleae and vestibules) are actually expressed in different cell types and, thus, may have different roles. For example, miR-182 and miR-199a-3p, which have similar temporal expression patterns in the developing mouse vestibule and cochlea and are very highly expressed in the newborn inner ear, are actually expressed in different cell types. While miR-182 is expressed exclusively in the inner-ear hair cells and the cochlear spiral ganglion neurons, miR-199a-3p is expressed in many other inner-ear tissues but not in these tissues. Although recently miR-199a-3p was reported as highly expressed in neuronal tissues in rats (Hua et al. 2009), its expression was not limited to the spiral or vestibular ganglia in our newborn mouse inner ears. Other miRNAs, with a lower expression level in the whole inner ear (i.e., miR-15a, miR-18a, miR-30b, and miR-99a), are expressed in many inner-ear tissues, including the sensory epithelia. In addition, the distribution of some miRNAs may be different in the cochlea and the vestibule. For example, miR-30b, which has a similar expression level in the newborn mouse cochlea and vestibule, is expressed in both cochlear hair and supporting cells, but in the vestibule it is expressed only in hair cells. We also found two miRNAs, miR-99a and miR-199a-3p, with a mirroring spatial expression, suggesting that these two miRNAs are involved in a negative control loop, most probably indirectly. All the miRNAs detected in inner-ear hair cells were also expressed in the spiral ganglion, the cochlear sensory nerve cells. This result is not surprising since hair cells are neurosensory cells that exhibit some neuronal-like characteristics. However, miR-18a, which is also expressed in

inner-ear hair cells, was found to have a higher expression level in the spiral (cochlear) ganglion (Friedman et al. 2009a). Weston et al. (2006) also studied the spatial expression pattern of several miRNAs by in situ hybridization in mouse P0 and P5 inner ears. In addition to the miR-183 family members, they published the spatial expression patterns of miR-100 and miR-124a, both of which were predominantly (miR-100) or exclusively (miR-124a) expressed in the spiral and vestibular ganglia. miR-124a is known as a neuronal miRNA (e.g., Lukiw 2007), and miR-18a was recently reported as playing a role in the rodent central nervous system (Uchida et al. 2008).

Conditional knockout of Dicer1 as a tool to study roles of miRNAs in the developing mouse inner ear

Dicer1 knockout mice die at an early embryonic stage, prior to inner-ear development (Bernstein et al. 2003). To study the roles of miRNAs in a specific tissue it is possible to develop viable offspring that do not express miRNAs specifically in this tissue by knocking out *Dicer1* conditionally (Harfe et al. 2005). Our group (Friedman et al. 2009a) and Soukup et al. (2009) used the *cre-loxP* recombination system to knock out *Dicer1* conditionally in the mouse developing inner ear. The $Dicer1^{flox}$ allele contains *loxP* sites at both sides of exon 24 of mouse *Dicer1* gene (Harfe et al. 2005). This exon encodes most of the second RNaseIII domain, which is crucial for the ability of Dicer1 to produce mature miRNAs from pre-miRNAs. The Cre recombinase cuts *loxP* sites and thus exon 24 of *Dicer1* is discarded from the genome only in cells that express the Cre recombinase. Each group expressed *Cre* under the regulation of a different promoter and blocked miRNA maturation in different inner ear cells and developmental stages, leading to different phenotypes.

Soukup et al. (2009) expressed the *Cre* gene under regulation of the *Pax2* promoter. *Pax2* is one of the earliest markers of the otic placode, from which both the inner ear and its sensory ganglia are developed. *Pax2-Cre* is expressed in all mouse otic placode cells from E8.5, and its expression in the developing inner ear continues beyond birth (Burton et al. 2004). In newborns *Pax2-Cre* is expressed in many inner-ear tissues, including the cochlear spiral ganglion, all cell types in the cochlear and vestibular sensory epithelia, and the other nonsensory epithelial tissues surrounding the cochlear scala media. It is also expressed outside the inner ear, mainly in the central nervous system and kidney (Ohyama and Groves 2004; Soukup et al. 2009). Thus, a conditional knockout of *Dicer1* under the regulation of the *Pax2* promoter (*Pax2-Cre/*$Dicer1^{flox/flox}$ or *Pax2-Cre Dicer* KO mice) prevents miRNA maturation in most inner-ear tissues, including the neurons of the inner-ear sensory ganglia, along their development from E8.5 (Soukup et al. 2009).

Pax2-Cre Dicer KO mice died in utero around E18.5, most probably due to CNS defects resulting from *Dicer1* knockout in several brain regions. Therefore, the hearing of these mice could not be evaluated. Nevertheless, defective development of their inner ears could be detected. Inner-ear development was severely flawed, and at E17.5 the cochlea was truncated and the anterior and horizontal cristae, as well as the horizontal semicircular canal connecting them, were missing. The saccule and utricle were also affected. Interestingly, *Dicer1* knockout reduced but did not prevent the development of inner-ear hair cells in this mouse. However, in the cochlea and utricle, the positions of kinocilia and the organization of stereocilia in developed hair cells were abnormal. This mouse contained an internal natural control since Cre was not expressed in part of the supporting cells in the vestibule's posterior crista. Indeed, the expression of miR-183 was completely or partially lost in all the sensory epithelia of the mutant inner ear, except for the posterior crista. While inner-ear development was severely damaged and anterior and horizontal cristae were missing, the posterior crista developed normally and its hair cells exhibited a normal organization of kinocilia and stereocilia. Although the afferent innervation of the inner-ear sensory epithelia began to develop normally, a short time after Cre expression it became severely defective, with a complete loss of posterior crista innervation, suggesting that the defective innervation of the vestibule resulted from the knockout of *Dicer1* in neurons of the inner-ear sensory ganglia rather than from the defective development of the sensory epithelia. However, in the cochlea, loss of hair cell miRNAs may contribute to decreased innervation since mutant hair cells expressed less Bdnf than wild-type hair cells (Soukup et al. 2009). Release of the neurotrophin Bdnf from hair cells is known to be crucial for guiding their afferent innervation (Agerman et al. 2003). Additional findings in the *Pax2-Cre Dicer* KO mouse suggest that the absent development of the anterior and horizontal cristae, and of the semicircular canal connecting them, may derive from loss of Fgf10 expression (Soukup et al. 2009).

Friedman et al. (2009a) expressed the *Cre* gene under the regulation of the *Pou4f3* promoter. In the inner ear, *Pou4f3* is expressed only in hair cells and only after their initial differentiation. Thus, it is expressed in vestibular hair cells from E12.5 and in cochlear hair cells from E14.5 (Xiang et al. 1998). Indeed, *Pou4f3-Cre* expression in the inner ear is limited only to the sensory epithelia, including the cochlear and vestibular hair cells, and ectopic expression in some cochlear supporting cells (Sage et al. 2006). In addition, *Pou4f3-Cre* expression was detected in many additional organs in the mouse. Thus, a conditional

knockout of *Dicer1* under the regulation of *Pou4f3* promoter [*Pou4f3-Cre/Dicer1*$^{flox/flox}$ or *Dicer-PCKO* (*Pou4f3-Cre*-induced Conditional Knock-Out) mice] prevents miRNA maturation in the inner ear only from a relatively late developmental stage, specifically in the inner-ear sensory epithelia cells after their initial differentiation. *Dicer-PCKO* mice also expressed Cre in many organs outside the inner ear, leading to several abnormalities in addition to the inner-ear defects. Perinatal death was common; however, some *Dicer-PCKO* mice survived. Therefore, it was possible to measure their hearing and follow the phenotype that developed in their inner ears (Friedman et al. 2009a).

Unlike *Pax2-Cre Dicer* KO mice that exhibited defective embryonic development of the inner ear and its innervation (Soukup et al. 2009), *Dicer-PCKO* mice were born with apparently normal inner ears. However, cochlear hair cells were gradually malformed and degenerated after birth, and the mice were totally deaf at P38. While the hair cell stereocilia were normally organized in newborn mutants, P38 *Dicer-PCKO* were totally deaf and some cochlear hair cells lost their stereocilia, while others exhibited disorganized stereocilia and fusion of adjacent stereocilia to form heavy projections (Fig. 3) (Friedman et al. 2009a). Interestingly, all utricular hair cells of *Dicer-PCKO* mice survived at P38 but displayed abnormal organization of stereocilia, resembling the utricular hair cells of *Pax2-Cre Dicer* KO mice (Friedman and Avraham, unpublished), suggesting that hair cell miRNAs play a role

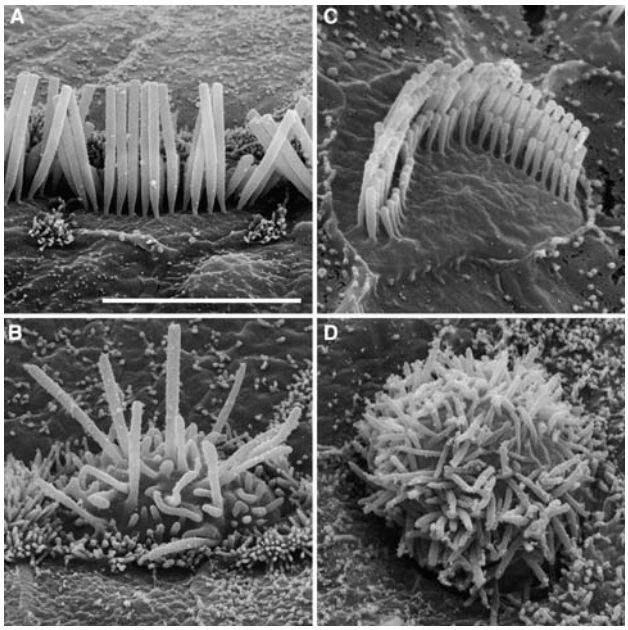

Fig. 3 High-resolution scanning electron microscopy of inner-ear hair cell bundles from inner (**a, b**) and outer (**c, d**) hair cells derived from wild-type (**a, c**) and *Dicer-PCKO* mutant (**b, d**) mice. Scale bar = 5 μm

in development and maintenance of stereocilia. Numerous factors are known to affect stereocilia development and survival in inner-ear hair cells, and the expression of some of them may be regulated by miRNAs. While *Dicer1* was knocked out only in hair cells in the vestibule of *Dicer-PCKO* mice, it was knocked out in both supporting and hair cells in the cochlea (Sage et al. 2006). The massive degeneration of hair cells in the cochlea but not in the utricle of *Dicer-PCKO* mice suggests that miRNAs in supporting cells are required for survival of hair cells (Friedman et al. 2009a).

While *Pax2-Cre Dicer* KO mice showed that miRNAs are required for the proper development of the mouse inner ear and for its normal innervation, *Dicer-PCKO* mice proved that miRNAs are also required for survival of functional hair cells in the inner ear after normal development. Therefore, these two mutant mice produce complementary data regarding the role of miRNAs in the mammalian inner ear.

Inner ears of *Pax2-Cre Dicer* KO mice had a prominent abnormal phenotype at E17.5, 9 days after the beginning of Cre expression (Soukup et al. 2009), while *Dicer-PCKO* mice inner ears appeared normal 9 days after the beginning of Cre expression (newborns) (Friedman et al. 2009a). To explain the delay in phenotypic development, we proposed that mature miRNAs produced before the beginning of Cre expression, or Dicer transcribed before Cre expression, may survive and function in inner-ear sensory epithelia for an unknown time in *Dicer-PCKO* mice (Friedman et al. 2009a). In contrast, *Pax2-Cre Dicer* KO mice display a complete depletion of mature miR-124a 3 days after the start of Cre expression in sensory neurons, and a defective innervation of the inner ear is observed a day later. However, in the same mouse, some hair cells still express miR-183 at least 6 days after *Dicer1* is knocked out, suggesting that the rate of miRNA or Dicer depletion may be variable in different cell types (Soukup et al. 2009). Such depletion may be more rapid in proliferating or rapidly growing cells than in differentiated quiescent cells due to dilution of the residual miRNAs. Alternatively, the effect of miRNA loss after the development of inner-ear sensory epithelia may be slower than such loss during early inner-ear development.

Mutations in MIR96 gene linked with hearing loss: roles of miR-96 in the inner ear

The miR-183 family of miRNAs is considered to be expressed specifically in the inner-ear hair cells and the eye retina in mammals (Ryan et al. 2006; Sacheli et al. 2009; Weston et al. 2006; Xu et al. 2007). The mature miRNAs produced from these three genes are very similar (Fig. 4). During the last year, two groups linked point mutations in

the seed region of the *Mir96* gene with autosomal dominant progressive NSHL in humans (Mencia et al. 2009) and mice (Lewis et al. 2009). This is the first (and thus far, only) example of a mutation in a single Mir gene that is linked with a human inherited phenotype.

In human, two different mutations of two sequential nucleotides (+13G>A and +14C>A) of the *MIR96* gene segregated with dominant progressive hearing loss in two unrelated Spanish families (locus DFNA50 on 7q32). These mutations affect the fifth and sixth nucleotides in the mature miR-96 and are included in its seed region (Fig. 4). The hearing loss in the two families was not identical. While in the first family (+13G>A) all frequencies were similarly affected, the second family (+14C>A) presented a more severe hearing loss at high frequencies. These mutations significantly impaired the biogenesis of mature miR-96 or increased its degradation, since the level of the mature mutated miRNA was approximately 20% from the wild-type miR-96 level in transfected cells. In addition, these mutations changed the target mRNA population, and the mutated miR-96 had a lower ability to downregulate the translation of several mRNAs that are targeted by wild-type miR-96. Other substitutions in the pre-miRNA sequences of *MIR96* and *MIR182* genes, outside the mature miRNA sequences, were found in 10 and 22 families, respectively, but did not segregate with the hearing loss phenotype (Mencia et al. 2009).

N-ethyl-*N*-nitrosourea (ENU) has been used to induce random mutations in mice (Acevedo-Arozena et al. 2008). The diminuendo (*Dmdo*) mouse carries an ENU-induced A>T point mutation in the *Mir96* seed, at the seventh nucleotide of the mature miR-96 (Fig. 4). Homozygotes exhibited deafness and a vestibular phenotype from an early age. Their hair cell stereocilia were already abnormally arranged at P4-P5, and massive hair cell degeneration began a few days later. Heterozygotes presented a progressive hearing loss that became severe at the age of 4-6 weeks, although their sensory epithelia still included many hair cells at this age. Their hair cells exhibited normally arranged stereocilia at P5, but by P28 the stereocilia began to lose their normal organization. At 4-6 weeks, part of the hair cells were degenerated, particularly in the basal coil of the cochlea that is responsible for sensing high-frequency sounds (Lewis et al. 2009). A more prominent hair cell degeneration was also found in the basal coil of the cochlea of the *Dicer-PCKO* mice described above (Friedman et al. 2009a). However, *Dmdo* heterozygotes presented more severe stereocilia disorganization and fusion in the vestibule (Lewis et al. 2009) than did *Dicer-PCKO* mice (Friedman and Avraham, unpublished). These results suggest that miRNAs in general, and miR-96 in particular, are essential for survival of functional hair cells in the mammalian cochlea after these hair cells are normally developed, and that basal cochlear hair cells are more sensitive to loss of miRNAs than apical cochlear hair cells.

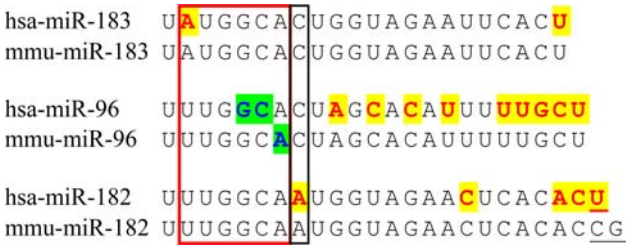

Fig. 4 Sequences of human and mouse mature miRNAs from the miR-183 family. Seeds are surrounded by the red (nucleotides 2-7) and black (nucleotide 8) squares. Sequence differences between human miRNAs are shown as red bold letters on a yellow background. Sequence differences between human and mouse miRNAs are shown as underlined letters in the mouse sequences. The miR-96 nucleotides that are mutated in DFNA50-linked hearing loss in humans and in diminuendo mice are labeled in blue bold letters on a green background. The sequences are as in miRBase release 13.0

The *Dmdo* mouse serves as a useful model to study the role of miR-96 in the mammalian inner ear in vivo. The mutant miR-96, as well as miR-182 and miR-183, is expressed in *Dmdo* inner-ear hair cells (Lewis et al. 2009). In the two articles describing *Mir96* seed mutations (Lewis et al. 2009; Mencia et al. 2009), five mRNAs were identified as miR-96 potential targets, and their translation was inhibited more efficiently by wild-type miR-96 than by mutant miR-96 in non-inner-ear cell lines. However, these five protein-coding genes were similarly expressed or exhibited only a slight expression difference in inner-ear sensory epithelia from wild-type and *Dmdo* mice (Lewis et al. 2009). Yet, protein expression detection by immunohistochemistry may not be sensitive enough to detect small expression differences, and miR-96 may finely tune the expression level of these putative targets.

Another approach to studying miR-96 roles in the inner ear used microarrays to compare mRNA expression in wild-type and *Dmdo* inner ears. It was commonly thought that most miRNAs in animals do not induce degradation of the target mRNA but rather block mRNA translation, but recent studies suggest that they can indeed degrade targets (Baek et al. 2008; Selbach et al. 2008). Indeed, in the population of mRNAs that are overexpressed (upregulated) in the *Dmdo* inner ears compared to wild-type inner ears, mRNAs with binding sites for miR-96 were enriched, suggesting that miR-96 induces degradation of many targets in parallel. Moreover, the mutated miR-96 in *Dmdo* homozygotes downregulated new targets with complementary sequences to the mutated seed that are not regulated by wild-type miR-96. mRNA microarrays also detected indirect effects of the miRNA. Dozens of mRNAs without miR-96 binding sites were up- or downregulated in

Dmdo inner ears compared to wild-type inner ears. These indirect effects included reduced expression of oncomodulin (*Onc*), *Ptprq*, prestin (*Slc26a5*), and *Gfi1* mRNAs in the *Dmdo* inner ears (Lewis et al. 2009). Oncomodulin is known to be expressed in inner-ear hair cells (Sakaguchi et al. 1998), and *Ptprq* is required for the development of normal stereocilia (Goodyear et al. 2003). Prestin (Liberman et al. 2002; Liu et al. 2003) and *Gfi1* (Hertzano et al. 2004) are known to have important roles in the function of inner-ear hair cells and are linked with hair cell degeneration and hearing loss in humans or mice.

miR-96 may indirectly recruit epigenetic mechanisms by its direct or indirect effects on mRNA expression. For example, Gfi1 is a transcription factor that may induce histone modifications [for details, see the Chromatin Remodeling section below and Duan et al. (2005); McGhee et al. (2003)]. The higher expression of *Gfi1*, induced indirectly by wild-type miR-96, may suggest that miR-96 indirectly induces histone modifications and regulates the transcription of specific genes. Indeed, binding sites for Gfi1 were found in the promoters of many of the genes upregulated in the *Dmdo* inner ears (Lewis et al. 2009). Induction of histone modifications by miR-96 was shown in the mantle cell lymphoma cell line (Pal et al. 2007).

The seed sequence (nucleotides 2-8) of the mature miRNA determines which mRNAs may be regulated by this miRNA. If the first base of the target site on the regulated mRNA is adenosine (A), complementation of nucleotides 2-7 of the mature miRNA to the target site is considered essential and sufficient for target regulation. A match of the eighth base increases the affinity of the miRNA to its target and adds a higher level of specificity, but target sites without a match in their eighth nucleotide can still be regulated by the miRNA (Lewis et al. 2005). The seed sequence of miR-182 differs from the miR-96 seed only in the eighth nucleotide, while the miR-183 seed differs also in the second nucleotide (Fig. 4). Therefore, miR-96 and miR-182 most probably share most of their targets, although each may also have a subset of specific targets that are not regulated by the other miRNA [compare the targets predicted for these miRNAs by the TargetScanHuman algorithm, release 5.1, April 2009: http://www.targetscan.org/; this algorithm includes additional determinants in addition to those described above (Friedman et al. 2009c; Grimson et al. 2007)]. Thus, theoretically, miR-182 may partially compensate for the loss of miR-96 and vice versa. In a recent review, Soukup (2009) proposed that the miR-183 family miRNAs, including miR-96, may downregulate the expression of supporting cell markers and thus induce the hair-cell phenotype. The author mentioned unpublished data that support the hypothesis that *Sox2* mRNA is a direct target of miR-182. However, the progressive nature of hearing loss in both humans and mice heterozygous for *Mir96* seed mutations, with normal hearing at young ages, suggests that high levels of wild-type miR-96 are not required for the late development of inner-ear hair cells, but rather are essential for survival of functional hair cells. Since miR-182 may compensate for part of the loss of miR-96 function in mice and humans with *Mir96* mutations, miR-183 family miRNAs may yet have a role in the initial differentiation of hair cells.

Recently, knocking out *Mir182* in mice resulted in an apparently normal phenotype, without any eye defect (Jin et al. 2009); however, the authors did not examine the inner ears. In this case, only the *Mir182* gene was deleted and the mice still expressed wild-type *Mir96* and *Mir183*. Accordingly, *Mir96* seed mutations were not reported as responsible for any eye abnormalities, although they induced hearing loss (Lewis et al. 2009; Mencia et al. 2009). Therefore, miR-182 and miR-96 may not be essential in the mouse eye or may compensate for the absence of each other, at least in the eye. On the other hand, *Mir96* seed mutations induced progressive hearing loss in humans and mice as well as stereocilia malformation and hair cell loss in mice, although the other cluster genes (*Mir182* and *Mir183*) were not mutated. While a redundancy gene, such as *Mir182*, may compensate for reduced biogenesis and loss of function of the mutated gene, it cannot compensate for gain-of-function mutations. *Mir96* seed mutations induce reduced biogenesis of miR-96 in humans (Mencia et al. 2009) and may reduce regulation of mRNAs that are targeted by wild-type miR-96 in both humans and mice. These effects may be partially compensated by miR-182. However, miR-96 seed mutations may also serve as gain-of-function mutations because the mutated miRNAs may downregulate mRNAs that are not targeted by wild-type miR-183 family miRNAs (Lewis et al. 2009; Mencia et al. 2009). Theoretically, such a gain of function cannot be compensated by miR-182. Actually, such a gain of function was reported in mice (Lewis et al. 2009). From the data published thus far it is not clear whether the miR-183 family miRNAs are less essential for the eye than for the inner ear, or, alternatively, if knockouts of the whole Mir gene are less harmful than seed mutations due to the compensation by other miR-183 cluster members.

Clues for roles of additional miRNAs in the vertebrate inner ear

To understand the function and role of a miRNA, its target mRNAs should be identified, but these targets are not yet known in most cases. Since miRNA binding to its target does not require a perfect complementation, the identification of miRNA targets is not trivial. Some clues about

the identification of miRNA targets have been published (Doench and Sharp 2004; Enright et al. 2003; John et al. 2004; Kertesz et al. 2007; Kiriakidou et al. 2004; Lewis et al. 2003, 2005; Stark et al. 2003), and computational algorithms to identify potential miRNA targets in mammals, including human, have been developed [e.g., TargetScan (Lewis et al. 2003), PicTar (Krek et al. 2005), miRanda (John et al. 2004), PITA (Kertesz et al. 2007)]. However, such bioinformatics-based predictions, which look mainly for conserved 3′UTR sequences that are complementary to the studied miRNA seed, yield hundreds of targets for each miRNA; it is still challenging to predict which are the best targets to pursue experimentally.

miRNAs can regulate the translation of coexpressed mRNAs. However, not all the predicted targets are coexpressed with the miRNA in the same cell at the same time. To select putative targets for further study, we used microarrays to profile the mRNAs expressed in the mouse cochlear and vestibular sensory epithelia at an early postnatal age (Friedman et al. 2009a). Then we selected miRNAs that are expressed in the inner-ear sensory epithelia, according to in situ hybridization experiments, and used several target prediction algorithms to look for putative targets that are also expressed in inner-ear sensory epithelia. Consequently, we selected three putative targets for miR-15a in the inner-ear sensory epithelia: *Slc12a2*, *Cldn12*, and *Bdnf* mRNAs, and confirmed experimentally in a non-inner-ear cell line that miR-15a can downregulate the expression of the translated proteins. Thus, the unique spatial and temporal expression pattern of each inner-ear miRNA may help to reveal its targets and roles.

Seed sequences of miRNAs are conserved in mammals and zebrafish, and their functions may be conserved as well. The zebrafish is good for developmental and genetic studies in vertebrates because of its short generation time, external fertilization, large number of transparent accessible embryos, and remarkably rapid embryogenesis. An individual miRNA can be knocked down easily in the zebrafish embryo by injecting antisense morpholino-modified oligonucleotides against the mature miRNA or pre-miRNA into one-cell-stage embryos (the fertilized eggs). Anti-miRNA morpholinos can specifically inhibit the formation of mature miRNAs or their activities for at least 3 days. During this period the inner-ear sensory epithelia begin to develop. Thus, the knocking down of a specific individual miRNA in the zebrafish embryo may be used to discover the roles of this miRNA in inner-ear development. Zebrafish embryos were used to expose the roles of miR-15a and miR-18a in inner-ear development. These miRNAs were selected because they are expressed in the mouse inner ear, in both sensory epithelia and additional tissues. In the zebrafish, each miRNA had a distinct spatial expression pattern and their inhibition led to different phenotypes. Knocking down miR-15a interfered with the development of the canal pillars from which the semicircular canals develop, while knocking down miR-18a interfered with the development of the anterior otolith, a gelatinous matrix above one of the inner-ear sensory epithelia. Both miRNAs support sensory epithelia development, since hair cell counts decreased following their reduction. Thus, miR-15a and miR-18a were found to be essential for the early development of the inner ear and its sensory epithelia in zebrafish (Friedman et al. 2009a).

Alternative splicing can result with several different mRNAs from the same protein-coding gene. This mechanism is also used to create transcripts with different 3′UTRs, which may include different miRNA binding sites and thus are regulated by different miRNAs. Comparison of the relative expression of such transcripts along the inner-ear development may shed light on the roles of miRNAs in sensory epithelia differentiation and maturation. An example of alternative transcripts that are differently regulated by miRNAs came from work on the human *COL9A1* gene, which is expressed in the fetal cochlea. A *COL9A1* transcript with a new 3′UTR, which contains a conserved binding site for miR-9, was identified in the sensory epithelium and in additional tissues of the human fetal cochlea (20 weeks gestational age), suggesting that miR-9 may have a role in regulating the translation of this transcript (Sivakumaran et al. 2006).

Identification of miRNAs that are involved in hair cell regeneration in animals that naturally regenerate hair cells after inner-ear damage, such as fish, amphibians, and birds, may help to identify regulatory points to which therapeutics may be targeted in order to force hair-cell regeneration in mammalian inner ears. Newts can regenerate inner-ear hair cells, after their loss due to aminoglycoside antibiotic treatment, by transdifferentiation of supporting cells. The first step in hair cell regeneration is dedifferentiation of terminally differentiated supporting cells. Members of the let-7 family of miRNAs were found to be downregulated during this process (Tsonis et al. 2007). The expression of many additional inner-ear miRNAs is changed during hair cell regeneration.

The eye and the inner ear appear to share similarities with respect to miRNAs. The miR-183 family miRNAs are expressed in both the inner-ear hair cells and the eye retina in mammals (Ryan et al. 2006; Xu et al. 2007). Additional miRNAs, including miR-181a, miR-184, and miR-124a, that are highly expressed in the mouse inner ear are also highly expressed in the developing mouse eye (Karali et al. 2007). A comparative study of the roles of these miRNAs in these two sensory organs may help us understand their evolutionary development.

Finally, studies from other systems can serve as a guide in deciphering the roles of inner-ear miRNAs. Some are

upregulated in cancers and may induce proliferation and angiogenesis [e.g., the miR-17-92 cluster that includes miR-18a (Mendell 2008)], while others are downregulated following exposure to carcinogens [miR-99a (Izzotti et al. 2008) and miR-199a-3p (Kalscheuer et al. 2008)]. miR-15a and miR-199a-3p are known to have anticarcinogenic and apoptotic effects (Cimmino et al. 2005; Rane et al. 2009), but in other cases miR-199a was linked with carcinogenesis (Garzon et al. 2008; Lee et al. 2008). However, for most miRNAs expressed in the mouse inner ear, a role in normal development and function has not been extensively studied. miR-15a has been linked to pancreas regeneration (Joglekar et al. 2007). miR-18a is downregulated during differentiation of chondrocytic cells to bone cells (Ohgawara et al. 2009). Both miR-18a and miR-124a downregulate the translation of the glucocorticoid receptor and were linked to the response of neurons to stress (Vreugdenhil et al. 2009). miR-199a-3p was reported to regulate the response of cardiac myocytes to hypoxia (Rane et al. 2009). A knockout of the Mir199a-2 cluster, induced by replacement of its gene (*Dnm30s*) by a *lacZ* gene, affected normal skeletal development and body and muscle growth. The inner ears of these mice have not been examined thus far (Watanabe et al. 2008).

DNA methylation and 5-methyl-cytosine spontaneous deamination

Methylation of DNA at position 5 of the cytosine ring is catalyzed by DNA methyltransferases, and 5-methyl-cytosines comprise 1-6% of the nucleotides in the mammalian genome. CpG dinucleotides are preferred substrates of DNA methyltransferases, and in the mammalian genome cytosine methylation occurs at most CpG dinucleotides (Lister and Ecker 2009). 5-Methyl-cytosines in the DNA are mutational hotspots and therefore genetically unstable due to the high rate of spontaneous [or mutagenic-induced (Pfeifer 2006)] deamination of this base to thymine and ammonia, resulting in a G/T mismatch. While deamination of unmethylated cytosine creates uracil, which is recognized and removed from the DNA by uracil DNA glycosylase, a C→T mutation is not easily recognized and repaired, leading to depletion of methylated CpG sequences from the genome (Walsh and Xu 2006). Therefore, CpG sequences are rarer than expected in the genome. Nevertheless, clusters of CpG sequences, termed CpG islands, appear in the genome, particularly in promoter regions of protein-coding genes, and sometimes flank the first exon. Approximately half of the human protein-coding genes contain CpG islands in their promoters. Although most of the CpG dinucleotides in the mammalian genome are methylated, methylation of CpG islands is rare and strictly regulated (Cross and Bird 1995). Cytosine methylation of CpG islands in gene promoters represses gene transcription by inhibition of transcription factors binding to the methylated promoter, by recruitment of methyl-CpG-binding proteins (MeCP), or by inducing histone modifications and chromatin remodeling to a more condensed chromatin, leading to interference with transcription initiation or elongation (reviewed in Colot and Rossignol 1999; Klose and Bird 2006). The methylation level of CpG islands in some gene promoters changes during embryogenesis, development (e.g., Ginder et al. 2008), aging, and disease (e.g., Richardson 2002; Wilson et al. 2007), and thus may have an important role as an epigenetic marker that silences transcription in specific cell types and developmental stages. DNA hypermethylation and histone modifications also cooperate to induce imprinting, silencing of specific genes and loci from maternal or paternal origin, and inactivation of one of the two X chromosomes in mammalian female cells (reviewed in LaSalle 2007). Cytosine methylation is important not only for regulation of gene expression but also for maintenance of DNA stability because it normally occurs mainly in areas of repeated DNA elements, including transposons, endogenous retroviruses (mainly LTRs), retrotransposons (including LINEs and SINEs), simple short repeats arranged in tandem (termed DNA satellites), and additional repetitive sequences. At these sites, methylation prevents transcription of the transposable elements, induces C→T mutations that destroy many transposons, and induces chromatin conformation that prevents chromosomal rearrangements and facilitates normal replication (Dillon and Festenstein 2002; Hassan et al. 2001; Vilain et al. 2000).

Although cytosine methylation has important consequences, the identification of all the methylated cytosines in the genome became technically possible only recently (reviewed in Lister and Ecker 2009). A systematic study of cytosine methylation in inner-ear tissues has not been performed yet, and aberrant DNA methylation has not been found to be associated with NSHL. Recently, the promoter region of *DFNA5*, a NSHL-related gene (Van Laer et al. 1998), was found to be methylated in several cancers, leading to its silencing (Akino et al. 2007; Kim et al. 2008a, b). *DFNA5* is ubiquitously expressed, although mutations in *DFNA5* were linked to autosomal dominant progressive NSHL without any additional phenotype. All of the deafness-linked *DFNA5* mutations result in the skipping of exon 8, suggesting a specific gain-of-function effect. Indeed, transfection of mammalian cells with *DFNA5* led to cell death (Van Laer et al. 2004, 2007), while knockout mice for this gene displayed normal hearing but with an abnormal number of hair cells (Van Laer et al. 2005). Nevertheless, aberrant methylation of this gene has been studied only in cancers thus far, and it is unknown

if cytosine methylation of the *DFNA5* promoter plays a role in the inner ear and hearing.

Aberrant CpG methylation has been linked to a few inherited syndromes that include hearing loss (reviewed in Provenzano and Domann 2007). Although genes that control cytosine methylation are expected to be expressed and crucial in all the mammalian body cells, surprisingly, mutations in such genes do not affect all body systems and they may induce different phenotypes. There are two syndromes that involve CpG methylation and include sensorineural hearing loss, at least in some patients.

Stickler syndrome type I (STL1; OMIM #108300) may involve deamination of methylated cytosines (Wilkin et al. 2000). This syndrome (Stickler et al. 1965) includes progressive sensorineural hearing loss, progressive myopathy, blindness due to vitreoretinal degeneration and retinal detachment, premature degenerative changes in various joints with abnormal epiphyseal development, vertebral abnormalities, osteoarthritis, and sometimes unusual facial features and cleft palate. Stickler syndrome type I derives from mutations in the *COL2A1* (collagen type II, alpha-1) gene (reviewed in Donoso et al. 2003). COL2A1 is an important component of the inner ear's tectorial membrane, the eye's vitreous, and cartilage. Since the developing inner ear has a cartilage cover, which plays an important role in its embryogenesis, *COL2A1* mutations affect not only the inner ear's tectorial membrane but also its global structure and development (Berggren et al. 1997; Maddox et al. 1998). Many different dominant mutations in the *COL2A1* gene have been linked with Stickler syndrome type I thus far. Most of them are point mutations resulting in a premature TGA stop codon (e.g., Ahmad et al. 1993; Freddi et al. 2000) or deletions of single nucleotides resulting in a frameshift and a premature TGA stop codon (e.g., Ahmad et al. 1995; Brown et al. 1992; Freddi et al. 2000; Ritvaniemi et al. 1993); however, point mutations responsible for replacement of a single amino acid by another or longer deletions were also reported. Because this syndrome is characterized by extensive intrafamilial and interfamilial phenotypic variability, epigenetic regulation was proposed. Due to the relatively large incidence of CGA-to-TGA point mutations in Stickler syndrome type I, Wilkin et al. (2000) proposed that these highly mutated sites are methylated cytosines that are deaminated to thymines. To evaluate their hypothesis, they sequenced the ten in-frame CGA codons in *COL2A1* in 40 unrelated Stickler syndrome type I patients. Twenty percent of the patients exhibited C-to-T transition in five different CGA codons, leading to a premature TGA stop codon. Four of these patients also exhibited sensorineural hearing loss (Wilkin et al. 2000). These results suggest that methylated CGA codons are mutational hotspots in the *COL2A1* gene. Thus, circumstantial evidence supports the theory that deamination of methylated cytosines is responsible for a significant fraction of Stickler syndrome type I cases.

Aberrant hypomethylation of a specific DNA repeat array may be part of facioscapulohumeral muscular dystrophy type 1A (FSHMD1A; OMIM #158900). The most prominent phenotype of this autosomal dominant syndrome is muscle weakness due to dystrophy, typically beginning in face, shoulder, and upper-arm muscles but can descend later to hip and leg muscles. This syndrome also includes, as integral parts, sensorineural hearing loss and abnormalities of retinal vessels, although these phenotypes do not appear in all patients. In most cases, hearing is lost only at high frequencies (mainly between 4000 and 6000 Hz), but in some cases the hearing loss is progressive and with time lower frequencies may be also affected (Brouwer et al. 1991; Voit et al. 1986). The exact mechanism for sensorineural hearing loss in this syndrome is not yet clear. FSHMD1A is characterized by DNA hypomethylation and contraction of the polymorphic *D4Z4* tandem repeat array at the subtelomeric human chromosomal locus 4qter (4q35). This array contains many repeats of the *D4Z4* sequence, 3.3 kb in size. Each repeat contains a single open reading frame, *DUX4*, encoding a putative double homeobox gene (reviewed in Tawil 2008), but *DUX4* transcription has not been detected yet in standard cDNA libraries. While healthy subjects exhibit 11-100 *D4Z4* repeats in both chromosome 4qter loci, FSHMD1A patients carry a shorter array of 1-10 units on one of their 4qter. Similar contractions of a homologous *D4Z4* array in the subtelomeric region of chromosome 10 are not pathogenic (Lemmers et al. 2002), but an ectopic recombination between chromosome 4 and chromosome 10 arrays was suggested as being responsible for chromosome 4 *D4Z4* array contraction (van der Maarel et al. 2000; van Deutekom et al. 1996). The severity of FSHMD1A syndrome is related not only to the length of chromosome 4 *D4Z4* array but also to its methylation level, with a more severe phenotype and earlier onset in patients displaying a more prominent *D4Z4* hypomethylation and array sizes of 10-20 kb (i.e., < 6 repeats) (van Overveld et al. 2003, 2005). It has been proposed that contraction of the chromosome 4 array leads to its demethylation and that hypomethylation rather than array shortening is responsible for the phenotype since the array contraction is not sufficient to induce the syndrome (Lemmers et al. 2002), and affected individuals that carry the same size deletion, even within the same family, can exhibit different phenotype severities (Tupler et al. 1996). Moreover, the FSHMD1A-like phenotype appears in individuals that exhibit hypomethylation of the *D4Z4* array in chromosome 4 without array contraction (van Overveld et al. 2003). A 10-20-kb polymorphic segment exists immediately distal to the D4Z4 array at 4qter, and two possible alleles (A and B) with almost equal

distributions were reported for this segment. However, only allele A was found distally to contracted chromosome 4 *D4Z4* arrays in FSHMD1A patients, while asymptomatic individuals with chromosome 4 *D4Z4* array contractions displayed both alleles (Lemmers et al. 2002). Therefore, an epigenetic mechanism involving *D4Z4* hypomethylation that controls or is regulated by an unknown gene in allele A may be suggested. However, the contribution of this distal DNA segment to the FSHMD1A phenotype is not yet understood, and the reason for *D4Z4* hypomethylation in FSHMD1A has not yet been elucidated.

Chromatin remodeling

A role for histone acetylation in regeneration and loss of inner-ear hair cells

The nucleosome consists of approximately 147 bp of DNA wrapped around a histone octamer, consisting of two copies each of the core histones H2A, H2B, H3, and H4. Dozens of different post-translational modifications of histone proteins are known, including acetylation, methylation, phosphorylation, and ubiquitination of their tails or cores. In addition, two of the histones (H2A and H3) can be replaced by different variants. These modifications alter chromatin structure and the organization of the nucleosome, serve as docking sites for recruitment of chromatin-associating proteins, affect the DNA accessibility for DNA-binding enzymes and proteins, and, as a result, affect gene transcription and regulation. Acetylation of histone tails is particularly enriched around promoter and transcription start site (TSS) regions of genes, which are thought to regulate transcriptional initiation. Acetylated histones are linked with a "relaxed" chromatin configuration and increase the DNA accessibility for transcription or for binding of additional proteins (reviewed in Bhaumik et al. 2007; Jiang and Pugh 2009).

The sensory epithelia of the newborn mouse inner ear retain many progenitor cells, which compose a subpopulation of the supporting cells. These progenitor cells can proliferate and transdifferentiate into new hair cells in vitro and may compensate for lost hair cells in vivo. Unfortunately, the ability to regenerate hair cells declines sharply a few days after birth, and the adult murine inner ear has no such ability in the cochlea and only limited regeneration ability in the vestibule (Gu et al. 2007; Savary et al. 2007). Primary organ cultures of the utricle, one of the five sensory epithelium patches of the vestibule, are commonly used to study vestibular hair and supporting cells. Inhibitors of histone deacetylases increase histone acetylation and gene transcription and are known to inhibit proliferation and cell cycling in many cell types. Recently, it was suggested that histone deacetylation is required for proliferation of utricular supporting cells from mouse (Lu and Corwin 2008) and bird (Slattery et al. 2009) inner ears.

Sodium butyrate, an inhibitor of histone deacetylases, sharply decreases the proliferation rate of supporting cells cultured from mouse P3 utricles (Lu and Corwin 2008). The transcription factor Snail can recruit histone deacetylases and corepressor Sin3A to target promoters and thus downregulate expression of target genes (Peinado et al. 2004). Indirect evidence suggests that Snail may downregulate E-cadherin (Cdh1) expression in mouse utricular supporting cells by inducing histone deacetylation of the *Cdh1* promoter. Reduction of Snail level and upregulation of E-cadherin accompanied the loss of ability of utricular supporting cells to proliferate, perhaps due to enhanced histone acetylation at the *Cdh1* promoter (Lu and Corwin 2008). However, additional sites of histone deacetylation may be crucial for maintenance of progenitor cell phenotype in the mouse inner ear.

While the ability of inner-ear hair cells to regenerate is very limited in mammals, avian inner ears regenerate lost hair cells quickly. Similarly, the activity of histone deacetylases was reported as essential for normal proliferation of supporting cells in primary cultures from avian utricles. Following treatment of cultures with the ototoxic antibiotic streptomycin, many hair cells die and the number of proliferating supporting cells increases dramatically in order to regenerate lost hair cells. Histone deacetylase inhibitors inhibited both the basal low proliferation and the post-damage high proliferation rates. Inhibition of class I histone deacetylases was sufficient to prevent proliferation, and high doses of inhibitors even induced apoptosis of supporting cells. However, while histone deacetylases were found to be required for proliferation of supporting cells, proper activity of these enzymes was not required for differentiation of new hair cells from existing supporting cells (Slattery et al. 2009).

Although the main role of histone deacetylases is to decrease acetylation of histones, these enzymes have additional roles. They can also regulate the activity of transcription factors by inducing their deacetylation (reviewed in Bolden et al. 2006). Therefore, the results described above suggest that histone deacetylases are required for proliferation of progenitor cells in the mouse and avian utricles, but they do not prove that histone acetylation is responsible for the reduced proliferation.

Alternatively, histone deacetylation may contribute to the ototoxic damage induced by aminoglycoside antibiotics, leading to death of inner-ear hair cells. In the experiments discussed below, the levels of acetylated histone proteins were measured directly by specific antibodies.

A systematic administration of kanamycin to mice in vivo induced downregulation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) expression and upregulation of

phosphatidylinositol-4,5-bisphosphate (PIP_2) in cochlear outer hair cells (OHC). Some of the upregulated PIP_2 was translocated to the cells' nuclei, where it was immunoprecipitated with histone H3 and reduced its acetylation (Jiang et al. 2006). In another study, gentamicin-induced deacetylation of many histone proteins in vitro, followed by hair cell death, was reported in primary organ cultures of mouse cochlear sensory epithelia. This effect was mediated by overexpression of several histone deacetylases, while histone acetyltransferases (the enzymes that acetylate histone proteins) were not affected. Moreover, inhibition of histone deacetylases attenuated hair cell death (Chen et al. 2009). Not only aminoglycosides insult hair cells by inducing histone deacetylation. The ototoxic effect of the anticancer drug cisplatin may also result from histone deacetylation in hair cells, since inhibition by sodium butyrate of histone deacetylases in guinea pigs in vivo completely prevented hearing loss after cisplatin administration. Since histone deacetylase inhibitors are also anticancer drugs, they may be suitable candidates for cotherapy with cisplatin in order to prevent ototoxic damage to patients (Drottar et al. 2006).

One candidate for upstream regulation of histone acetylation in inner-ear hair cells is the transcription factor Gfi1, known as a transcriptional repressor that promotes cell proliferation and prevents apoptosis (Duan and Horwitz 2003). Our group reported that in the mouse inner ear, *Gfi1* expression may be regulated by the deafness-related transcription factor Pou4f3 (Vahava et al. 1998), and both Pou4f3 and Gfi1 expression in the mouse inner ear is limited to hair cells. A knockout of *Gfi1* in mice led to progressive deformation and loss of outer hair cells, while residual hair cells lost their stereocilia or displayed abnormal or immature stereocilia (Hertzano et al. 2004). Although Gfi1-induced histone modifications have not been studied in the inner ear yet, it was shown in other human cell lines that Gfi1 can recruit histone deacetylases and induce histone deacetylation (Duan et al. 2005; McGhee et al. 2003), and it can recruit histone lysine methyltransferase G9a and induce methylation of histone H3 (Duan et al. 2005). Moreover, Gfi1 was found in a complex with histone lysine methyltransferase G9a and histone deacetylase 1 on the promoter of *CDKN1A* ($p21^{Cip/WAF1}$), which in known to induce cell cycle arrest. As a result, Gfi1 repressed *CDKN1A* expression in a transfected human cell line (Duan et al. 2005). Thus, Gfi1 can repress transcription of target genes by inducing histone modifications at their promoter sites.

Other chromatin-related proteins linked with syndromic sensorineural hearing loss

Townes-Brocks syndrome (TBS; OMIM #107480) is an autosomal dominant syndrome with sensorineural hearing loss and several body malformations of the anus, thumbs, hands, feet, and ears. This syndrome derives from mutations in the *SALL1* (Sal-like 1) gene that encodes for a putative transcription factor. Localization of the GFP-SALL-1 fusion protein in transiently transfected NIH-3T3 cells revealed that this protein is colocalized with heterochromatin, particularly in chromocenters (dense clusters of pericentromeric heterochromatin), but also in smaller heterochromatin foci. Heterochromatin is tightly packed chromatin, and the transcription of genes packaged in heterochromatin is known to be repressed. Indeed, SALL1 was found to be a strong transcriptional repressor in transfected cells. Moreover, its effect does not depend on histone acetylation since treatment of transfected cells with histone deacetylase inhibitor did not relieve the transcriptional suppression. Therefore, it was proposed that SALL1 plays a role in regulating chromatin structure (Netzer et al. 2001).

CHARGE syndrome (OMIM #214800) includes coloboma of the eye, heart defects, atresia of the choanae, retardation of mental and somatic development, microphallus, external ear abnormalities, and sensorineural hearing loss. In most cases, CHARGE syndrome results from mutations in the gene *CHD7* (chromodomain helicase DNA-binding protein-7) (Vissers et al. 2004), which encodes for an ATP-dependent chromatin-remodeling enzyme. Recently, chromatin immunoprecipitation of CHD7 and hybridization of precipitated sequences with tilling microarrays were used to profile the binding sites of CHD7 in several human cell lines (but not in inner-ear cells). CHD7 was found to be a specific chromatin-binding protein because it binds specifically to histone H3 methylated at lysine 4 (H3K4me). CHD7 binding sites are discrete in different cell types, and most of them are distal to transcription start sites (TSS) and hypersensitive to DNase I digestion, suggesting that they are located in an open chromatin environment. Finally, CHD7 binding was associated with increased gene expression. Thus, the H3K4me-binding CHD7 may function as an enhancer that upregulates transcription of genes labeled with the histone modification H3K4me, and CHARGE syndrome may result from alterations in transcription of tissue-specific genes regulated by CHD7 (Schnetz et al. 2009).

Hearing-related genes implicated in both DNA methylation and histone modifications

DNA methylation and histone modification are dynamic epigenetic marks that change extensively during cellular differentiation and cooperate to induce transcription or gene silencing. DNA methylation labels sites at which the histone should be modified and the chromatin should be

rearranged (Klose and Bird 2006; Meissner et al. 2008). For example, the maintenance DNA methyltransferase DNMT1, which is responsible for placing methyl groups in the daughter DNA stand during replication only in sites that are methylated in the original strand, interacts with histone methyltransferases and methyl-CpG-binding proteins (MeCPs) that mediate chromatin remodeling (reviewed in Klose and Bird 2006). Two genes crucial for normal hearing in humans and mice, *MECP2* and *Rb1*, link DNA methylation and histone modifications in the mammalian inner ear.

Rett syndrome (OMIM #312750), a dominant X-linked neurodevelopmental disorder, is one of the most common causes of mental retardation in girls and often lethal in males due to encephalopathy (Ellaway and Christodoulou 1999). Approximately 85% of cases derive from mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene at chromosomal locus Xq28 (Amir et al. 1999), and more than 200 different mutations in this gene have been identified in Rett patients (Hoffbuhr et al. 2002; Matijevic et al. 2009). Most common mutations are C-to-T transitions at eight CpG dinucleotides that may derive from deamination of methylated cytosines (Matijevic et al. 2009). Typical Rett patients with *MECP2* mutations develop normally until they are 6-18 months old, then their development is arrested, leading to regression of acquired skills, loss of speech, stereotypical movements (classically of the hands), microcephaly, seizures, and mental retardation (Hagberg 1995). In mice, deficiency of MeCP2 after the normal development of immature neurons results in defective postnatal neuronal maturation, synaptogenesis, and maintenance of synapses and neurons, leading to dysfunctionality and apoptosis of neurons (Francke 2006; Palmer et al. 2008; Smrt et al. 2007). Sensorineural hearing loss is relatively common in Rett patients ($\sim$17%; Pillion et al. 2003), suggesting that MeCP2 is necessary for the development, maintenance, or function of both the nervous system and the inner ear. MeCP2 binds selectively to methylated CpG dinucleotides and acts as a transcriptional repressor (Nan et al. 1997) that induces histone deacetylation, mostly of core histones H3 and H4, by recruiting the transcriptional silencer Sin3A and histone deacetylase 1 (HDAC1) to the methylated CpG site (Jones et al. 1998; Nan et al. 1998). Indeed, Rett *MECP2* mutations induce histone hyperacetylation in blood cells (Wan et al. 2001). MeCP2 induces additional histone modifications indirectly, in particular, methylation of core histone H3 (Fuks et al. 2003; Kaufmann et al. 2005) mainly during the critical postnatal stage of neuronal maturation (Thatcher and LaSalle 2006). Thus, MeCP2 links two epigenetic modifications, DNA methylation and histone methylation or deacetylation, and reinforces a repressive chromatin state. MeCP2 has additional epigenetic roles (reviewed in Singh et al. 2008): The MeCPG/Sin3A/HDAC1 silencing complex can bind transcription factors and thus competitively inhibit their binding to promoters (Suzuki et al. 2003); it interacts with transcription factor IIB (TFIIB) to prevent assembly of the preinitiation complex (Kaludov and Wolffe 2000); and it may target transcripts in addition to methylated DNA (Buschdorf and Stratling 2004). Moreover, MeCP2 may affect X chromosome inactivation (XCI). Since the *MECP2* gene is located on chromosome X, heterozygote females that carry mutations in this gene may be protected from disease by nonrandom (skewed) inactivation of the X chromosome carrying the mutant *MECP2*. However, XCI is random in most Rett cases, leading to many female patients, and different mosaicism of XCI may result in different phenotypes in female individuals that carry the same mutation, even in the same family. However, skewed XCI was found in patients that exhibit a less severe phenotype compared to their monozygotic twins (Hoffbuhr et al. 2001; Ishii et al. 2001; Migeon et al. 1995), and some healthy women that carry the mutation responsible for Rett syndrome in their daughters may also derive from skewed XCI (Hoffbuhr et al. 2002; Sirianni et al. 1998). These findings suggest that MeCP2 may affect the XCI mechanism. However, some other cases with phenotype differences in the same family could not be explained by skewed XCI, suggesting that additional mechanisms may be responsible for phenotypical differences (Takahashi et al. 2008; Xinhua et al. 2008). XCI requires hypermethylation of the inactivated chromosome and maintenance of this hypermethylation. Although it is not clear yet whether MeCP2 participates directly or indirectly in the XCI machinery, recent evidence suggests that MeCP2 is required for parental imprinting of two specific genes, *UBE3A* (Makedonski et al. 2005) and *DLX5* (Horike et al. 2005), while it is not required for imprinting of other imprinted genes (Balmer et al. 2002). The possibility that MeCP2 is required for imprinting of *UBE3A* is particularly interesting since *UBE3A* is known to be imprinted in neurons only after birth. Studies suggesting that MeCP2 induces XCI or imprinting of specific genes should be taken with caution because most of them detected XCI or imprinting in blood leukocytes from Rett patients, and the effect of MeCP2 on a specific target gene may be different at different cells or tissues (LaSalle 2007), such as the nervous system or the inner-ear sensory epithelia. MeCP2 expression peaks in mature neurons and it regulates the expression of a relatively small set of genes (Francke 2006; Singh et al. 2008; Tudor et al. 2002), including *BDNF* (brain-derived neurotrophic factor). The effect of MeCP2 on *BDNF* expression is different in different tissues. For example, MeCP2 binds selectively to *BDNF* promoter III and represses *BDNF* expression in neuronal cultures (Chen et al. 2003), while it enhances *Bdnf* expression in mouse

brain (Chang et al. 2006). The gene *Bdnf* was linked to hereditary hearing loss in mice (Bianchi et al. 1996) and is expressed in vestibular hair cells and in cochlear hair and supporting cells in newborn rodents (Farinas et al. 2001; Pirvola et al. 1992). Bdnf is released from inner-ear hair cells to guide their afferent innervation (Agerman et al. 2003), which is required for survival of vestibular and spiral ganglia neurons, and has a role protecting spiral ganglion cells following exposure to ototoxic antibiotics and acoustic trauma (Staecker et al. 1998).

The second protein known to affect both DNA methylation and chromatin remodeling of inner-ear genes is the transcriptional repressor Rb1 (retinoblastoma 1). Rb1 can directly interact with histone modification enzymes and induce chromatin remodeling (reviewed in Giacinti and Giordano 2006). In addition, the Rb1/E2F1 complex can bind the DNMT1 promoter and increase the expression of this DNA methylation enzyme (McCabe et al. 2005). *Rb1* expression itself is regulated by histone modifications (reviewed in Moss and Wallrath 2007) and DNA methylation (Morey et al. 2006) and is required for normal hearing in mice. A conditional knockout of *Rb1* in the mouse inner-ear sensory epithelia induced degeneration of outer hair cells in the cochlea, and, as a result, the organ of Corti collapsed and all the cochlear hair cells were degenerated, inducing hearing loss that gradually progressed to profound deafness in adult mice. Thus, Rb1 is required for maturation and survival of cochlear outer hair cells. In contrast, most of the vestibular hair cells survived and functioned normally in these mice (Sage et al. 2006). Two alternative hypotheses may explain these results. First, *Rb1* was knocked out from both supporting and hair cells in the cochlea, but only from hair cells in the vestibule. Perhaps its expression in the supporting cells is required for hair cell survival. Alternatively, this protein may be less crucial in the vestibular sensory epithelia.

Integration between miRNAs and epigenetic mechanisms

There are several interactions between miRNAs and epigenetic mechanisms. miRNAs can inhibit the translation of enzymes required for DNA methylation or histone modifications and thus affect gene transcription indirectly. For example, it was suggested that the miR-29 family of miRNAs directly inhibits the translation of DNA methyltransferases DNMT3A and DNMT3B and thus inhibits both maintenance of methylation and *de novo* DNA methylation (Fabbri et al. 2007). In another example, the miR-290 cluster miRNAs indirectly affect histone modifications and reduce the transcription of DNA methyltransferases (Benetti et al. 2008; Sinkkonen et al. 2008). Such transcriptional regulation may be considered a more permanent method of gene silencing than the post-transcriptional effect. Direct effects of miRNAs or other Dicer products on gene transcription were also suggested. *Dicer* knockout cells exhibit reduced expression of DNA methyltransferases, reduced DNA methylation, and aberrant phenotype (reviewed in Guil and Esteller 2009). However, the level of DNA methyltransferases was not reduced in other *Dicer* knockdown cells (with a defective hypomorphic *Dicer* gene). These cells exhibited demethylation of promoters of specific genes rather than along the whole genome, with an unknown mechanism (Ting et al. 2008). Dicer is responsible for production of both miRNAs and natural short interfering RNAs (siRNAs). siRNAs (at least exogenous siRNAs) are known to control DNA methylation, histone modifications, and transcription of specific genes in mammals (Kawasaki and Taira 2004; Morris et al. 2004 and reviewed in Morris 2005). Recently, it was proposed that miRNAs may also function as siRNAs and affect DNA methylation and histone modifications of specific sites in the genome. The proposed mechanism includes binding of siRNAs and perhaps also miRNAs to complementary sequences on the DNA [or on the transcribed mRNA during transcription, as reported in plants (Bao et al. 2004)] and induction of chromatin modification (Verdel et al. 2004) or *de novo* DNA methylation (reviewed in Bayne and Allshire 2005) downstream to the binding sites. As a result, the transcription of the target gene is inhibited. This mechanism was reported initially in plants and yeasts and subsequently in mammals [siRNAs (Bayne and Allshire 2005) and miRNAs (Tan et al. 2009)].

Mir genes are also subjected to epigenetic regulation. The transcription of a Mir gene may also be regulated by cytosine methylation in its promoter region (Guil and Esteller 2009; Saito and Jones 2006) or by histone acetylation (e.g., Lee et al. 2009), and aberrant methylation of Mir genes was shown to contribute to tumorigenesis and metastasis (Lujambio et al. 2008). Chromatin-modifying drugs were also reported to affect miRNA expression (Saito et al. 2006). Moreover, DNA methylation of Mir gene promoters is associated with histone modifications that underlie a closed chromatin structure and repressed expression of the encoded miRNA. Such epigenetic regulation of cancer-related miRNAs was associated with survival rates in humans (Roman-Gomez et al. 2009). The interactions between miRNAs, DNA methylation, and chromatin remodeling were recently reviewed (Guil and Esteller 2009).

Conclusions

The complex sensory transduction of the inner ear, and primarily the cochlea, the organ of hearing, is dependent on a

tremendous amount of synchronized processes and mechanisms that require a large battery of protein-coding genes and regulatory elements. While in many cases NSHL is hereditary, hearing loss may also result from nonhereditary causes such as degeneration of inner-ear hair cells following exposure to intense auditory stimuli. However, the sensitivity to these stimuli may also be influenced by genetic or epigenetic factors. Study of the factors and pathways that lead to malformation or degeneration of the inner-ear sensory epithelia may help to identify regulatory intersections to which therapies can be directed. For example, gene therapy may be used to induce cochlear supporting cells to transdifferentiate to hair cells in adult mammals, although such a process does not occur naturally (Izumikawa et al. 2005). While the protein-coding genes that have been implicated in hearing and deafness are widely studied, there are only a few reports describing hearing loss-related miRNA or epigenetic regulation in the mammalian inner ear.

A systematic study of the expression and roles of miRNAs in the mammalian inner ear has begun in the last 3 years, as described above. However, technical difficulties have precluded a systematic study of cytosine methylation or histone modification sites in inner-ear tissues. The profiling of cytosine methylation and histone modification sites in the mammalian inner ear and the comparison of these epigenetic marks in wild-type and deaf animals is complicated by the fact that the inner ear is a very complex organ, composed of many different tissues and cells. For example, the mouse cochlear sensory epithelium includes up to 15,000 hair cells and several times more supporting cells, arranged in a way that it is very difficult to separate surgically (see Fig. 1). A strict separation of different cell types is required to compare methylation or histone modification sites in deaf and hearing mice, since these epigenetic markers are expected to differ in different cell types, and if genomes from several cell types are pooled together, important hair cell-specific markers may be masked. Although recent articles describe flow cytometry-based separation of hair and supporting cells from dissected inner-ear sensory epithelia (Lumpkin et al. 2003; Savary et al. 2007; White et al. 2006), it is difficult to obtain with these techniques a sufficient number of separated cells for profiling epigenetic markers. With the development of new technologies for the efficient separation of inner-ear cell types and for the screening of DNA methylation and histone modifications in their genome, hopefully it will become possible in the near future to study these epigenetic mechanisms in the inner ear. Such studies may open new horizons for understanding inner-ear normal function, development, and pathology.

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