Molecular mechanisms underlying inner ear patterning defects in *kreisler* mutants

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Abstract

Prior studies have shown that *kreisler* mutants display early inner ear defects that are related to abnormal hindbrain development and signaling. These defects in *kreisler* mice have been linked to mutation of the *kr/mafB* gene. To investigate potential relevance of *kr/mafB* and abnormal hindbrain development in inner ear patterning, we analyzed the ear morphogenesis in *kreisler* mice using a paint-fill technique. We also examined the expression patterns of a battery of genes important for normal inner ear patterning and development. Our results indicate that the loss of dorsal otic structures such as the endolymphatic duct and sac is attributable to the downregulation of *Gbx2*, *Dlx5* and *Wnt2b* in the dorsal region of the otocyst. In contrast, the expanded expression domain of *Otx2* in the ventral otic region likely contributes to the cochlear phenotype seen in *kreisler* mutants. Sensory organ development is also markedly disrupted in *kreisler* mutants. This pattern of defects and gene expression changes is remarkably similar to that observed in *Gbx2* mutants. Taken together, the data show an important role for hindbrain cues, and indirectly, *kr/mafB*, in guiding inner ear morphogenesis. The data also identify *Gbx2*, *Dlx5*, *Wnt2b* and *Otx2* as key otic genes ultimately affected by perturbation of the *kr/mafB*–hindbrain pathway.

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Introduction

In the United States, congenital permanent hearing loss has been estimated to affect as many as 1–3 infants per 1000 births (1991; 2000; Mehl and Thomson, 1998). Reports have also estimated that upwards of 50–60% of cases of congenital hearing loss have a genetic etiology (Brookhouser, 1993). The presence of a hearing loss at birth suggests that many cases may result from a perturbation of inner ear development that makes normal hearing impossible. Accordingly, it is important to study the genetic processes involved in inner ear development that may have relevance to such a common congenital disorder.

First described in the early 1940s by Hertwig and then later by Deol and Ruben, the *kreisler* mouse was generated by X-ray mutagenesis and identified by its circling behavior and deafness noted in offspring of the irradiated founders (Hertwig, 1944). Morphological studies described the earliest defect in *kreisler* mutant inner ears as a failure of the endolymphatic duct to differentiate from the otocyst (Deol, 1964; Ruben, 1973). Subsequently, the inner ears developed into cystic, poorly differentiated structures in mutant mice. Roughly 50 years after the original description of the *kreisler* strain by Hertwig, Cordes and Barsch (1994) identified the genetic defect as a chromosomal microinversion affecting a zinc finger, leucine zipper transcription factor referred to as the *kr/mafB* gene (kreisler/musculoaponeurotic fibrosarcoma B). *kreisler* mutants fail to develop rhombomeres 5 and 6 of the embryonic hindbrain and this neural tube defect is associated with a loss of normal *kr/mafB* expression in these rhombomeres. Studies also suggest that those regions of the neural tube that fail to differentiate into rhombomeres 5 and 6 appear to adopt a rhombomeres 4 identity and are subsequently eliminated by programmed cell death (McKay et al., 1994). Intriguingly,
expression studies have confirmed that \textit{kr}/\textit{mafB} is not normally expressed in the early developing ear itself but is instead normally restricted to those rhombomeres (5 and 6) immediately adjacent to the otic region between embryonic day 8.5–10. As a result, investigators have proposed that loss of hindbrain \textit{kr}/\textit{mafB} expression in mutant mice results in an altered inductive signal from the hindbrain to the developing ear that ultimately leads to the inner ear malformations. However, the potential molecular targets of this hindbrain signaling pathway have yet to be identified. We focused on otic gene expression patterns to examine the molecular processes in the developing inner ear that are ultimately affected by perturbation of the \textit{kr}/\textit{mafB}–hindbrain pathway. While a complex cascade of genetic events in the hindbrain is clearly involved prior to effects observed in the inner ear, we focused our attention on identification of early otocyst-specific genes. Our data demonstrate that the absence of rhombomeres 5 and 6 and loss of normal hindbrain \textit{kr}/\textit{mafB} activity ultimately results in a loss of \textit{Gbx2}, \textit{Dlx5} and \textit{Wnt2b} expression in the dorsal part of the otic epithelium that eventually gives rise to the endolymphatic duct. \textit{Otx2}, a gene important for cochlear patterning, shows a medially-expanded expression pattern suggesting an inhibitory role of \textit{kr}/\textit{mafB} in its regulation. Later expression of otic markers (such as lunatic fringe, \textit{Lfng}, Neurofilament 68, \textit{Nf68}, Pendred syndrome gene, \textit{Pds} and Tyrosinase-related protein 2, \textit{Trp2}) is also altered in \textit{kreisler} mutants suggesting that global ear patterning is perturbed by abnormal hindbrain patterning and \textit{kr}/\textit{mafB} mutation.

Materials and methods

Mice

The \textit{kreisler} mouse colony described in this study was maintained and handled according to an IACUC-approved protocol. Homozygous mutants were generated by mating male homozygotes with female heterozygotes. The morning a vaginal plug was observed was designated as embryonic day 0.5. A PCR-based genotyping method was performed as previously described (Frohman et al., 1993).

Paint filling

Mouse embryos were harvested at each embryonic day between E10 and E17 and processed for paint filling as previously described (Choo et al., 1998; Martin and Swanson, 1993; Morsli et al., 1998).

Whole mount and serial section situ hybridization

Embryos between E8.5 and E11.5 were processed for wholemount in situ hybridization with older embryos processed for cryosections as previously described (Choo et al., 1998; Morsli et al., 1998, 1999). Sense and antisense riboprobes were generated from plasmids for: \textit{Pan2} (Torres et al., 1996), \textit{Gbx2} (Liu and Joyner, 2001; Millet et al., 1999), \textit{Otx2} (Morsli et al., 1999), \textit{Dlx5} (Depew et al., 1999), \textit{Pds} (Everett et al., 1999), \textit{Trp2} (Zhao and Overbeck, 1999), \textit{Bmp4}, \textit{Nf68} and \textit{Lfng} (Morsli et al., 1998), \textit{Gata3} (Karis et al., 2001) and \textit{Wnt2b} (Ng et al., 2002).

Assay for programmed cell death and cell proliferation

Terminal dUTP Nick End Labeling (TUNEL) was performed to assay for cells undergoing programmed cell death using an Apoptag® Plus kit (Oncor, Inc) as previously described (Choo et al., 1998). At least 5 \textit{kreisler} and control embryos at each embryonic day between E9.5 and E13.5 were examined by TUNEL assay (n = 5 at E9.5, n = 6 at E10.5, n = 5 at E11.5, n = 5 at E12.5 and n = 5 at E13.5).

An immunohistochemical technique using a biotinylated mouse monoclonal antibody against Proliferating Cell Nuclear Antigen (PCNA, Zymed Labs, Inc.) was utilized as an assay of cell proliferation (Takahashi et al., 1994; Tsue et al., 1994; Umemoto et al., 1995). Cryosections from \textit{kreisler} mutant and control embryos between E9.5 and E13.5 were incubated with the anti-PCNA-antiserum for 30–60 min after preblocking for 10 min according to manufacturer’s recommended guidelines. A manufacturer-supplied secondary antibody and diaminobenzidine (DAB)–peroxidase reaction was then used to detect the antibody along with a hematoxylin counterstain. Total counts of TUNEL- and PCNA-labeled cells were taken from serial sections of the inner ear from an equal number of \textit{kreisler} and control embryos between E9.5 and E13.5 (n = 5 at E9.5, n = 6 at E10.5, n = 4 at E11.5, n = 4 at E12.5 and n = 4 at E13.5).

Results

The \textit{kreisler} adult ear phenotype

The \textit{kreisler} strain used in these studies was a congenic line derived in a C3H background (Cordes and Barsch, 1994). This differed from the CBA strain previously described by Ruben (Ruben, 1973) as well as the B6CBAc strain at Jackson Laboratories (Bar Harbor, ME). Accordingly, we confirmed that the adult behavioral, hearing loss and inner ear phenotype in our C3H strain was identical compared to that reported in earlier studies. Hyperactive circling behaviors and the absence of Preyer’s reflex, auditory brainstem responses and an endocochlear potential were all consistent with previously reported \textit{kreisler} phenotypes. Serial semithin plastic sections of 4 adult mutant mice also showed the same poorly differentiated inner ear structures and dilated cochlear morphologies consistent with earlier reports of \textit{kreisler} mice. These functional and morphologic data confirm previous reports of the \textit{kreisler} phenotype and are not shown here.

Developmental morphology of the \textit{kreisler} inner ear

Because of the intricate anatomy of the mouse inner ear and the potential for missing subtle anomalies even with meticulous serial sectioning, we analyzed developing \textit{kreisler} inner ears using a paint-fill technique from the otocyst stage through embryonic day 17 (E17). Prior morphologic studies of \textit{kr}/\textit{mafB}−/− mutants in different background strains relied on histologic sections without any 3-dimensional reconstruction to define the phenotype (Deol, 1964; Ruben, 1973). The paint-fill technique provided a rapid and sensitive method for demonstrating the \textit{kr}/\textit{mafB}−/− phenotype. More than 60 \textit{kreisler} mutant embryos from E10 to E17 were paint-filled to evaluate the developmental morphology. Seven to 10 homozygous \textit{kreisler} embryos (along with an equal number of heterozygous control littermates) were analyzed at each embryonic day.

The earliest difference between \textit{kr}/\textit{mafB}−/− and control littermates could be demonstrated at E9–E9.5 when the position of the early otic cup could be noted in a more lateral
displaced position relative to the neural tube (data not shown). Fig. 1A shows an E10.5 kr/mafB−/− paint-injected specimen along with a control littermate (Fig. 1B). The arrowed brackets in Fig. 1A indicate the abnormal separation between the otocyst and the neural tube edge (nt). At this stage, 9 of 10 kr/mafB−/− embryos showed an absence of the early endolymphatic duct (ed, endolymphatic duct in Fig. 1B) at the dorsal surface of the otocyst. By E10.75, the kr/mafB−/− otocysts show a ventral elongation indicating initial outgrowth of a cochlear projection (Fig. 1C). This rudimentary cochlea continues to expand ventrally through E11 and E12 with a circumferentially distended cochlear duct noted in most specimens at E11.5 and E12.5 (arrows in Figs. 1D, E and F; n = 6/8 for E11.5; n = 7/9 for E12.5) compared to the E12.5 control (Fig. 1G, arrows). In some kr/mafB−/− E12.5 specimens, an initial coiling of the distal cochlear projection could be noted (see Fig. 1E).

The vertical canal plate at E11.5 is frequently smaller compared to controls (Fig. 1D, n = 6/8). Semicircular canal morphology showed greater variability than the early cochlear projection at E11 or E12. As demonstrated in Figs. 1E and F, canal plates showed varying degrees of central resorption (arrowheads in Fig. 1E) or even absence of recognizable vertical canal plates (Fig. 1F). The lateral canal showed less tendency for perturbation with 7/9 E12 mutant specimens showing at least a rudimentary lateral canal plate (lcp in Fig. 1F).

By E15.5, the kr/mafB−/− specimens typically showed an obvious cochlear duct with a minimal amount of coiling (Figs. 1H–J). Note that a poorly differentiated saccule-like structure can be seen in some specimens at E15 (see Fig. 1H; sac, saccule). Although canal malformation is variable, the lateral canal is the least commonly affected and is identifiable in 18/22 specimens between E15 and E17. Notably, ears that did not develop a lateral canal displayed a more severe overall ear phenotype with grossly malformed cochlear ducts and no evidence of a common crus, other semicircular canals or the endolymphatic duct and sac (EDS). It is noteworthy that 2 embryos showed interaural differences indicating the potential for variability of the ear phenotype within a given specimen. This intra-specimen variability could also be observed in terms of the degree of cochlear coiling. Among all paint-filled embryos (between E13 and E17, n = 37), differences in semicircular canal and/or cochlear morphology from one ear to the other (within one embryo) were noted in 4 specimens. A
heterozygous \((kr/mafB^{+/−})\) E15.5 paint-filled inner ear is shown in Fig. 1K.

Based upon the paint-fill data of embryos between E15 and E17, we grouped the 22 kreisler specimens into 3 phenotypic categories (I, II and III) based upon the pattern of inner ear structures affected (for summary, see Table 1). The predominant defect in kreisler mutant ears is an absence of the EDS and common crus (CC) with dilated cochleas and variable defects of the anterior and posterior semicircular canals. Type I inner ears showed the specific absence of the EDS and CC \((n = 7)\). The Type II phenotype included the EDS and CC defects along with abnormalities of the anterior and posterior canals \((n = 10)\). The Type III phenotype was the most severe with no EDS, CC and all semicircular canals affected \((n = 5)\). In all phenotypes, the cochlear ducts were abnormal and showed globular and nominally-coiled appearances. In one Type III specimen, a ventral cochlear projection could not be identified.

**A loss of early endolymphatic duct and sac markers in kreisler mutants**

One of the earliest developmental aberrations of kreisler mice is the failure of the endolymphatic duct to evaginate from the dorsal tip of the otocyst. Based upon this morphologic observation, we examined genes expressed in the dorsal and medial aspects of E9–11 otocysts that could serve as molecular markers of the developing endolymphatic duct (such as Wnt2b). We also studied the expression patterns of genes such as Dlx5 and Gbx2, whose null mutant phenotype included an EDS phenotype (Depew et al., 1999; Lin et al., 2005).

At E9 (~20 somites), the otic epithelium diffusely expresses Dlx5 and is comparable in kreisler mutants and controls (data not shown). However, at E9.5, Dlx5 signal in the dorsal–medial otocyst is down-regulated (compare arrows in Figs. 2A and E) while the dorsal–lateral signal is unchanged (arrowheads in Figs. 2A and E). Similarly, Gbx2 expression is absent in E9 \(kr/mafB^{+/−}\) embryos as evidenced by the loss of signal at the dorsal rim of the otocyst (arrows in Fig. 2C and arrowheads outlining the otocyst in Fig. 2G). Acknowledging that Gbx2 is also expressed in the mid–hindbrain junction (in addition to the otocyst), we carefully examined the neural tube expression of Gbx2 in kreisler mutants. Gbx2 expression in the mid–hindbrain region (arrows in Figs. 2D and H), is unchanged in kreisler mutants. The only change in kreisler mutants is the loss of Gbx2 signal in the dorsal otocyst (arrowheads in Figs. 2D and H).

At E11 (Figs. 2B, I, K), control specimens show a well-defined ED and substantial elongation along the dorsal–ventral axis. Dlx5 expression persists in the \(kr/mafB^{+/−}\) inner ears and can be observed along the dorso-medial otic epithelium and continues laterally in the epithelium that eventually gives rise to the semicircular pouch (arrows, Fig. 2B). In \(kr/mafB^{+/−}\) specimens (Figs. 2F, M and O), the otocyst is noticeably smaller than control inner ears and no ED outpouching is observed. However, Dlx5 expression in the dorsal half of the mutant otocyst appears comparable to the expression seen in controls (arrows, Fig. 2F). In contrast, Gbx2 expression is absent in the kreisler E11 ears (Fig. 2M) with no Gbx2 mRNA detected in other regions of the otocyst \((n = 10)\). Arrows in Fig. 2M mark the approximate regions of the medial otocyst where the endolymphatic duct should be forming and where Gbx2 expression is noted in the control ear (compare to arrows in Fig. 2I). Gbx2 expression continues to be absent at E12 and E13 in kreisler mutants (data not shown).

Similar to Gbx2, Wnt2b expression is absent at E9.5 in kreisler mutants \((n = 7, \text{Fig. 2N})\). The earliest Wnt2b expression demonstrable by in situ hybridization was E9.5 (20 somites, Fig. 2J). Loss of Wnt2b signal was also noted at E10 \((n = 5)\) and E11 \((n = 4)\) as well as later stages in \(kr/mafB^{+/−}\) otocysts that lack an endolymphatic duct (compare arrows in Figs. 2K and O). In E11 controls, Wnt2b is normally expressed in the epithelium marking the endolymphatic duct \((n = 4)\), arrows in Fig. 2K).

**An expansion of early Otx2 expression in kreisler mutants**

In the developing mouse central nervous system, Otx2 and Gbx2 interactions have been implicated in refining and/or positioning the midbrain–hindbrain boundary by means of a mutually antagonistic mechanism (Li and Joyner, 2001; Millet et al., 1999). Given that our data indicated a down-regulation of Gbx2 in the developing kreisler otocyst, we studied Otx2 in kreisler mutants to determine whether analogous Otx2–Gbx2 interactions were involved in inner ear patterning and if these were potentially perturbed by \(kr/mafB\) mutation and the hindbrain abnormalities in kreisler mice. At E11 (Figs. 2L and P), in situ data show a medial expansion of the Otx2 expression domain in the kreisler cochlear ducts \((n = 4)\). Normally restricted to the lateral epithelium of the early cochlea (arrows in Fig. 2L), Otx2 transcripts were noted to extend along the medial cochlear duct (arrowheads in Fig. 2P).

A similar expression pattern for Otx2 persists at E12.5 \((n = 2)\)
and E13.5 (n = 2) (data not shown). It is noteworthy that Gbx2 expression is not normally observed in the developing cochlear duct and the expression domains of Otx2 and Gbx2 do not abut each other as seen in the midbrain–hindbrain (Lin et al., 2005).

Sensory organ development in kreisler mutants

We also examined how patterning of the sensory organs (organ of Corti, cristae and maculae) as well as the cochlear–vestibular ganglia were affected by kreisler mutation. Using lunatic fringe (Lfnig) as a molecular marker for these sensory organs (Morsli et al., 1998), we performed in situ hybridization experiments on E15–16 kreisler mutants. These data show that Lfnig is expressed in scattered patches of epithelium in both the vestibular regions of kreisler inner ears as well as small patches in the cochlea (Figs. 3A–C). Seven kr/mafB−/− embryos probed for Lfnig expression were systematically analyzed for each sensory organ (results summarized in Supplementary Table 2). The data showed that the anterior and posterior cristae were variably affected and tended to be absent in the more severe (Type III) inner ears. In contrast, the lateral crista was almost always identifiable in all specimens regardless of the severity of the phenotype (for example, see crista in Fig. 3A). At least one (and usually 2) specific patches of Lfnig expression could be noted in the cochlear regions of kr/mafB−/− mutants (arrows in Figs. 3B and C). In addition, these patches typically expressed both Myo15a as well as Gata3; supporting their identity as sensory-hair cell related patches (Figs. 3E–G). A heterozygous E15.5 control specimen is shown in Fig. 3H.

Fig. 2. Early gene expression in kreisler. As early as E9.5 (A), Dlx5 expression is noted by in situ hybridization at the dorsal half of the otic epithelium (arrows indicate the dorso-medial expression while arrowheads indicate the dorsolateral expression in A). This pattern of expression along the entire dorsal half of the otocyst persists at E10.5 including the EDS (arrows in B). However, in kreisler mutants at E9.5 (E), the dorso-medial signal for Dlx5 is absent. At slightly later timepoints, a dorso-medial Dlx5 signal is seen (arrows in F) despite the absence of an EDS. Gbx2 expression is observed along the very dorsal rim of the otic epithelium at E9.5 (arrows in C and arrowheads in D) and is then restricted to the dorso-medial region of the otocyst and ED by E10.5 (arrows in I). All otic Gbx2 signal is absent in kreisler mutants at E9.5 and E10.5 (arrowheads in G and H, and arrows in M, respectively). Wnt2b expression in the dorsal rim of the E9.5 otocyst (arrows in J) and the dorso-medial epithelium at E10.5 (arrows in K) are downregulated in kreisler mutants (arrowheads in N outline the otocyst void of any signal while arrows in O mark the comparable area from K that lacks any Wnt2b signal). Normal Otx2 expression at E11.5 is demonstrated by the arrows in panel L. In kreisler mutants, an expansion of the Otx2 domain along the medial cochlear duct is indicated by the arrowheads in panel P. Orientation axes in A apply to B, E, F, I, K, L, M, O and P. Orientation axes in C apply to G, J and N. D and H are images taken from a directly dorsal view. Scale bars = 100 μm.
Four *kreisler* embryos at E15.5 were studied by in situ hybridization using a probe for *Gata3* as a means of assessing spiral ganglion development. All 4 specimens were either Type II or III based upon semicircular canal defects, the absence of an EDS and the typical uncoiled cochlear duct. In 3 mutant ears, a small spiral ganglion could be demonstrated by *Gata3* in situ hybridization (Fig. 3G). In the 4th specimen (Type III), a definitive spiral ganglion could not be identified.

Analogously, alternate sections from the above E15.5 specimens were probed with *Nf68* as a means of examining vestibular ganglion development. Distinct *Nf68* signal in proximity to the vestibular structures in *kreisler* mutants was observed in all specimens (gangl, Fig. 3I). Fig. 3J shows a control specimen with the anticipated *Nf68* expression in the vestibular ganglion.

**Cell proliferation and programmed cell death in kreisler inner ears**

An abnormality in regulated cell proliferation and/or programmed cell death during inner ear morphogenesis were considered potentially relevant to the generation of inner ear malformations in *kreisler* mice. Accordingly, we immunohistochemically labelled proliferating cells (using a monoclonal mouse anti-PCNA-antibody) and TUNEL-labelled those cells undergoing apoptosis using alternate serial sections from embryos between E9.5 and E13.5.

At E9.5, proliferating cells are observed diffusely throughout the otic epithelium of both control and *kreisler* mutant embryos (Figs. 4A and E). No statistically significant difference in the number of proliferating cells could be demonstrated between *kreisler* mutants (*n* = 5) and controls (*n* = 5) at this stage (*α* = 0.10). However, a significantly increased number of TUNEL profiles (*α* ≤ 0.05) was noted in *kreisler* otocysts (*n* = 6, Fig. 4B) compared to controls (*n* = 6, Fig. 4F) at E9.5. A particularly obvious increase in TUNEL-labelled profiles was observed in the lateral otocyst (bracketed arrows in Fig. 4B) as well as the geniculate ganglion region of *kreisler* mutants (arrowheads in Fig. 4B) compared to control specimens.

Between E10.5 and E13.5, no statistically significant changes in the number of PCNA-labelled or TUNEL-labelled cells could be demonstrated in the otic epithelium (*n* = 6 at E10.5; *n* = 4 at E11.5; *n* = 3 at E12.5; *n* = 3 at E13.5). Despite the size and shape of *kreisler* inner ears being different and smaller than control ears (see Fig. 1), the patterns of both PCNA-labelled and TUNEL-labelled cells in the inner ears were similar between *kreisler* and control specimens (E10.5 PCNA and TUNEL data shown in Figs. 4C, D, G and H). Specific attention was directed at areas such as the fusion plate.
region in the canal pouches at E11.5–12.5, which have previously been demonstrated as “hotspots” of PCD in normally developing inner ears (Fekete et al., 1997; Nishikori et al., 1999). No changes in TUNEL labeling could be observed in those areas between 11.5 and E13.5 (data not shown).

Discussion

Morphogenesis of the kreisler inner ear

This report provides new morphologic data on the developmental inner ear phenotype in kreisler generated by paint filling of the membranous labyrinth. We drew several conclusions regarding the effects of kr/mafB mutation and the loss of rhombomeres 5 and 6 on inner ear morphogenesis. First, the hindbrain perturbations in kreisler mutants globally affect inner ear development. The cochlea, vestibular structures, ganglia and EDS are all perturbed to some extent. Our observation that the EDS and common crus are almost always affected, while the lateral canal is least often affected, suggest that a more dorsal and medial (vs. lateral) defect is incurred by loss of normal kr/mafB function. The progressive dilation of the membranous labyrinth in kreisler mutants led us to consider that the early failure to form an EDS may contribute to this distended phenotype. Taking into consideration the proposed endolymph-regulating role of the EDS in the inner ear, it seems reasonable to speculate that absence of the EDS contributes to a failure of kreisler ears to develop or maintain a proper endolymph composition and volume. Correlative evidence of the EDS being relevant to inner ear morphogenesis can be drawn from Mansour’s report on Fgf3 null mutants (Mansour et al., 1993) and Foxi1 mutants (Hulander et al., 2003). In the Fgf3 mutant mice, a similar developmental failure to form an EDS results in a comparable (although notably less severe) distended inner ear phenotype. In the case of Foxi1 mice, a failure to form a properly functioning EDS results in a much milder, but still dilated overall inner ear phenotype. Accordingly, one possibility is that kr/mafB mutation results in a failure of the EDS to develop which then disrupts endolymph homeostasis and this abnormal endolymph volume/composition then disrupts normal morphogenesis of the entire inner ear. Given the dramatically abnormal molecular patterning in kreisler inner ears (elaborated on below), we find it difficult to attribute all of these changes to the absence of an EDS.

Semicircular canal defects in kreisler

Paint-fill data from kreisler mutants suggested that canal defects could potentially be due to altered cell proliferation during canal pouch formation and/or dysregulated apoptosis. The majority of kreisler E11.5 embryos (Fig. 2D) demonstrated smaller canal pouch compared to controls. This observation raised the possibility that an adequate canal pouch never developed in these kreisler mutants and subsequent defects of the canals are due to this failure to generate a requisite mass of canal epithelium. Based upon tritiated-thymidine experiments, Ruben reported that kreisler otocysts demonstrated a shortened cell cycle and, somewhat contradictorily, implicated a higher mitotic rate (Ruben, 1973). However, our PCNA experiments looking at cell proliferation in kreisler ears between E9.5 and E13.5 have not shown any obvious differences in cell proliferation compared to controls.

Similarly, TUNEL labelling of cells undergoing programmed cell death did not show any marked changes except at E9.5 when kreisler mutant otocysts showed a statistically significant increase in TUNEL profiles. The number of apoptotic cells also seemed to be particularly increased along
the lateral otocyst epithelium (Fig. 4B). Fate-mapping the rim of the otic cup in chicken has shown that a large region of the lateral otic epithelium gives rise to the vertical and lateral canal pouches (Brigande et al., 2000). Assuming mice have a similar fate map as that of chicken, the increased cell death in the lateral wall of kreisler otocysts at E9.5 could have contributed to the smaller size of the vertical canal pouch observed at E11. Whether the initial increase in cell death and the size reduction of the canal pouch contributed to the expanded and seemingly disorganized areas of resorption in the canal plates at later stages is not clear.

A loss of endolymphatic duct markers in kreisler mutants

We initially selected candidate otic targets of the kr/mafB–hindbrain pathway based upon their expression pattern in the dorsal and/or medial otocyst (which gives rise to the EDS) or based upon a similar EDS phenotype in null mutant mice. As an example, Dlx5 and Gbx2 were studied given the comparable absence of an EDS in some lateral otic epithelium gives rise to the vertical and lateral canal of the otic cup in chicken has shown that a large region of the lateral wall of kreisler otocysts at E9.5 could have contributed to the smaller size of the vertical canal pouch observed at E11. Whether the initial increase in cell death and the size reduction of the canal pouch contributed to the expanded and seemingly disorganized areas of resorption in the canal plates at later stages is not clear.

The expression domain of Otx2 in the otocyst is expanded in kreisler mutants, similar to the observations in Gbx2 knockout mice (Lin et al., 2005). Taken together with earlier reports on the complimentary expression boundaries of Otx2 and Gbx2 in the developing chick inner ear (Hidalgo-Sanchez et al., 2000; Sanchez-Calderon et al., 2002) and the antagonistic relationship between Gbx2 and Otx2 in the midbrain–hindbrain region, we propose that loss of kr/mafB expression and hindbrain signaling results in the downregulation of Gbx2 expression in the inner ear which then leads to an expansion of the Otx2 domain in the mutant cochleae. We further propose that such an altered Otx2 domain might then perturb the normal patterning of the cochlea and result in altered cell populations in the kreisler cochleae. The unusual and scattered hair cell patches in kreisler ears (as shown by Lfng, Mys15a and GATA3 in situ hybridization data, Figs. 3A–I) are all consistent with such a process.

Cochlear patterning in kreisler mutants

The expression domain of Otx2 in the otocyst is expanded in kreisler mutants, similar to the observations in Gbx2 knockout mice (Lin et al., 2005). Taken together with earlier reports on the complimentary expression boundaries of Otx2 and Gbx2 in the developing chick inner ear (Hidalgo-Sanchez et al., 2000; Sanchez-Calderon et al., 2002) and the antagonistic relationship between Gbx2 and Otx2 in the midbrain–hindbrain region, we propose that loss of kr/mafB expression and hindbrain signaling results in the downregulation of Gbx2 expression in the inner ear which then leads to an expansion of the Otx2 domain in the mutant cochleae. We further propose that such an altered Otx2 domain might then perturb the normal patterning of the cochlea and result in altered cell populations in the kreisler cochleae. The unusual and scattered hair cell patches in kreisler ears (as shown by Lfng, Mys15a and GATA3 in situ hybridization data, Figs. 3A–I) are all consistent with such a process.

Fig. 5. Genes likely involved in the kr/mafB–hindbrain pathway and early inner ear patterning. In the posterior hindbrain, a complex interaction of Fgf5 and Hox genes likely regulates kr/mafB expression. Binding sites for MysD and kr/mafB have been identified in the promoter of kr/mafB suggesting a role in kr/mafB (auto)regulation. Data from Lin et al. also suggest that Gbx2 in the hindbrain negatively regulates kr/mafB expression. Our data focusing on the early otocyst show that normal cues from the kr/mafB–hindbrain pathway are required for Gbx2 expression in the ear. Dorsomedial Dlx5 and Wnt2b expression at E9.5 is also dependent upon normal hindbrain cues while ventral expression of Otx2 in the otocyst is negatively regulated by the kr/mafB–hindbrain pathway. Arrows from the hindbrain to the otocyst do not imply direct interactions or relationships.
kr/mafB and hindbrain cues for patterning the inner ear

The putative pathways involved in early otocyst differentiation are schematically presented in Fig. 5. Fully acknowledging gaps in our understanding of the hindbrain-to-ear signaling pathways, this model provided a template into which certain components in the distal pathway could be inserted.

At the level of the hindbrain, expression of kr/mafB is likely autoregulated by its own product as well as MyoD. Both kr/mafB and MyoD have been shown to activate Maf recognition elements (MAREs) in the kr/mafB promoter region (Huang, 1999). Various Hox genes have also been demonstrated to affect hindbrain segmentation and kr/mafB expression in rhombomere 5 (Barrow et al., 2000) while Fgf8s have been shown in chick embryos to regulate kr/mafB expression (Marin and Charnay, 2000). Hox proteins also interact with kr/mafB to inhibit its DNA binding, transactivation and transforming activities (Kataoka et al., 2001). And as reported by Lin et al., Gbx2 likely plays a negative inhibitory role in hindbrain kr/mafB expression (Lin et al., 2005). As a result, a myriad of molecular interactions involving kr/mafB are ongoing in the posterior hindbrain in a manner that is temporally and spatially relevant to early inner ear patterning.

At the otocyst level, our data as well as the data from Lin et al. suggest that Gbx2 is one of the key otic targets of hindbrain signaling. Furthermore, in the dorsal and medial regions of the otocyst, disruption of normal hindbrain development and cues by kr/mafB mutation perturbs Dlx5 and Wnt2b which appears to be associated with failure of the EDS to form. In the more ventral portion of the early otocyst, loss of normal hindbrain signals in kreisler mutants results in an expanded Otx2 expression domain. Given the negative regulatory effect of Gbx2 on Otx2 in the CNS, it seems plausible to consider similar mechanisms might be utilized in the ear and that loss of otic Gbx2 activity in kreisler mutants would then allow for the expansion of the Otx2 expression domain in kreisler cochleas.

With regards to the actual signals that convey the cues from the hindbrain to the inner ear (the schematic red arrows in Fig. 5), several molecules (Wnts and Fgf8s, for example) have been implicated as either inducing formation of the inner ear or providing cues for its differentiation (Anniko and Schacht, 1984; Ladher et al., 2000; Represa et al., 1991; Wright and Mansour, 2003). However, the exact signals from the hindbrain and the targets of these hindbrain signals in the inner ear have remained elusive.

It is also critical to acknowledge the additional signaling cues from ectoderm and mesoderm, as proposed by Ladher and Wright, (Ladher et al., 2000; Wright et al., 2004) that together with neural signals comprise the overall developmental program that guides inner ear morphogenesis. The loss of normal hindbrain cues in kreisler mutants may perturb not only the neural cues for inner ear development, but also the balance of signals from surrounding tissues that guide proper ear development. This area requires further investigation.

Gaps clearly exist in identifying additional components of the kr/mafB–hindbrain pathway that ultimately guides ear development. However, by focusing on the early otocyst, we have begun identifying differentially expressed genes in this pathway that may help us develop a more refined hypothesis of the “upstream” molecular pathways. Continuing experiments include array-based comparisons of early (E9–E10.5) kreisler and control transcripts in an attempt to identify other differentially expressed otic genes that are regulated by the hindbrain–kreisler pathway.

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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.ydbio.2005.10.007.

References


