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## Little but loud: small RNAs have a resounding affect on ear development

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### Abstract

The impact of small RNA function has resonated throughout nearly every aspect of eukaryotic biology and captured the varied interests of researchers, whether they are endeavoring to understand the basis of development and disease or seeking novel therapeutic targets and tools. The genetic regulatory roles of microRNAs (miRNAs) are particularly interesting given that these often highly conserved factors post-transcriptionally silence many complementary target genes by inhibiting messenger RNA translation. In this regard, miRNAs can be considered as counterpart to transcription factors, the ensemble of which establish the set of expressed genes that define the characteristics of a specific cell type. In this review, evidence supporting a resounding role for small RNAs in development and maturation of sensory epithelia in the mouse inner ear will be considered with an emphasis on the contribution of one hair cell miRNA family (miR-183, miR-96, and miR-182). Although there is much yet to be explored in this fledgling aspect of ear biology, the breadth of miRNA expression and functional requirement for ear development are already sounding off.

### Keywords

Hair cell; sensory epithelium; microRNA; Dicer; translational repression; genetic regulation

### 1. Introduction

Development of the mammalian inner ear requires coordinated transformation of a uniform sheet of cells to form an intricate labyrinthine structure that includes strategic positioning of vestibular and auditory sensory epithelia, and appropriate histological organization of epithelial supporting cells and mechanosensory hair cells. Many studies have revealed the importance of various regulatory proteins including morphogens and transcription factors on patterning, morphogenesis and histogenesis (reviewed in Fritzsche et al., 2007; Kelley, 2007), where coordinated expression and interaction contribute to precision in developmental transitions from precursor cells to differentiated cell types. Nevertheless, recent studies regarding the genetic regulatory roles of small RNAs (reviewed in Amaral et al., 2008) suggest that such developmental transitions in the inner ear are not exclusively orchestrated by the regulatory functions of proteins. Indeed, there is substantial evidence for the widespread importance of microRNAs (miRNAs) in post-transcriptional regulation of target gene expression affecting

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development, cell differentiation and maintenance, and disease (reviewed in Hobert, 2008; Makeyev and Maniatis, 2008). There are approximately 500 mammalian miRNAs representing about 2% of known genes and estimated to affect the expression of one-third of known protein-coding genes (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006). In this review, consideration will be given to the general function of miRNAs in post-transcriptional regulation of target gene expression and challenges to determining individual miRNA functions. Moreover, evidence for the expression and biological significance of miRNAs in development of the mouse inner ear are presented with a particular focus on specific miRNA families contributing to neurogenesis and innervation, epithelial development, and hair cell differentiation.

## 2. miRNA biogenesis and function

To best appreciate the role of miRNA-mediated gene regulation and the challenges to determining individual miRNA functions in development and maintenance of the inner ear, a brief review of miRNA biogenesis and function is warranted. The topic has been reviewed in detail from a number of interesting viewpoints including development and disease (Ambros, 2004; He and Hannon, 2004; Wienholds and Plasterk, 2005; Flynt and Lai, 2008; Stefani and Slack, 2008).

### 2.1. miRNA biogenesis

MicroRNA genes are expressed as capped and polyadenylated RNA polymerase II transcripts (Cai et al., 2004). Among human and mouse miRNA genes, approximately half reside within introns and are presumably co-expressed with known protein-coding genes, and approximately one-third reside in tandem with another miRNA gene(s) and exhibit coordinated expression (Saini et al., 2008). The production of functional miRNAs is mediated by the miRNA pathway (Fig. 1A). Unprocessed miRNA transcript, termed primary miRNA (pri-miR), contains the miRNA sequence within either side of a mostly base-paired stem-loop or hairpin structure. In the nucleus, DiGeorge syndrome critical region 8 (Dgcr8) protein is required to direct the activity of Drosha, a double-stranded RNA (dsRNA)-specific ribonuclease (RNase) III family member, to the pri-miR hairpin (Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Wang et al., 2007; Yi et al., 2008). Drosha cleavage of the pri-miR in this 'microprocessor' complex produces an approximately 70-nucleotide (nt) hairpin called a precursor miRNA (pre-miR), which is exported from the nucleus through a Ran-GTP/Exportin-5-dependent process (Kim, 2004).

In the cytoplasm, the pre-miR is further processed by Dicer, another dsRNA-specific RNase III family member, to produce a small dsRNA with approximately 21-nt strands that is similar to a small interfering RNA (siRNA) (Knight et al., 2001; Grishok et al., 2001; Harfe et al., 2005; Kanellopoulou et al., 2005). Indeed, both miRNA and siRNA biogenesis share Dicer and downstream components of the miRNA pathway (Tabara et al., 1999; Meister et al., 2004; Okamura et al., 2004; Chendrimada et al., 2005; Hasse et al., 2005). Selection of the mature miRNA strand from the complementary or so-called "star" strand is based on thermodynamic stability at each end of the dsRNA, where the miRNA strand is distinguished as that with its 5' terminus at the lower stability dsRNA end (Schwarz et al., 2003; Tomari et al., 2004). Nevertheless, in some cases both strands of a pre-miR can represent mature functional miRNAs. The mature miRNA finally associates with an Argonaute (Ago) protein within the RNA-induced silencing complex (RISC), the cellular apparatus that exerts post-transcriptional silencing upon complementary target mRNAs using the miRNA as a guide sequence (Tabara et al., 1999; Meister et al., 2004; Okamura et al., 2004).

## 2.2. miRNA function

Post-transcriptional silencing of miRNA target genes can occur through two RISC-mediated mechanisms dependent upon the extent of complementarity between the miRNA and target mRNA sequence. Perfect complementarity between a miRNA and target mRNA sequence, like that of an siRNA, can elicit ribonuclease activity and mRNA cleavage in Ago2-containing RISC complexes (i.e. “Slicer” activity; Liu et al., 2004; Meister et al., 2004; Okamura et al., 2004). However, partial complementarity results primarily in translational repression through mechanisms not yet well understood (reviewed in Eulalio et al., 2008; Stefani et al., 2008). In fact, the vast majority of mammalian miRNAs lack perfect complementarity to any genomic sequence (other than their own) and thus function by the latter mechanism. The task of predicting target mRNAs is therefore made challenging by the lesser extent of base-pairing with the miRNA, which relies foremost on the miRNA “seed” sequence comprised of nucleotides 2 through 7 or 8 (Fig. 1B) (Lewis et al., 2003; Lewis et al., 2005). Nevertheless, several algorithms including TargetScan, miRanda, and PicTar (Lewis et al., 2003; John et al., 2004; Krek et al., 2005) have been devised to predict target mRNAs based upon the presence and phylogenetic conservation of seed matches within the 3' untranslated regions (UTRs) of homologous genes.

Predicted miRNA target genes must be experimentally validated on a case-by-case basis or elucidated by other experimental approaches. The incorporation of miRNA target sequences within the 3' UTR of reporter gene constructs provides validation by demonstrating miRNA-mediated reporter gene repression in cell culture and in vivo (Doench et al., 2003; Zeng et al., 2003; Mansfield et al., 2004; Vella et al., 2004), where silencing is typically enhanced by the presence of multiple miRNA-binding sites (Johnson et al., 2005; Mayr et al., 2007). Moreover, microarray analysis of gene expression demonstrates that target mRNA levels can be reduced upon miRNA transfection in cell culture (Lewis et al., 2005; Lim et al., 2005). This effect is likely mediated by miRNA-directed mRNA deadenylation independent of translational repression (Giraldez et al., 2006; Wu et al., 2006), although it is not an absolute feature of miRNA regulation. Nevertheless, microarray analysis provides a relatively accessible means of determining miRNA target genes and assessing miRNA functions. Importantly, recent advances have been made toward biochemical isolation and identification miRNA-target transcripts by immunoprecipitation of silencing complexes from *C. elegans* (Zhang et al., 2007; Hammell et al., 2008), *Drosophila* (Easow et al., 2007), and human cell culture (Karginov et al., 2007; Landthaler et al., 2008). In conclusion, miRNAs can post-transcriptionally silence target genes through immediate effects upon mRNA stability and/or translation, and such interactions can be determined through the combination of bioinformatic prediction and experimental methodologies.

## 2.3. Challenges to determining function

The determination of individual miRNA functions faces many challenges. Although predicted target mRNAs can be ranked with regard to the likelihood of miRNA-mediated repression based on the number, type, and context of interactions (Hon et al., 2007; Lewis et al., 2005; Grimson et al., 2007; Nielsen et al., 2007), individual miRNAs might have as many as hundreds of predicted targets. Microarray analysis of predicted target gene expression is dependent upon the transcriptome of cell culture models suitable to assessment of a particular miRNA. Moreover, the effect of miRNAs on target mRNA levels does not reflect translational inhibition and does not necessarily coincide with miRNA regulation. Whereas proteomic analysis is more suitable to the task, it is considerably more laborious and technically challenging. Nevertheless, recent studies applying both transcriptome and proteome analyses have revealed that miRNAs indeed impact the expression of hundreds of genes enriched for predicted targets, but that miRNA-mediated regulation rarely accounts for effects on target gene expression exceeding 50% reduction (Baek et al., 2008; Selbach et al., 2008). Individual miRNAs thus appear to

function as “rheostats” that fine-tune the output of many expressed target genes more so than absolute genetic “switches” affecting one or a few target genes. Therefore, it becomes considerably more challenging to attribute a phenotypic outcome resulting from an individual miRNA's contribution to one or few determinable genetic mechanisms.

### 3. miRNA expression in the inner ear

As is the case with most regulatory genes of interest, considerable insight regarding their prospective functions can be gained by examining their tissue and cell-specific expression. Among the first studies of miRNA expression, cloning and sequencing revealed the existence of a large number of unique miRNAs, many of which are differentially expressed in various cell types or tissues (Lagos-Quintana et al., 2002; Houbaviy et al., 2003; Landgraf et al., 2007). With the further development of techniques and sequence-specific probes appropriate for microarray analysis (Krichevsky et al., 2003; Liu et al., 2004; Babak et al., 2004), quantitative RT-PCR (Schmittgen et al., 2004; Chen et al., 2005), and in situ hybridization (Weinholds et al., 2005; Kloosterman et al., 2006), studies have been facily extended to examine changes in miRNA expression coincident with temporospatial aspects of development, disease, and cellular responses to biological factors and stresses. Moreover, the generation of animal models wherein small RNA biogenesis and function is disrupted by deletion of *Dicer* has facilitated the analysis of miRNA effects in organisms including zebrafish and mouse (Giraldez et al., 2005; Harfe et al., 2005).

Microarray analysis of miRNA expression in the postnatal mouse inner ear has revealed that at least 100 or approximately one-fourth of known mouse miRNAs are present (Weston et al., 2006). Expression profiles are not substantially altered from the newborn mouse inner ear, through maturation and functional onset of hearing, and into adulthood, suggesting that miRNA expression is largely established in embryonic development. Nevertheless, the abundance of miRNAs implies a considerable contribution to the regulation of genetic programs amongst the various cell types that are important to inner ear development, maturation, and function. Moreover, the cell-specific expression of certain miRNAs demonstrated by in situ hybridization in this work and others predicts the relevance of such miRNAs to attaining mature cell characteristics in neurosensory epithelia. The following sections explore just a handful of miRNAs that appear to be important to the establishment of neurons, epithelial cells, and hair cells in the mouse inner ear.

#### 3.1. Neuronal miRNA expression

Many miRNAs have been demonstrated to contribute to neuronal development, plasticity, and disease (reviewed by Fiore et al., 2008). With regard to neurosensory development, miRNAs and their target genes demonstrably constitute genetic mechanisms that dictate sensory neuron cell fates and properties in *C. elegans*, *Drosophila*, and mouse. The establishment of left/right asymmetry in *C. elegans* taste receptor neurons is enforced by a double-negative feedback loop involving cell-specific miRNAs and transcription factor targets (Johnston and Hobert, 2003; Johnston et al., 2005). Whereas the left neuron expresses *die-1* transcription factor and *lys-6* miRNA which represses *cog-1* transcription factor expression, the right neuron expresses *cog-1* transcription factor and miR-273 which represses *die-1* transcription factor expression. In *Drosophila*, miR-279 expression in olfactory neurons prevents carbon dioxide (CO<sub>2</sub>) neuron development by repressing *nerfin-1* expression among other genes (Cayirlioglu et al., 2008). In mouse, neuronal miR-124 expression has been shown to represses REST silencing transcription factor (REST) and phosphatase SCP1, stifling the anti-neural function of the REST/SCP1 pathway (Conaco et al., 2006; Visvanathan et al., 2007), and to repress polypyrimidine tract binding protein 1 (PTBP1), a global repressor of alternative splicing in non-neuronal cells (Makeyev et al., 2007).

In the mouse inner ear, miR-124 is strongly expressed in sensory neurons derived from the otocyst (Weston et al., 2006). In addition to promoting neuronal cell differentiation and expression profiles (Lim et al., 2005; Krichevsky et al., 2006), miR-124 has been demonstrated to affect neurite outgrowth in differentiating mouse cells (Yu et al., 2008) and branching in *Drosophila* dendritic arborization sensory neurons through association with fragile X mental retardation protein 1 (dFMRP) and components of the miRNA pathway (Xu et al., 2008). Taken together, these studies suggest that miR-124 among other neuronal miRNAs figure prominently in neurogenesis and innervation of the inner ear.

### 3.2. Epithelial miRNA expression

The miR-200 family is comprised of five homologous miRNAs (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) that are coordinately expressed from two different genetic loci in epithelia including those of the inner ear in zebrafish (Wienholds et al., 2005), chick (Darnell et al., 2006), and mouse (Kloosterman et al., 2006; Weston et al., 2006). The miRNAs are key regulators of mesenchymal to epithelial transition (MET; reviewed by Gregory et al., 2008a), where they enforce epithelial cell fate by repressing expression of the E-cadherin transcriptional repressors zinc finger E-box binding homeobox 1 (Zeb1) and Smad-interacting protein 1 (Sip1/Zeb2) (Hurteau et al., 2006; Christoffersen et al., 2007; Gregory et al., 2008b; Korpál et al., 2008; Park et al., 2008). The miRNAs and their target genes constitute a double-negative feedback loop wherein Zeb1/Sip1 expression induced by the transforming growth factor beta 1 (Tgfb1) and bone morphogenic protein (Bmp) signaling pathway also represses miR-200 family transcription to promote epithelial to mesenchymal transition (EMT) (Bracken et al., 2008; Burk et al., 2008). Given that Bmp signaling and Smad proteins affect formation of the bony labyrinth and sensory epithelial cell differentiation (Chang et al., 2002; Pujades et al., 2006; Liu et al., 2007) and that hair cell specification requires the basic helix-loop-helix (bHLH) transcription factor Atonal 1 (Atoh1/Math1) which binds E-box motifs (Bermingham et al., 1999; Woods et al., 2004; Krizhanovsky et al., 2006), it is likely that the miR-200 family plays a key role in establishing prosensory epithelial domains in which E-box motifs are receptive to bHLH transcription factors.

### 3.3. Hair cell miRNA expression

The miR-183 family is comprised of three homologous miRNAs (miR-183, miR-96, and miR-182) that are coordinately expressed from a single genetic locus in vertebrates (Fig. 2A). Moreover, homologous miRNAs are found in *Drosophila* (miR-263b) and *C. elegans* (miR-228; Fig. 2A). Importantly, this highly conserved family of miRNAs demonstrates expression in ciliated neurosensory organs across phyla (Pierce et al., 2008). In *C. elegans*, miR-228 appears to be specifically expressed in various chemosensory and mechanosensory sensilla. In *Drosophila*, miR-263b is expressed in sensory organ precursors (SOPs) in the embryo (Aboobaker et al., 2005), and in chemosensory and mechanosensory segments of the adult antenna and the haltere (Pierce et al., 2008). In vertebrates, miR-183 family members are expressed in sensory cells of various organs including the olfactory epithelium, eye, neuromast, and ear of zebrafish (Wienholds et al., 2005), and in sensory neurons (cranial and spinal ganglia) and sensory cells of eye and ear in chicken and mouse (Darnell et al., 2006; Kloosterman et al., 2006; Weston et al., 2006). In the mouse eye, the miRNAs are expressed in photoreceptor, retinal bipolar, and amacrine cells (Xu et al., 2007), and their expression is perturbed in a mutant rhodopsin (Rho) model of retinitis pigmentosa (Loscher et al., 2007). In the mouse inner ear, the miRNAs are expressed in sensory neurons and hair cells throughout the vestibular end organs and organ of Corti (Fig. 2B; Weston et al., 2006). Although little is known regarding target genes affected by miR-183 family members, which will be explored in a following section, the high degree of sequence conservation and expression in neurosensory cells suggest these miRNAs play important if not varied roles in the development of functional sensory cells including inner ear hair cells.

## 4. miRNA function in inner ear development

MicroRNA function is typically assessed using genetic models for loss-of-function (LOF) or gain-of-function (GOF), although recent studies have employed antisense oligonucleotides termed antigomirs to abrogate specific miRNA function in vivo (Krützfeldt et al., 2005; Carè et al., 2007). In *Drosophila*, LOF and GOF studies have facilitated determination of miR-9a repression of *senseless* expression in regulating the formation of SOPs (Li et al., 2006), and miR-7 repression of *yan* expression in photoreceptor differentiation (Li and Carthew, 2005). In mouse, targeted deletions of certain miRNAs have recently revealed their specific roles in cardiac and immune functions (Rodriguez et al., 2007; Thai et al., 2007; van Rooij et al., 2007; Zhao et al., 2007; Ventura et al., 2008), but no specific miRNA deletions with relevance to neurosensory organs have yet been generated. Nevertheless, miRNA function as a whole can be assessed through the analysis of *Dicer* knockout (KO) models. Whereas *Dicer* KO in zebrafish has a demonstrated effect on ear development and otoconia formation (Giraldez et al., 2005), *Dicer* KO mice exhibit early embryonic lethality preceding otocyst development (Bernstein et al., 2003). However, the roles of miRNAs in development of specific mouse tissues can be assessed by conditional KO (CKO) of floxed *Dicer* alleles through Cre recombinase-mediated deletion (Harfe et al., 2005; Andl et al., 2006; Yi et al., 2006).

### 4.1 Effect of *Dicer* CKO on mouse inner ear

The significance of small RNAs to inner ear development has recently been examined in *Pax2-Cre;Dicer* CKO mice (Soukup et al., 2009). The *Pax2-Cre* transgene is transiently expressed in the otic placode and results in floxed allele deletion in most cells of the inner ear including sensory neurons, and supporting cell and hair cells in all sensory epithelia (Fig. 3A; Ohyama and Groves, 2004). The transgene is also expressed in the mid-hindbrain, olfactory bulb, and kidneys. Analysis of *Pax2-Cre;Dicer* CKO mouse inner ear demonstrates major defects in neurogenesis and innervation, morphogenesis, and sensory epithelial histogenesis (summarized in Fig. 3B). The effect of small RNA depletion in this model is similar in severity to LOF models for key regulators of prosensory development such as the SRY-box containing gene 2 protein (Sox2; Kiernan et al., 2005), suggesting a prominent role for miRNAs in the regulation of genetic programs that contribute to ear development.

In *Pax2-Cre;Dicer* CKO mouse inner ear, sensory neurons initially form but are rapidly depleted of miRNAs including miR-124 and undergo apoptosis. The few surviving neurons fail to properly project to targets in the hindbrain and inner ear, with the exception of some innervation of the utricular macula. The effect of miRNA depletion on surviving neurons is consistent with the apparent roles of miRNAs including miR-124 in neuronal differentiation and neurite outgrowth (Krichevsky et al., 2006; Yu et al., 2008). Morphogenesis and sensory epithelial development are variously affected by small RNA depletion, where there is a complete loss of anterior and horizontal cristae, loss or severe reduction of the saccular macula, reduction of the utricular macula, and severe truncation of the cochlea which typically exhibits two Sox2-positive sensory patches. Interestingly, the posterior crista is least affected in the model, where it was shown to retain some residual miR-183 expression despite *Dicer* deletion (Soukup et al., 2009), suggesting that *Dicer* mRNA and/or *Dicer* protein are relatively more stable in the development of this tissue.

### 4.2 Importance of hair cell miRNAs

The unexpected differential and residual miRNA expression in *Pax2-Cre;Dicer* CKO mouse inner ear sensory epithelia provides an interesting perspective on the role of hair cell miRNAs in differentiation. Whereas the production of mature miR-183 is evident in posterior crista hair cells, expression in the utricular macula and cochlea is weak or absent. Examination of hair cell apical specializations by scanning electron microscopy (SEM) revealed that hair cell

differentiation in this regard correlates with residual miRNA expression (Fig. 4), where hair cells in the posterior crista show well developed kinocilia and those in the utricular macula are notably bulbous and microvillous.

The range of effects in the *Pax2-Cre;Dicer* CKO mouse thus demonstrate the importance of small RNAs in inner ear development and cell differentiation. Loss of mid-hindbrain structures and sensory neurons are consistent with effects on proliferation and apoptosis observed in other *Dicer* CKO models (Davis et al., 2008), and might in part be attributable to loss of natural siRNA functions in *Dicer* null cells (Kanellopoulou et al., 2005). However, it is interesting to note that the specific depletion of miRNAs in *Dgcr8* CKO stem cells reveals similar defects in cell differentiation with little effect on proliferation (Wang et al., 2007), and that a recent model of *Dgcr8* CKO in mouse skin is indistinguishable from that of *Dicer* CKO (Yi et al., 2006; Yi et al., 2009). These studies suggest that the bulk of developmental effects observed in the *Pax2-Cre;Dicer* CKO inner ear are attributable to the depletion of miRNAs. Ongoing studies seek to examine the post-developmental effects of miRNA depletion on degeneration of sensory epithelia in the ear, as has recently been described for the mouse eye (Damiani et al., 2008).

## 5. Potential hair cell miRNA mechanisms

Among the many hundreds of predicted target genes for neurosensory/hair cell miR-183 family members reside several of particular interest to the development of hair cells from prosensory epithelia. The mutual exclusion model for miRNA and target gene expression (Flynt and Lai, 2008) predicts that miRNAs contribute to differentiating cell transitions by targeting genes expressed in precursor cells and/or alternative cell fates. Therefore, it is of interest to note the cadre of genes whose interactions contribute to supporting and hair cell fates that possess putative miR-183, miR-96, and/or miR-182 target sites (Fig. 5). These include genes encoding Sox2, which is required to establish prosensory domains and is downregulated in hair cells (Kiernan et al., 2005); Notch1, which through interactions with its ligands Jagged 2 (Jag2) and delta-like 1 (Dll1) expressed in hair cells is key to establishing supporting cell versus hair cell fate (Lanford et al., 1999; Kiernan et al., 2005; Brooker et al., 2006); other prosensory Notch ligands such as Jag1 (Brooker et al., 2006; Kiernan et al., 2006); and Notch1-regulated factors such as Hes1, which antagonizes the function of Atoh1 in specifying hair cell fate (Zheng et al., 2000; Zine and de Ribaupierre, 2002). Importantly, the 3' UTR of *Sox2* mRNA appears to be a bona fide target of miR-182, which mildly albeit significantly downregulates expression in a reporter assay (Weston and Soukup, unpublished data). Considering the rheostat-like function of miRNAs, the whole of these putative interactions suggest that miR-183 family members serve to reinforce hair cell fate and differentiation through a network of integrated effects.

Other insights to miR-183 family member function can be derived from analyses of their dysregulation in cancer cells. The upregulation of miR-183 family members in leukemia, hepatic, and colorectal cancer cells (Bandrés et al., 2006; Agirre et al., 2008; Ladeiro et al., 2008) has been postulated to downregulate anti-apoptotic factors including forkhead transcription factor (Fox) proteins (Bandrés et al., 2006; Pineles et al., 2007), thus suggesting a pro-apoptotic function for the miRNAs. In other hematopoietic disorders, upregulation of the miRNAs correlates with downregulation of heparin-binding epidermal growth factor (Hbegf; Guglielmelli et al., 2007; Bruchova et al., 2008), which among other mitogens effects the formation of supernumerary hair cells (Zheng et al., 1999). Other studies of leukemia and lymphoma cells (Pal et al., 2007; Wang et al., 2008a) demonstrate that miR-183 family members downregulate expression of protein arginine methyltransferase 5 (Prmt5), reducing histone methylation and upregulating expression of suppressor of tumorigenicity 7 (St7) and retinoblastoma (Rb) pocket proteins, the latter of which maintain post-mitotic differentiation

of hair cells (Rocha-Sanchez and Beisel, 2007). Moreover, Prmt5 methylation of p53 modulates responses to DNA damage (Jansson et al., 2008), where depletion of Prmt5 triggers p53-dependent apoptosis rather arrest. Lastly, miR-183 family members have been demonstrated in lung cancer to repress ezrin (Ezr; Wang et al., 2008b), a member of the ezrin/radixin/moesin (ERM) family of proteins that mediate cytoskeletal-membrane interactions and cell signaling (Niggli and Rossy, 2008), where radixin is crucial to hair cell maintenance (Kitajiri et al., 2004). Taken together, these studies suggest that miR-183 family members reinforce the post-mitotic differentiated state of hair cells, but might otherwise predispose hair cells to apoptosis particularly in response to oxidative stresses caused by ototoxic agents such as cisplatin (Rybak et al., 2007). Nevertheless, these putative roles for miR-183 family members in hair cell development and maintenance will require a substantial effort toward validation.

## 6. Conclusion

The astounding impact of miRNA function in development has been widely examined in model organisms, and the roles of many conserved miRNAs in specific cells, organs, and biological processes including disease are well appreciated. Although miRNA function in development and maintenance of the inner ear is just beginning to be explored, studies already suggest that their genetic regulatory properties are essential to the proper establishment of sensory neurons and sensory epithelial supporting and hair cells. Moreover, a study of miRNA expression during hair cell regeneration in newt sensory epithelia suggest a role for let-7 miRNAs in the process (Tsonis et al., 2007), and miR-9 function has been suggested in the regulation of a COL9A1 expression in human cochlea (Sivakumaran et al., 2006). Many questions remain regarding the functional contributions and mechanistic actions of individual miRNAs amidst the complexity of genetic and biochemical interactions that contribute to development, differentiation, and maintenance of specific cell types throughout the inner ear. Nevertheless, determination of individual miRNA functions is sure to enrich our understanding of pathways and factors important to such processes. Moreover, one can envisage miRNAs among other factors as targets or agents to affect cellular properties or cell differentiation in various therapeutic strategies including stem cell approaches to hair cell regeneration (Beisel et al., 2008).

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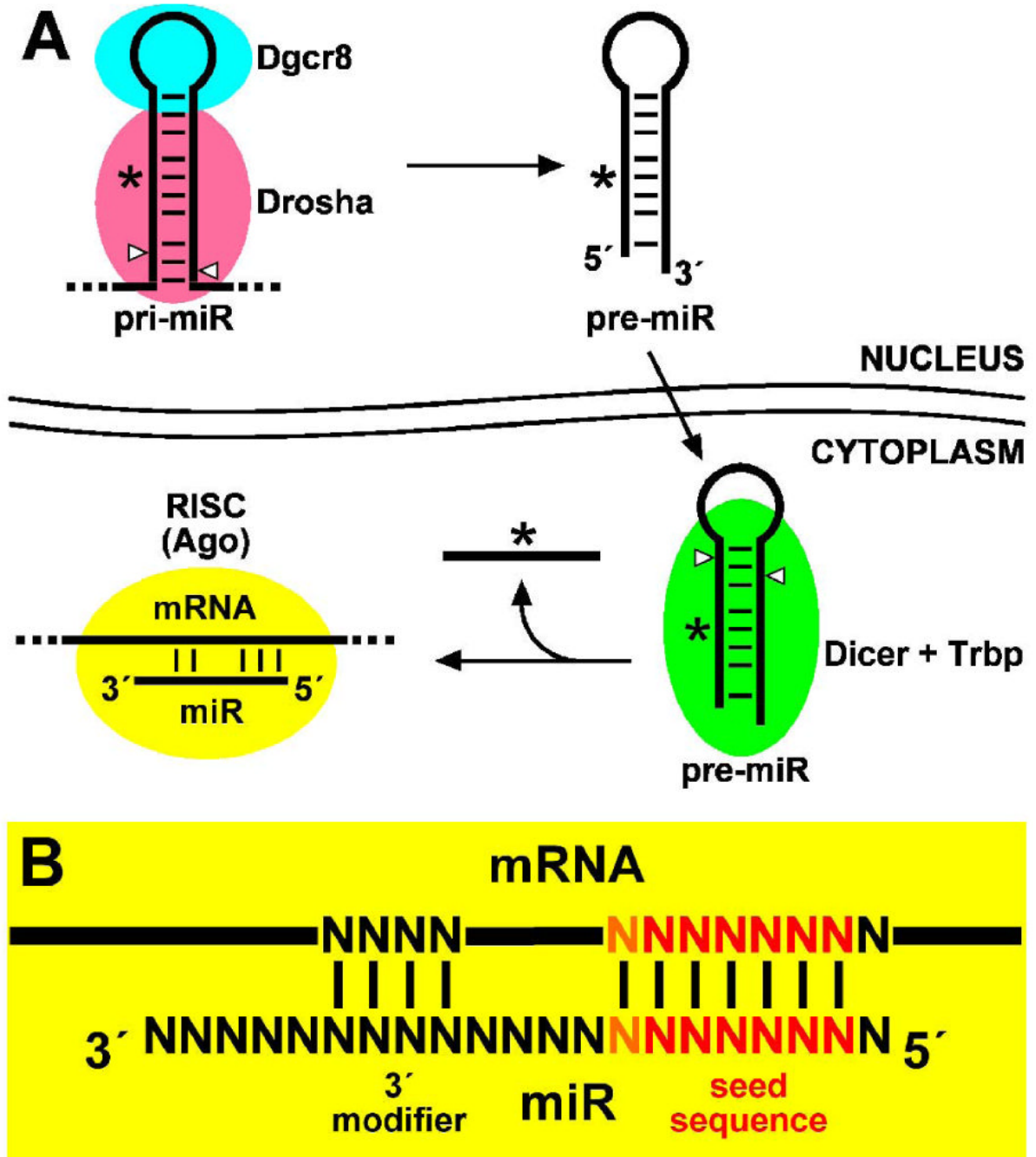
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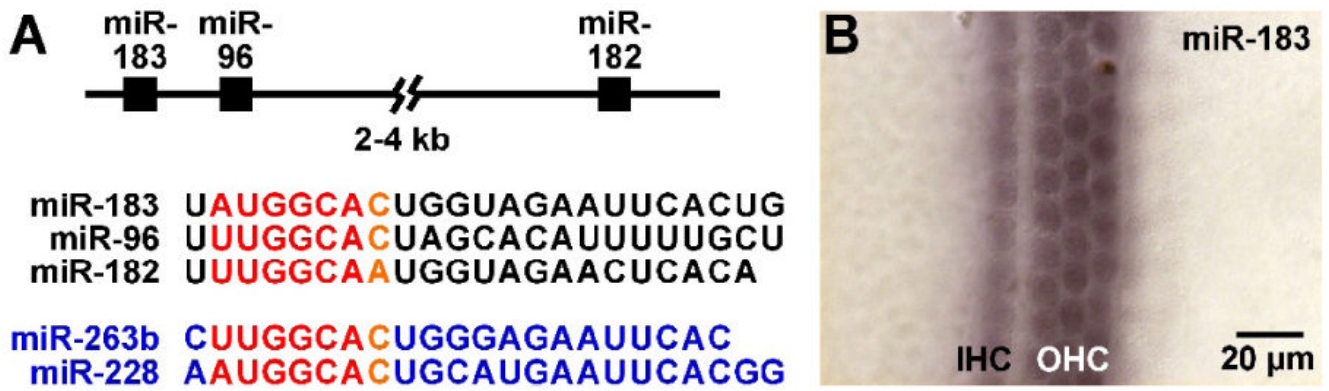
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**Fig. 1.** miRNA biogenesis and function. (A) The miRNA pathway. Primary miRNA (pri-miR) transcript processing in the nucleus requires Dgcr8 and the ribonuclease III family member, Drosha. The short hairpin precursor miRNA (pre-miR) is exported from the nucleus and subsequently processed by another ribonuclease III family member, Dicer, with Trbp. The mature miRNA strand is separated from the complementary strand (so-called “star” strand denoted by an asterisk) and is associated with an Argonaute (Ago) protein within the RNA induced silencing complex (RISC). RISC utilizes the miRNA as a guide sequence to discriminately bind target mRNAs and primarily effects translational repression. (B) miRNA-target mRNA interaction. Target mRNA is primarily discriminated through Watson-Crick base

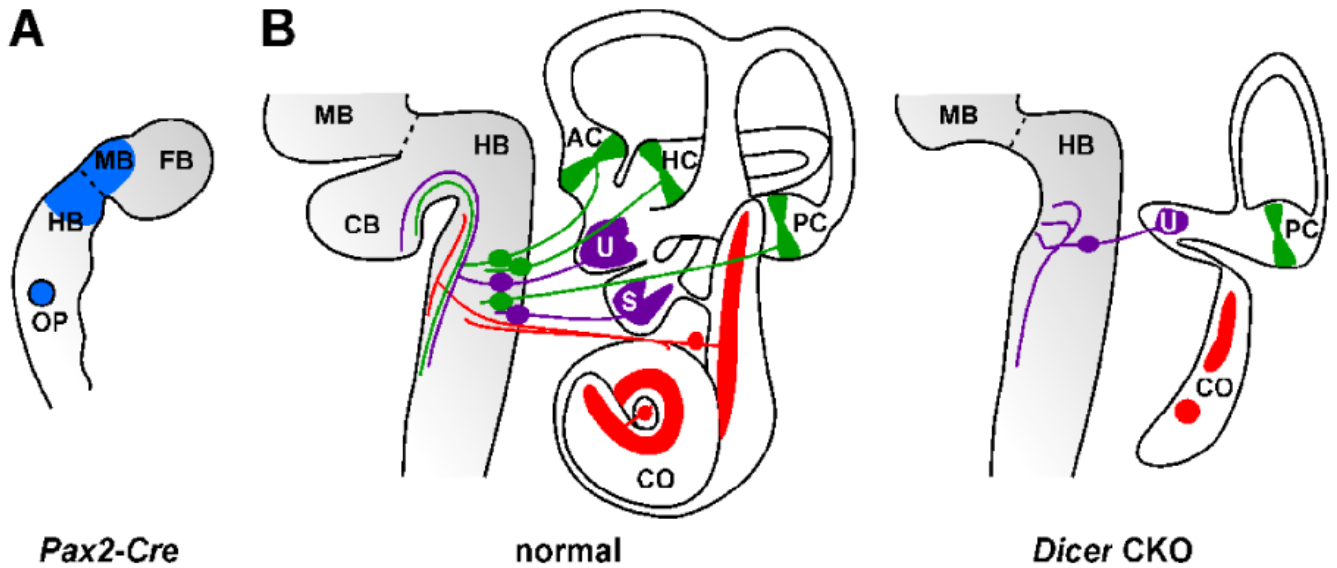


pairing to the miRNA “seed” sequence comprising nucleotides 2 through 7 (red) or 8 (orange). Other base-pairing interactions near the 3' end of the miRNA can contribute further specificity and/or affinity.

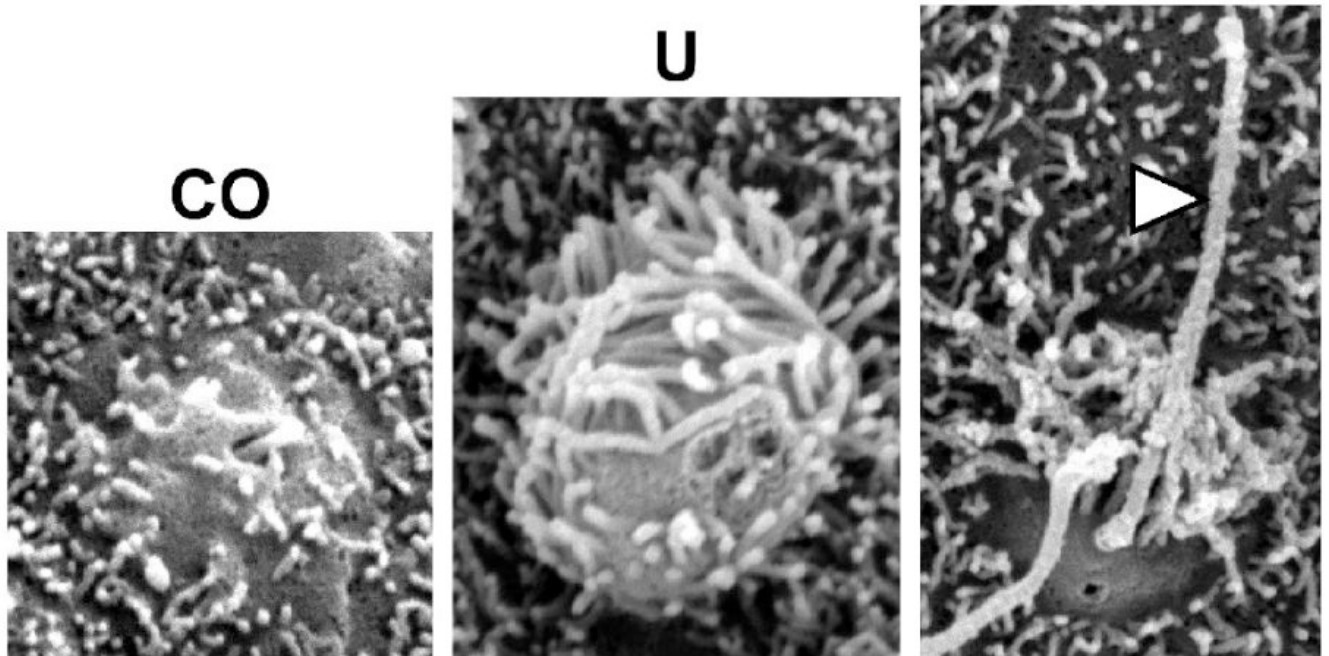


**Fig. 2.**

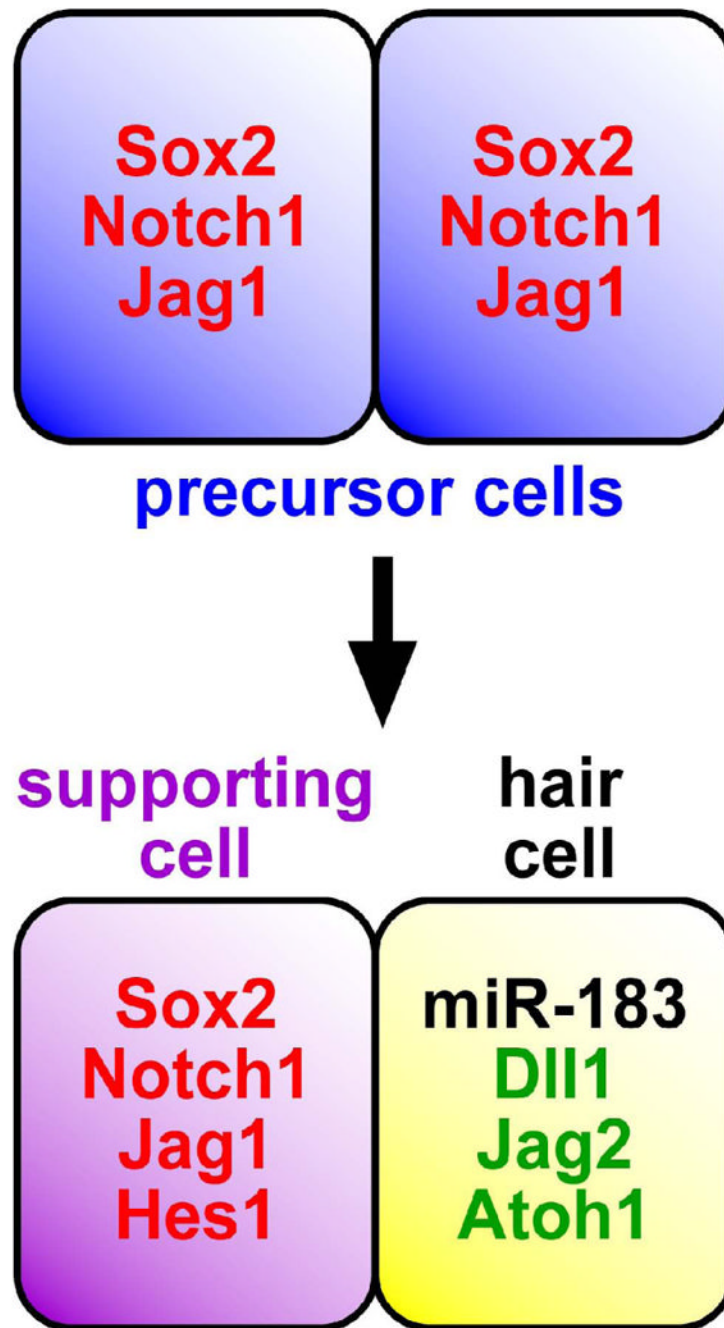
Neurosensory miR-183 family. (A) miR-183 family member sequence and homology. miR-183, miR-96, and miR-182 are clustered within 2-4 kb in vertebrate genomes and co-expressed from a single pri-miR transcript. The vertebrate miRNAs (black) share substantial sequence homology, particularly with the seed sequence (red/orange). Other members of the neurosensory miRNA family include homologous miR-263b and miR-228 (blue) respectively found in *D. melanogaster* and *C. elegans*. (B) miR-183 expression in newborn mouse cochlea. miR-183 is detected specifically in inner and outer hair cells (IHC and OHC, respectively) in the organ of Corti by in situ hybridization using a digoxigenin-labeled LNA probe.



**Fig. 3.** *Dicer* conditional knockout (CKO) and affect of miRNA depletion on inner ear development. (A) Tissue-specific *Cre* recombination in transgenic *Pax2-Cre* mouse embryo (~8.5 days post coitus). *Cre*-mediated recombination supports conditional reporter gene expression (blue) in the midbrain (BM), anterior hindbrain (HB), and otic placode (OP). Also indicated are the mid-hindbrain boundary (dashed line) and forebrain (FB). (B) Overview of developmental defects in *Pax2-Cre Dicer* CKO inner ear (~17.5 days post coitus). Schematically depicted is normal morphology, histology and innervation of the inner ear and brain (left) versus that of the *Dicer* CKO (right). *Dicer* CKO and loss of miRNAs results in a substantial loss of mid-hindbrain structure including the cerebellum (CB), auditory and vestibular neurons, and certain sensory epithelia of the inner ear (colored) are variously affected. Remaining fibers innervating the utricular macula fail to project properly to the brain. AC, anterior crista; HC, horizontal crista; PC, posterior crista; U, utricular macula; S, saccular macula; CO, cochlea.

**Dicer CKO****PC****residual  
miR-183**

**Fig. 4.** Residual miRNA expression correlates with extent of hair cell differentiation in *Dicer* CKO inner ear sensory epithelia (~17.5 days post coitus). Absence or presence of apical specializations for presumptive hair cells in the cochlea (CO), utricular macula (U), and posterior crista (PC) correlate with observed loss or retention of residual miR-183. Hair cells in the utricular macula appear as bulbous microvillus cells whereas those in the posterior crista exhibit well-developed kinocilia (arrowhead).



**Fig. 5.** Mutually exclusive expression of miR-183 family members and predicted target genes. Precursor and/or supporting cells express predicted target genes (red) including Sox2, Notch1, Jag1, and Hes1, whereas hair cells expressing miR-183 family members (black) exclude such factors and otherwise express Dll1, Jag2, and Atoh1 (green). Genetic and/or biochemical interactions among these factors are key to establishing supporting versus hair cell fates, suggesting that miR-183 family members help tip the balance in favor of hair cell differentiation.