Residual microRNA expression dictates the extent of inner ear development in conditional Dicer knockout mice

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Abstract

Inner ear development requires coordinated transformation of a uniform sheet of cells into a labyrinth with multiple cell types. While numerous regulatory proteins have been shown to play critical roles in this process, the regulatory functions of microRNAs (miRNAs) have not been explored. To demonstrate the importance of miRNAs in inner ear development, we generated conditional Dicer knockout mice by the expression of Cre recombinase in the otic placode at E8.5. Otocyst-derived ganglia exhibit rapid neuron-specific miR-124 depletion by E11.5, degeneration by E12.5, and profound defects in subsequent sensory epithelial innervations by E17.5. However, the small and malformed inner ear at E17.5 exhibits residual and graded hair cell-specific miR-183 expression in the three remaining sensory epithelia (posterior crista, utricle, and cochlea) that closely corresponds to the degree of hair cell and sensory epithelium differentiation, and Fgf10 expression required for morphohistogenesis. The highest miR-183 expression is observed in near-normal hair cells of the posterior crista, whereas the reduced utricular macula demonstrates weak miR-183 expression and develops presumptive hair cells with numerous disorganized microvilli instead of ordered stereocilia. The correlation of differential and delayed depletion of mature miRNAs with the derailment of inner ear development demonstrates that miRNAs are crucial for inner ear neurosensory development and neurosensory-dependent morphogenesis.

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Introduction

The vertebrate ear’s ability to extract sound and angular and linear acceleration from mechanical stimuli requires an intricate three dimensional geometry that includes the strategic positioning of sensory epithelia and the appropriate histological organization of epithelial hair cells and supporting cells. In recent years, molecular analyses have revealed many factors that influence patterning, morphogenesis and histogenesis. For example, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) are essential factors that transduce early patterning signals into specific regional gene expression in developing prosensory epithelia, which in turn governs the morphogenesis of semicircular canals (Chang et al., 2004; Pauley et al., 2003, 2006; Nichols et al., 2008). Likewise, molecular steps in histogenesis of the sensory epithelia have emerged (Fritzsch et al., 2006; Kelley, 2006) and indicate that coordinated transitions of Eya1, Sox2, and possibly Isl1 and Neurog1 gene expression are required to up-regulate Atoh1 in postmitotic precursors for hair cell differentiation (Kiernan et al., 2005; Matei et al., 2005; Radde-Gallwitz et al., 2004; Raft et al., 2007; Zhou et al., 2008). Such developmental transitions are not only orchestrated by the regulatory functions of morphogens and transcription factors. Indeed, growing evidence demonstrates the widespread importance of non-coding RNAs in transcriptional and post-transcriptional regulation of eukaryotic gene expression (Amaral et al., 2008). In particular, the ~500 mammalian microRNAs (miRNAs) appear to play a substantial role in development, cell maintenance, and disease (Hobert, 2008; Makeyev and Maniatis, 2008).

Our prior work has shown that ~100 miRNAs are expressed in development and maturation of the inner ear (Weston et al., 2006). In addition, we have recently shown that hair cell-specific miRNAs are highly conserved across phyla and exhibit expression in known mechanosensory cells and neurosensory organs from C. elegans to mammals (Pierce et al., 2008), consistent with previous notions on the ancestry of mechanosensors suggested by the conservation of transcription factors (Fritzsch et al., 2007). If indeed these and other miRNAs play crucial roles in development as indicated by their abundant expression in the developing central nervous system (CNS)
and mammalian inner ear (Kapsimali et al., 2007; Weston et al., 2006), it is reasonable to hypothesize that major histogenetic and morphogenetic defects will result when small RNA production is abrogated using a conditional approach to eliminated the RNA processing enzyme Dicer (Harfe et al., 2005).

We have therefore examined the effect of conditional Dicer knockout (KO) in Pax2-Cre transgenic mice (Ohyama and Groves, 2004), which enable deletion of floxed Dicer alleles in ear, kidney, and mid-hindbrain. Moreover, the early expression of Pax2-Cre in the otic placode provides for a broad examination of the importance of Dicer and its small RNA products in inner ear development. The data demonstrate that small RNAs, including miRNAs, are required for inner ear development, maintenance of sensory neurons, and differentiation of sensory epithelia. Of particular interest is the finding that residual mature miRNAs appear to enable partial hair cell differentiation, suggesting that no neurosensory component of the ear can form in the complete absence of miRNAs. These data are consistent with our hypothesis that miRNAs are integral components of inner ear developmental programs, although the model cannot differentiate the biological impact of miRNAs and other small RNA products of Dicer (i.e. siRNAs). Nevertheless, the data validate to an extent previously unknown the essential function of small RNAs in neurosensory organ development.

Materials and methods

Generation of conditional Dicer knockout mice

Mice carrying floxed Dicer alleles (Dicer\textsuperscript{lox/lox}; Harfe et al., 2005) were mated to mice carrying a Pax2-Cre transgene (Ohyama and Groves, 2004) to generate Pax2-Cre/Dicer\textsuperscript{lox/wt} mice. Pax2-Cre; Dicer\textsuperscript{lox/wt} mice were subsequently mated to Dicer\textsuperscript{lox/lox} mice to generate Pax2-Cre; Dicer\textsuperscript{lox/lox} mutant embryos. Offspring were genotyped by PCR analysis of tail DNA using Cre-specific primers (5′-CCCTGCTACTACCGTCCATGGCAAAGA and 5′-GTGGCA-GATGGCGCGGCAACCATT) that produce a 726 bp product, and Dicer-specific primers (5′-CCTGACAGTACGTCCCAAAGG and 5′-CATGACCTCTCACAATCAGTACGTCCCAAAGG) that produce a 420 bp product from the Dicer\textsuperscript{lox} allele and a 351 bp product from the Dicer\textsuperscript{wt} allele. Mice carrying a Dicer\textsuperscript{wt} allele or lacking the Pax2-Cre transgene were used as control littermates. Embryos were harvested at E11.5, E12.5, E14.5, E17.5, and E18.5 from timed pregnant females counting from noon on the day the vaginal plug was found as E0.5. Embryos were perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) and tissues were isolated for analysis.

Primers used to discriminate the Dicer\textsuperscript{lox} allele and the deletion allele (Dicer\textsuperscript{Δ}) by PCR amplification of DNA isolated from inner ear tissues were 5′-CCCTGACAGTACGTCCCAAAGG and 5′-CTGACCTCTCAATCAGTACGTCCCAAAGG as previously described (Harfe et al., 2005).

In situ hybridization (ISH)

Whole-mount ISH detecting miRNA expression was performed as previously described (Weston et al., 2006) using locked nucleic acid (LNA) probes (Integrated DNA Technologies) labeled with digoxigenin (DIG; Roche). Briefly, tissues fixed with 4% PFA in 0.1 M phosphate buffer (pH 7.4) were deaffeted with ethanol and digested with proteinase K prior to hybridization of 12 pmol LNA probe, washing and RNase A digestion, and probe detection using alkaline phosphatase (AP) conjugated sheep anti-DIG Fab fragment and BM Purple AP Substrate (Roche). Tissues were whole mounted in glycerol and viewed by differential interference contrast microscopy on a Nikon Eclipse 800 microscope. Two ears or brains were analyzed at each time point described.

LNA probes antisense to miR-124 (5′-GGCTACCCGCTGCCGT, miR-183 (5′-TGTTGAATCTCAGGTGCACT), or miR-183* (5′-TTATGGCCCTTCGTAATC) contain LNA modifications at every third nucleotide position from the 5′ end. Such LNA probes have previously been shown to provide exceptional miRNA hybridization specificity that is sensitive to 1 or 2 nucleotide mismatches (Kloosterman et al., 2006; Pierce et al., 2008).

Whole-mount ISH detecting Fgf10 mRNA was similarly performed using a 552 nt riboprobe generated by in vitro transcription of PCR products derived from an Fgf10 cDNA clone using primers 5′-...
CACATTGTGCTCAGCGCTTC and 5′-TAATACGACTCAGTTAG-3′. Two ears or brains were analyzed at each time point.

**Neuronal tracing**

Lipophilic dye-soaked filter strips were inserted into the brainstem to label afferents and efferents separately, or in the ear to fill afferents from the ear or discrete sensory epithelia to the brain as previously described (Jensen-Smith et al., 2007). Ears or brains were mounted in glycerol and viewed using a Zeiss LSM 510 confocal microscope. From two to six specimens were analyzed at each time point described.

**Immunocytochemistry (ICC)**

Antibodies for acetylated tubulin (Sigma), BDNF (R&D Systems), Sox2 (Millipore), MyoVIIa (Proteus Biosciences), and Caspase 3 (Chemicon) were used for ICC as previously described (Matei et al., 2005; Pauley et al., 2006). Briefly, tissues fixed with 4% PFA in 0.1 M phosphate buffer (pH 7.4) were defatted with ethanol, blocked with goat serum, and incubated for 1 day with primary antibody. Secondary antibody was conjugated to either Alexa 543 or Alexa 648. Tissues were mounted in glycerol and viewed using a Zeiss LSM 510 confocal microscope. From two to six specimens were analyzed at each time point described.

**Scanning and transmission electron microscopy (SEM and TEM)**

Three E17.5 ears and one E18.5 ear were post-fixed in 0.5% glutaraldehyde, followed by 0.5% osmium tetroxide. Ears were either embedded in epoxy resin to obtain ultrathin sections for TEM (E18.5) or critical point dried for SEM (E17.5). Critical point dried ears were mounted on stubs, sputter coated and viewed in a JEOL JSM-840A SEM. Ultrathin sections were counterstained with uranyl acetate and lead citrate and viewed in a JEOL JEM-1011 TEM as previously described (Ma et al., 2000).

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**Fig. 2.** Defects in inner ear morphogenesis and sensory epithelial development of Pax2-Cre Dicer KO mice. (A) Stylized representation of normal inner ear morphology and sensory epithelia (colored). Indicated are the anterior, horizontal, and posterior cristae (AC, HC, and PC, respectively), utricular (U) and saccular (S) maculae, and organ of Corti in the cochlea (CO). Arrows indicate dorsal (D) and posterior (P) orientation for all images. (B–D) Morphology of an E17.5 Pax2-Cre Dicer KO mouse inner ear. Invariably, each KO mouse inner ear lacks recognizable anterior and horizontal cristae and a horizontal canal, has a well-formed posterior crista and canal, and forms a utricle and an abbreviated cochlea consisting of approximately one half-turn. Rarely observed is a small saccule and misformed anterior canal. The utricle (U) may contain a single otoconial mass, and the saccule (S) if present may contain a misshapen otoconial mass. (E) Normal sensory epithelial development indicated by X-gal staining of hair cells in Atoh1-LacZ mouse inner ear (i.e. Atoh1/lacZ/wt; Fritzsch et al., 2005). The inset depicts to scale a conditional Dicer KO ear. (F) Sensory epithelial development and innervation of the KO mouse inner ear. Sensory epithelia indicated by ICC detecting Sox2 invariably show a well-developed posterior crista, a utricular macula, and one or two sensory patches within the cochlea. Innervation shown by ICC detecting tubulin reveals fibers to the utricle, but not posterior crista. Few fibers innervate the cochlea but fail to target the sensory patch. Similar labeling of the normal cochlea is provide in Fig. S4D.
Results

**Conditional Dicer knockout mouse model**

To investigate the function of small RNAs in mouse inner ear development, we have generated Pax2-Cre conditional Dicer knockout (KO) mice (i.e. Pax2-Cre;Dicer^{flox/flox}). KO mice have a number of developmental defects owing to Dicer deletion in Pax2-Cre expression domains that likely contribute to late embryonic lethality at approximately E18.5. The Pax2-Cre transgene is strongly expressed in the mid-hindbrain, kidney, and otic placode at ∼E8.5 (Ohyama and Groves, 2004). KO mice thus exhibit notable defects in mid-hindbrain development that include ablation of the cerebellum and tectum (Figs. 1A and B) and reduced kidney size (data not shown). Interestingly, KO mice also demonstrate obvious defects in craniofacial and eyelid development (Fig. 1A) outside the expected domain of Pax2-Cre expression. These defects are currently being investigated to determine whether they are direct or indirect effects of Pax2-Cre conditional Dicer KO.

To confirm the loss of functional Dicer and depletion of miRNAs, neuron-specific expression of miR-124 was examined in E11.5 KO mice and control littermates (e.g. Pax2-Cre;Dicer^{flox/wt}). In situ hybridization (ISH) using a locked nucleic acid (LNA) probe for miR-124 demonstrates that expression is abundant in the control mouse throughout the mid-hindbrain and in sensory neurons of the trigeminal ganglia and vestibulocochlear ganglia (Fig. 1C). However, miR-124 expression is absent in the KO mouse throughout the caudal midbrain and anterior hindbrain (i.e. rhombomere 1), and in the vestibular and acoustic ganglion neurons that derive from the otocyst. Importantly, hindbrain miR-124 expression remains in the hindbrain posterior to rhombomere 1, suggesting that the model does not directly perturb vestibular and cochlear efferents from rhombomere 4 (Bruce et al., 1997), or known effects of rhombomeres 5 and 6 on early patterning of the otocyst (Bok et al., 2007). These data suggest that functional Dicer and neuron-specific miRNAs are largely depleted within three days from the time of Pax2-Cre expression in the mid-hindbrain and otic placode. Moreover, the otocyst of the KO mouse is notably smaller at this early point in inner ear development (Fig. 1C).

To ensure that Pax2-Cre transgene expression and floxed allele recombination precisely recapitulates that previous observed (Ohyama and Groves, 2004) in the context of conditional Dicer KO, tissues from E17.5 control and KO mice carrying a Rosa26-LacZ reporter (R26R) allele (Soriano, 1999) were examined by X-gal staining. As expected, X-gal staining shows that Pax2-Cre expression supports recombination throughout the mid-hindbrain (Fig. 1B) and the inner ear (Fig. S1) of control mice and any remaining brain and ear...
tissues of KO mice. In particular, X-gal staining is observed in all sensory epithelia of control and KO mouse inner ears including the posterior crista as previously reported (Ohyama and Groves, 2004). However, it should be noted that some cells in the dorsoposterior part of the otic vesicle and non-sensory cells of the posterior crista may be reporter-negative (Ohyama and Groves, 2004). Nevertheless, the data suggest that Pax2-Cre expression domains retain their definition in the inner ear and midbrain of the Dicer KO model.

Overview of effects on inner ear development

The inner ear of mice consists of six sensory epithelia located within the vestibular and auditory organs that include the anterior, horizontal, and posterior cristae, utricular and saccular maculae, and the organ of Corti that runs the length of the cochlea (Fig. 2A). Conditional Dicer KO mice demonstrate a significant truncation of most inner ear structures at E17.5 (Fig. 2B). Although the KO inner ear exhibits a well developed posterior crista and canal, the horizontal crista and canal and the anterior crista are always absent, and the anterior canal is either absent or substantially reduced. A reduced utricle is always present (Figs. 2B and C) and a small saccule is sometimes present (Figs. 2B and D), either of which rarely contain misshapen otoconial masses. The cochlea presents only as a comma-shaped structure with no coils.

A comparison of normal inner ear and conditional Dicer KO inner ear provides perspective to the overall reduction in KO inner ear development. A developmentally normal inner ear at E18.5 from an Atoh1-LacZ mouse (i.e. Atoh1LacZ/wt; Fritzsch et al., 2005) shows X-gal staining of hair cells throughout the sensory epithelia (Fig. 2E) compared to scale with an E17.5 Dicer KO inner ear (Fig. 2E; inset). The truncation of KO inner ear sensory epithelial development is demonstrated by immunocytochemistry (ICC) detecting Sox2, which is specifically expressed in prosensory epithelia and in supporting cells following hair cell differentiation (Kiernan et al., 2005; Dabdoub et al., 2008). The E17.5 KO inner ear thus exhibits a well-developed posterior crista and relatively small utricular and cochlear patches of sensory epithelium development (Fig. 2F). Moreover, labeling of nerve fibers by ICC detecting tubulin shows that innervation of the E17.5 KO inner ear is severely reduced (Fig. 2F). Whereas a few fibers targeting the utricular macula remain, there is no innervation of the posterior crista and any fibers remaining to the cochlea fail to target the developing sensory epithelium (Fig. S2). These data demonstrate that conditional Dicer KO in the developing mouse inner ear results in profound morphogenic, histogenic and innervation defects.

Defects in sensory epithelial histogenesis

The histological development of sensory epithelia in the E17.5 KO mouse inner ear was further evaluated by ICC detecting brain-derived neurotrophic factor (BDNF) and myosin VIIa (MyoVIIa) markers for hair cells. The sensory epithelium of the cochlea expressing Sox2 most often appears as two distinct basal and apical patches (Fig. 3A).

Fig. 4. Fine structure of Pax2-Cre Dicer KO mouse inner ear sensory epithelia. (A) SEM of E17.5 KO mouse inner ear sensory epithelial apical specializations. Presumptive hair cells of the cochlea (CO), posterior crista (PC), and utricular macula (U) depicted at relative low (left) and high (right) magnification show disorganized microvillus extensions with only posterior crista hair cells exhibiting kinocilia and normal organization of stereocilia. (B) Thin section TEM of E18.5 KO mouse inner ear sensory epithelia. Depicted are sections of each sensory epithelium at various magnification as indicated. Supporting cells and hair cells of the cochlea lack the distinctive normal cellular organization of the organ of Corti into inner and outer hair cell rows separated by pillar cells. Hair cells in the posterior crista show normal organization. The utricular macula exhibits presumptive hair cells showing mostly disorganized microvilli and is the only epithelium to show evidence of synapse formation with pre-synaptic vesicles (asterisk).
Relative few cells within each patch demonstrate detection of BDNF (Figs. 3B and C), a documented hair cell factor that attracts nerve fibers (Tessarollo et al., 2004). Moreover, these presumptive hair cells fail to assume the precise organization of inner and outer hair cell rows seen in the normal organ of Corti (Fig. 3D). However, many cells within each patch appear MyoVIIa positive (Fig. 3E), suggesting they might represent developing hair cells.

The Sox2-positive sensory epithelia of the utricle and posterior crista demonstrate detection of relatively more BDNF positive and MyoVIIa positive cells (Figs. 3F–J). The utricular macula shows a circular morphology rather than the typical kidney-shaped sensory epithelium, whereas the posterior crista demonstrates a normal separation of the two hemicristae by a cruciate eminence. The sensory epithelia of the KO inner ear therefore demonstrate a range of morphological and histological definition from the well-developed posterior crista to the poorly-developed cochlea. Interestingly, the absence or presence of BDNF in developing hair cells does not strictly correlate with the lack of innervation indicated by tubulin ICC (Fig. 2F), suggesting that the inability of nerve fibers to properly target sensory epithelia is intrinsic to the neurons rather than a failure of developing hair cells to provide neurotrophic support. Overall, the conditional Dicer KO mouse phenotype is highly penetrant, with variation amongst specimens observed only in regard to the absence or presence of a discernible saccule, and the presence of 1 or 2 sensory epithelial patches in the cochlea.

Defects in hair cell development

To further examine developing hair cells in KO inner ear sensory epithelia, the detailed cellular anatomy was examined by tubulin ICC and electron microscopy. Tubulin ICC shows that most developing hair cells possess kinocilia (Fig. S2). The positioning of kinocilia shows that near normal hair cell polarity develops in the posterior crista and in the cochlea for one case where the organ of Corti demonstrates some...
organization into inner and outer hair cells. However, there was no evidence for formation of striolar polarity reversal in the utricular macula, where kinocilia positioning appeared mostly random.

Examination of E17.5 and E18.5 inner ear sensory epithelia by scanning electron microscopy (SEM) and transmission electron microscopy (TEM; Figs. 4A and B, respectively) shows that apical specializations were found to be fairly normal in the posterior crista. However, the utricular macula shows unusual organization of apical specializations presenting as multiple elongated microvilli in stark contrast to normal stereocilia development (Fig. S3). If developed, cochlear hair cells show unusual stereocilia organization having multiple clustered rows (Fig. 4B). Additionally, TEM demonstrates that both afferent and efferent synapses form in the utricular macula (Fig. 4B), but no innervation is detected in developing hair cells of the posterior crista or cochlea consistent with the absence of nerve fibers observed by tubulin ICC.

**Defects in innervation**

Our initial examination of the E17.5 conditional Dicer KO inner ear suggests a near absence of innervation (Fig. 2F), where only the utricular macula receives specific projections that contact developing hair cells (Fig. 4B). The data also show that neuron-specific miR-124 expression is absent by E11.5 (Fig. 1C). Therefore, sensory neuron development was further examined (Fig. 5) using recently improved lipophilic dyes (Jensen-Smith et al., 2007) as in previously published studies on ear development (Farinas et al., 2001; Tessarollo et al., 2004). At E11.5, near normal innervation of the KO inner ear was observed with fibers extending toward the posterior crista (Fig. 5A), albeit in a less directed growth pattern than the control littermate (Fig. 5B). At E12.5, fibers do not progress in their projection to the posterior crista of the KO inner ear as they do in the control (Figs. 5C and D), and few fibers extended toward the utricle and the missing anterior and horizontal cristae in the KO inner ear (Fig. 5C; inset). Similarly, central projections showed near normal vestibular fiber elongation along the hindbrain and toward the cerebellum at E11.5 that do not progress beyond rhombomere 1 into the cerebellum at E12.5 (Figs. 5E and F). These data suggest that the growth and pathfinding properties of neurons are affected within one day from loss of neuronal miRNA expression, which is evident approximately three days following conditional Dicer KO.

The sparse innervation of the KO inner ear is further demonstrated by tubulin ICC (Figs. S2 and S4). At E17.5, few fibers are observed to reach the sensory epithelium of the utricle or, if present, the saccule.

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**Fig. 6.** Brainstem development in E12.5 control and conditional Dicer KO mice. Whole-mounted brains show the distribution of Fgf10 expression by ISH in branchial motoneurons and efferents (A, B). Indicated is expression in trigeminal (V), facial (FBM), olivo-cochlear efferents (OC), and nucleus ambiguous branchial motoneurons (NA). The cerebellum (CB) shows Fgf10 expression in the control, but not in the KO brain. The KO cerebellum instead exhibits numerous birefringent cells in dark field illumination (C). ICC detecting caspase 3 with Hoechst staining of nuclei shows that these cells are macrophages that contain several nuclei of dying cells (inset). Note the absence of apoptosis in the floor plate of rhombomere 1, indicating that only proliferating and/or differentiating neurons are affected by Dicer KO.
Fibers to the cochlea are much reduced and extend only in the base toward developing hair cells. The apical fibers typically extend along the cochlea without targeting the developing sensory epithelium. Most obvious is the complete absence of any innervation of the posterior crista, which is otherwise the most normal developed sensory epithelium of the KO inner ear.

A closer examination of E17.5 KO inner ear innervation indicated the presence of very few vestibular neurons to the utricle (∼ 15–35 in two different mutants) that could be traced after lipophilic dye injection into the brainstem (Figs. 5G–I). We also examined the number of neurons labeled after lipophilic dye injection into the ear and found ∼ 30 small neurons scattered along the nerve fibers reaching toward the brain. Only an occasional efferent fiber was found at this late stage (E17.5) to reach to the inner ear (Fig. 5J), implying near complete loss of efferent innervation in the KO inner ear (Bruce et al., 1997; Fritzsch, 1999; Simmons, 2002). The central projection of KO inner ears showed vestibular afferent fibers with a normal projection along the descending vestibular nucleus (Fig. 5K).

Consistent with the fact that Pax2-Cre is not expressed in the hindbrain rhombomeres from which facial branchial motoneurons and efferent neurons originate (Karlis et al., 2001), an examination of E12.5 control and conditional Dicer KO mouse brainstem by ISH shows similar sensory neuron expression of fibroblast growth factor 10 (Fgf10) mRNA (Fig. 6). Accordingly, lipophilic dye staining of hindbrain efferents shows normal development at this early stage (Figs. 7A and S5), although there is a severe reduction of afferent and efferent fibers in the auditory nerve (Figs. 7B–D). Further examination of the ear shows that conditional Dicer KO in auditory afferents initially has a more profound effect on the afferents than hindbrain efferents projecting to the ear (Figs. 7E–G). These data suggest that loss of afferent neuronal miRNA expression affects cues along sensory

Fig. 7. Efferent and afferent development in the brainstem and ear of E12.5 control and conditional Dicer KO mice. A. Absence of effects on ear-related hindbrain development in Dicer KO mice demonstrated by normal labeling of the facial nerve (VII root) and facial branchial motoneurons (FBM) in rhombomeres 4–6 (∗4–6). Left and right sides are labeled green and red, respectively. B–D. Labeling of the auditory nerve afferents (VIII root) and efferents (arrow) in E12.5 control (B) and Dicer KO (C, D) mice. Backfilling from the ear shows a large afferent and efferent component in the control (B), whereas the Dicer KO shows a severe reduction of afferents extending anterior–posterior (horizontally) in the hindbrain (C) and a severe reduction in the efferent fiber bundle (compare arrow in B to those in panels A, C, D). E–G. Sensory neuron development in E12.5 control (E) and Dicer KO (F, G) inner ear. KO ears show a severe reduction in afferent fibers (red) of the inferior and superior vestibular ganglia (IVG and SVG, respectively) with only some fibers extending toward the posterior crista (PC). There is also a reduction of efferent fibers (green) projecting to the ear, albeit less profound than afferent reduction, and normal labeling of the facial nerve (VII root). Bars represent 200 μm in panel A and 100 μm in B–G. Vd, descending tract of the trigeminal; SS, superior salivatory nucleus; IN, intermediate nerve.
neurons that are required for efferent fiber growth (Fritzsch et al., 1998a,b; Matei et al., 2005). Moreover, analysis of cell death by ICC detecting caspase 3 shows that regions of Pax2-Cre expression and conditional Dicer KO in the cerebellum (Fig. 6C; inset), midbrain (Figs. 8A–C), and inner ear (Figs. 8D and E) exhibit a substantial number of apoptotic cells consistent with analysis in other conditional Dicer KO models (Davis et al., 2008).

In summary, the data demonstrate a near normal initial formation and growth of sensory neurons with rapid and near complete loss of all innervation after the loss of afferent neuronal miRNA expression at E11.5 in the conditional Dicer KO inner ear. Defects in sensory neuron projection and loss of sensory neurons result from either loss of target structures or fibers required for neuronal pathfinding if not directly from conditional Dicer KO. Whether the few remaining afferent neurons at E17.5 (Fig. 5G–K) are impervious to the loss of miRNA expression or retain low levels of some miRNAs remains unclear.

**Defects in morphogenesis**

Prior work has shown that canal formation hinges on the expression of fibroblast growth factor 10 (Fgf10) and bone morphogenic proteins (BMPs) in canal crista epithelia (Pauley et al., 2003; Chang et al., 2004). Given that canal/crista formation is most affected in conditional Dicer KO inner ears (Figs. 2B and F), Fgf10 mRNA expression was examined throughout development by ISH (Fig. 9A). Fgf10 expression in the E11.5 control inner ear is prominent in the delaminating vestibular neurons, utricle, and each canal crista. KO inner ears likewise show expression in the vestibular neurons, utricle, and posterior canal crista. However, there is only limited expression in a poorly defined anterior canal crista, and no expression suggestive of a horizontal canal crista is observed. At E12.5, dramatic differences in Fgf10 expression are seen between KO and control inner ears. Whereas the control shows expression in sensory neurons and all sensory

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**Fig. 8.** Cell death in the brain and ear of an E12.5 conditional Dicer KO mouse. Depicted is ICC detection of caspase 3 and tubulin with Hoechst staining of nuclei in the midbrain (A–C) and ear (D, E). There is a thinning of the midbrain in areas of caspase 3 expression (A, B) where caspase 3-positive cells exhibit multiple pycnotic nuclei (C–C″). The inferior vestibular ganglion (IVG) shows clusters of caspase 3-positive cells that are tubulin-negative (D). Observed throughout the nerve trajectory to the posterior crista (PC) are fragments of caspase 3-positive and tubulin-positive fibers.
epithelia, the KO demonstrates a loss of expression in sensory neurons and retains only strong expression in the posterior canal crista and utricle, and weak expression in the cochlea. By E14.5, differences in Fgf10 expression are even more pronounced between KO and control inner ears. While the control continues to demonstrate expression throughout sensory neurons and epithelia, the KO shows only strong expression in the posterior crista and weak expression in the utricle. The rapid loss of Fgf10 expression in sensory neurons of the conditional Dicer KO inner ear corresponds well to the early depletion of neuronal miRNAs (Fig. 1C), loss of sensory neurons through apoptosis (Fig. 8), and decline of neuronal differentiation (Fig. 5). Moreover, the respective absence, gradual loss, or persistence of Fgf10 expression in the posterior crista and weak expression in the utricle.

Fig. 9. Correlation of Pax2-Cre Dicer KO inner ear morphogenesis with developmental gene expression and residual mature miRNA expression. (A) Progressive loss of Fgf10 expression in KO inner ear. At E11.5, the KO inner ear exhibits diminished but discrete expression of Fgf10 in sensory epithelia (PC, posterior crista; AC, anterior crista; HC, horizontal crista; U, utricle; CO, cochlea). At E12.5, Fgf10 expression is observed only in the posterior crista and utricular macula, and expression is entirely lost in vestibular ganglia (VG) and the developing cochlea. By E14.5, Fgf10 expression remains only in the posterior crista with faint expression in the utricular macula, in sharp contrast to extensive expression in all sensory epithelia and delaminating neurons of control littermate inner ear. (B) ISH detecting miR-183 and miR-183* in KO and control littermate inner ear. Throughout the control inner ear, miR-183 expression is apparent in statoacoustic ganglia (SAG) and hair cells of developing sensory epithelia, whereas mir-183* does not accumulate as detectible pre-miRNA or mature miRNA. In the KO inner ear, miR-183 is detected primarily in the posterior crista (PC; filled arrowhead) whereas miR-183* is not (open arrowhead), thus suggesting that miR-183 detection results from residual mature miRNA production rather than detection of accumulated pre-miRNA. Labels are as indicated in the legend to Fig. 2.
expression in horizontal, anterior, or posterior crista correlates with the observed degree of canal formation driven by epithelial Fgf10 signaling (Fig. 2B). It is also interesting to note that the overall size and morphology of the Dicer KO and control inner ears are somewhat synchronous up to E12.5 in contrast to obvious differences at E17.5 (Fig. 2E), suggesting that cell proliferation and growth are largely affected after E12.5.

Residual mature miRNA expression

To understand why there exist such variation between the degree of sensory epithelial and hair cell development in the conditional Dicer KO inner ear, we examined whether Pax2-Cre transgene expression, floxed Dicer deletion, or residual miRNA expression might underlie the observed phenotype. Importantly, R26R expression did not indicate any deviation from the reported expression domain of the Pax2-Cre transgene (Ohyama and Groves, 2004) in conditional Dicer KO or control mice (Fig. S1). We therefore investigated whether any residual mature miRNA expression could explain the variability of the model. Notably, rapid and thorough depletion of miR-124 in the mid-hindbrain and delaminating vestibular ganglia of the KO mouse (Fig. 1C) suggests efficient Dicer deletion and miRNA depletion in prosensory precursors. Accordingly, ISH detecting miR-183, which is normally expressed in all hair cells and sensory neurons of the mouse inner ear (Weston et al., 2006; Pierce et al., 2008), shows absence of miR-183 in remaining sensory neurons of the E17.5 KO inner ear (Fig. S6A). However, some miR-183 is surprisingly observed in KO inner ear sensory epithelia, most prominently in the posterior crista and sometimes weakly in the utricle and cochlea.

To establish whether detection of miR-183 represents mature miRNA expression versus detection of accumulated miRNA precursors, we performed ISH to detect miR-183*, the complementary strand to miR-183 in the precursor miRNA hairpin, reasoning that it should be detected at the same level as miR-183 if detection represents precursor miRNA accumulation. At E14.5, miR-183 detection is robust throughout the sensory neurons and epithelia of the control inner ear, but present only in the posterior crista of the KO inner ear (Fig. 9B). However, miR-183* was undetectable in either control or KO inner ears, consistent with miR-183 detection representing genuine mature miRNA expression. This residual mature miRNA expression persists despite confirmation that the floxed Dicer allele is indeed deleted in the conditional KO inner ear by PCR analysis of DNA isolated from the posterior crista (Fig. S6B).

The data therefore suggest that Dicer mRNA and/or Dicer protein must persist for at least 6 days to generate mature miRNAs in developing hair cells at E14.5 in some sensory epithelia of the Pax2-Cre conditional Dicer KO inner ear, but not sensory neurons. Once formed, mature hair cell miRNAs persist for at least 3 more days until detection at E17.5. Importantly, the differential capacity for residual miRNA expression within and between different ears correlates well with the extent of inner ear neurogenesis and innervation, Fgf10 expression and morphogenesis, sensory epithelial histogenesis, and hair cell development observed in the conditional Dicer KO mouse.

Discussion

Our data show that progressive reduction of miRNAs through the conditional KO of Dicer using Pax2-Cre results in progressive loss of neurosensory gene expression, arrested neurosensory development and loss of sensory neurons, loss or reduction of sensory epithelia, and associated disruption of morphogenesis (Fig. 10). While delayed and apparently incomplete by E18.5, the oldest viable embryos we could obtain, the depletion of miRNAs demonstrates to a degree unknown in other systems the dependence of neurosensory development on miRNA expression.

Technical considerations of the conditional KO model

One of the striking features of our conditional KO model is the apparent differential retention of some Dicer in certain hair cell precursors but not in others. It is likely that hair cells up-regulate specific miRNAs (e.g. miR-183) only after exit from cell cycle, which shows specific differences across various epithelia (Ruben, 1967). In contrast, Dicer is likely expressed in all cells of early embryos, as Dicer null mutants exhibit early lethality by E7.5 (Bernstein et al., 2003). Therefore, residual Dicer in our conditional KO model would be required to generate mature forms of hair cell-specific miRNAs. In support of these presumptions, we validated that floxed Dicer is indeed eliminated and showed that miRNA detection represents active mature forms rather than inactive precursor accumulation in remaining hair cells. We were unable to immunohistochemically confirm the presence of Dicer protein, but consider the detection of residual mature miRNAs as proof of residual Dicer protein activity in developing hair cells. Consistent with recent observations regarding the effect of conditional Dicer KO and miRNA depletion in post-mitotic cerebellar Purkinje cells (Schaefer et al., 2007), it appears that there can be substantial differences in the rates at which Dicer protein, Dicer mRNA, and/or mature miRNAs are metabolized. Such issues have to our knowledge not been observed in other conditional Dicer KO models, where progressive loss of miRNAs might contribute to a

Fig. 10. Overview of developmental defects in Pax2-Cre Dicer KO inner ear. (A) Normal morphology, histology and innervation of wild type inner ear and brain. (B) Morphology, histology and innervation of the KO inner ear and brain. The remaining sensory epithelia of the inner ear are variously affected by a loss of miRNAs, and the only remaining fibers innervating the utricular macula fail to project properly to the brain.
degree of randomness that characterizes cell fate decision in general (Losick and Desplan, 2008).

Neuronal development

Sensory neurons are among the first cells of the ear to exit cell cycle and initiate differentiation (Ma et al., 1998, 2000; Matei et al., 2005). Analysis shows that these neurons are initially positive for factors such as Fgf10 and send processes toward specific sensory epithelia of the ear and into the brain. However, as early as E12.5 the expression of neuron-specific factors including Fgf10 and mir-124 are lost, extension of both peripheral and central processes stalls, and there is a near-complete loss of neurons by E18.5. Vestibular neurons and their fibers to the end organs exhibit caspase 3 expression, a reliable marker for cell death. At later stages, the few remaining neurons synapse only on utricular hair cells. Interestingly, the most normal developed sensory epithelium of the ear, the posterior crista, shows no trace of innervation at any stage although fibers initially extend toward it.

It remains unclear whether the general loss of neurons and innervation relates exclusively to rapid cell death caused by Dicer KO which is known to have variable effects dependent upon neuron type and/or time of Dicer deletion (Schaefer et al., 2007; Cuellar et al., 2008; Davis et al., 2008). Alternatively, such effects might reflect miRNA-mediated phenotypic differences (Caçarolígu et al., 2008) or miRNA-mediated lack of sensory epithelial differentiation and loss of epithelial neurotrophins that maintain neurons (Farinas et al., 2001; Fritzsch et al., 2004). It is possible that the earliest neurons to exit the cell cycle at E8.75 retain enough Dicer and miRNAs to complete near normal differentiation and extend peripheral processes to the nearest sensory epithelium (i.e. the utricular macula). In contrast, more distant or later differentiating epithelia receive little to no innervation possibly because the neurons are depleted of miRNAs before they reach their target (e.g. posterior crista) or because the target has not completed differentiation (e.g. organ of Corti). Conditional deletion of Dicer using inducible neuron-specific or prosensory-specific Cre expression driven by promoters for Neurog1 (Raft et al., 2007) or Is11 (Lin et al., 2006), respectively, is needed to distinguish between direct neuronal effects and sensory epithelia defects. The disorganized central projection that develops after normal onset of projection suggests direct effects as Pax2-Cre is not expressed in rhombomeres 2–6. However, the near complete loss of the cerebellum, which derives from the midbrain and rhombomere 1 (Hatten and Heintz, 1995), could produce secondary effects in the remaining brainstem.

Sensory epithelial development

As with neurons, the initial up-regulation of sensory epithelial specific factors such as Fgf10 is near normal at E11.5 in conditional Dicer KO mice. However, Fgf10 expression is entirely lost within one day in the anterior and horizontal crista and is down-regulated in the utricular macula. It is noteworthy that only the posterior crista retains high levels of Fgf10 expression, and it is the only near normal sensory epithelium developed at E17.5 as evidenced by hair cell and supporting cell specific gene expression (e.g. MyoVIIa, BDNF, Sox2) and cytoarchitecture. As previously demonstrated in Neurog1 null mice (Ma et al., 2000) and neurotrophin and neurotrophin receptor null mice (Fritzsch et al., 1997; Rocha-Sanchez et al., 2007), hair cell differentiation is cell-autonomous and continues in the absence of innervation (Fritzsch et al., 1998a,b).

With similarity to several transcription and growth factor mutant mice (Pirvola et al., 2000; Pauley et al., 2003, 2006; Kiernan et al., 2005) and notch signaling mutant mice (Daudet and Lewis, 2005; Kiernan et al., 2006), we find in conditional Dicer KO mice that anterior and horizontal cristae do not differentiate despite an initial expression of crista-specific factors such as Fgf10 (Pauley et al., 2003). Certain sensory epithelia are therefore highly sensitive to precise timing and expression levels of factors that are integral to the regulation of normal epithelium development from prosensory domains. Even where sensory epithelia form in conditional Dicer KO mice, overall cellular organization is disrupted as exemplified by the cochlea where discontinuous patches or multiple rows of inner and outer hair cells form as reported for several transcription and growth factors mutants (Pirvola et al., 2002; Kiernan et al., 2005; Pauley et al., 2006). Which one of these factors, or yet to be described factors, is primarily affected by miRNA depletion remains to be demonstrated.

Hair cell development

Consistent with our recent description of the ancestry of transcriptional regulatory machinery in hair cells (Fritzsch et al., 2007), including the ancestry of miRNAs (Pierce et al., 2008), are specific defects of hair cells that include incomplete transformation of microvilli into stereocilia. With similarity to factors that lead to patchy distribution of hair cells or formation of multiple rows, numerous factors are known to affect stereocilia development (Leibovic et al., 2005; Ahmed et al., 2006; Friedman et al., 2007). More work is needed to understand which of these factors requires precise levels of certain miRNAs for normal function. Nevertheless, the fact that hair cells form but cannot fully differentiate underscores the critical role of miRNAs in the normal development of these highly specialized cells. The general requirement for miRNAs to achieve differentiation is supported by other models of small RNA or miRNA depletion in embryonic stem cells (Kanellopoulou et al., 2005; Wang et al., 2007) and conditional Dicer KO tissues (Muljo et al., 2005; Andl et al., 2006; Lynn et al., 2007).

Summary

miRNAs and other small RNAs are among the best understood non-coding functional RNAs produced by the mammalian genome. In recent years, miRNAs have been shown to be involved in multiple aspects of disease, development, and maintenance (Amaral et al., 2008; Couzin, 2008), and they exhibit regulatory functions that parallel the importance of the better known transcription factors (Hobert, 2008; Makeyev and Maniatis, 2008). These regulatory functions combined with the increased number of miRNAs in vertebrates have prompted speculations about the possible involvement of miRNAs in the evolution of complexity (Heimberg et al., 2008). Our data further the appreciation of such known and presumed functions by demonstrating that specific levels of miRNAs are necessary for neurosensory development of the inner ear and, directly or indirectly, morphogenesis of the labyrinth. Importantly, we demonstrate a 3–10 day delay between elimination of Dicer and depletion of specific miRNAs, implying that the extent of inner ear development is reflective of a hypomorphic miRNA condition rather than complete miRNA abrogation. Our data therefore suggest that critical levels of miRNAs are needed to achieve any extent of neurosensory inner ear development. An earlier onset of floxed Dicer deletion through pre-placodal Cre expression at least one day prior to Pax2 (Streit, 2007) is needed to eliminate miRNAs before formation of the otocyst to fully demonstrate the requirement of miRNAs for inner ear development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdybio.2009.01.037.

References


