Gbtx2 is required for the morphogenesis of the mouse inner ear: a downstream candidate of hindbrain signaling
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Accepted 28 February 2005

Summary
Gbtx2 is a homeobox-containing transcription factor that is related to unplugged in Drosophila. In mice, Gbtx2 and Otx2 negatively regulate each other to establish the midhindbrain boundary in the neural tube. Here, we show that Gbtx2 is required for the development of the mouse inner ear. Absence of the endolymphatic duct and swelling of the membranous labyrinth are common features in Gbtx2+/− inner ears. More severe mutant phenotypes include absence of the anterior and posterior semicircular canals, and a malformed saccule and cochlear duct. However, formation of the lateral semicircular canal and its ampulla is usually unaffected. These inner ear phenotypes are remarkably similar to those reported in kreasler mice, which have inner ear defects attributed to defects in the hindbrain. Based on gene expression analyses, we propose that activation of Gbtx2 expression within the inner ear is an important pathway whereby signals from the hindbrain regulate inner ear development. In addition, our results suggest that Gbtx2 normally promotes dorsal fates such as the endolymphatic duct and semicircular canals by positively regulating genes such as Wnt2b and Dlx5. However, Gbtx2 promotes ventral fates such as the saccule and cochlear duct, possibly by restricting Otx2 expression.

Key words: Gbtx2, Otx2, kreasler, Hindbrain signaling, Inner ear development, Otic vesicle, Mouse

Introduction
The mammalian inner ear develops from a thickening of the ectoderm adjacent to the hindbrain known as the otic placode, which invaginates to form the otocyst. The teardrop-shaped otocyst then undergoes a series of morphogenetic events to give rise to a structurally complex inner ear, consisting of vestibular and auditory components. In the vestibular component, three semicircular canals and their associated sensory tissues (cristae) housed within the ampullae, are responsible for detecting angular head movements. Two additional sensory tissues, the macula of the utricle and the macula of the saccule, are responsible for sensing gravity and linear acceleration, respectively. The auditory component, the cochlear duct, is a coiled structure in mammals. The molecular mechanisms that govern the development of these various components of the inner ear are largely unknown.

Tissues surrounding the inner ear, such as the hindbrain, mesoderm and endoderm, have been implicated in conferring signals required for inner ear development (for reviews, see Fekete, 1999; Kiernan et al., 2002). The importance of the hindbrain in this process is evident from analyses of mutant mice such as the Hoxa1 knockout and kreisler (for a review, see Kiernan et al., 2002). Both kreisler/Mafb and Hoxa1 are expressed in the hindbrain but not the inner ear, yet inner ears of mice with these genes mutated are abnormal. The inner ear defects of these mutant mice are attributed, in particular, to defects in rhombomere 5 (r5), a region of the hindbrain juxtaposing the developing otic placode (Kiernan et al., 2002).

Although the inner ear phenotypes in these mutants are variable, they often include the absence of the endolymphatic duct and an enlargement of the membranous labyrinth. The enlarged membranous labyrinth could be secondary to the loss of the endolymphatic duct, which has been shown to be important in maintaining fluid homeostasis within the membranous labyrinth (Everett et al., 2001; Hulander et al., 2003). In addition to the kreisler and Hoxa1 mutants, knockout of Raldh2 (retinaldehyde dehydrogenase 2) also results in otocyst malformations that are attributed to a defective hindbrain (Niederreither et al., 2000).

As both kreisler/Mafb and Hoxa1 are transcription factors, their effects on inner ear development are likely to be mediated via the regulation of signaling molecules. Several lines of evidence suggest that FGF3 might be one of these hindbrain-derived signals that mediate inner ear development. First, inner ears of Fgf3 knockout mice show a similar phenotype to those of kreisler and Hoxa1 mutants (Mansour et al., 1993). Second, FGF3 and kreisler/Mafb are thought to positively regulate each other in the hindbrain (Marin and Charnay, 2000; Theil et al., 2002). Consistent with these results, the expression of Fgf3 in r5 and r6 is absent in kreisler mutants, whereas Fgf3 expression in the mutant inner ears is present (McKay et al., 1996). Third, knockout of a receptor for FGF3, Fgfr2(IIIb), results in severe inner ear malformations that include an absence of the endolymphatic duct (Pirvola et al., 2000). However, FGF3 may not be the only signaling factor from r5 that mediates inner ear development, as the inner ear phenotypes of Fgf3 knockout
mice are relatively milder and lower in penetrance when compared with inner ears of kreisler, Hoxa1, Raldh2 and Fgfr2(IIIb) mutant mice. Furthermore, another Fgf3 knockout mouse strain that was recently generated has no apparent inner ear phenotype (Alvarez et al., 2003). Therefore, additional signaling factors from the hindbrain including other members of FGF family could be involved in mediating inner ear development. Nevertheless, to date, specific downstream otic genes that are activated by these signaling molecules from the hindbrain remain elusive.

Gbx2 is a homeobox gene that is related to Drosophila unplugged (Chiang et al., 1995). The expression of Gbx2 in the midbrain-hindbrain junction of vertebrates is conserved among several species (Bouillet et al., 1995; Shamim and Mason, 1998; Su and Meng, 2002; von Bubnoff et al., 1996), and knockout of Gbx2 in mice affects the normal positioning of this junction in the brain (Wassarman et al., 1997). Gbx2 is also expressed in the otic placode of several species (Bouillet et al., 1995; Shamim and Mason, 1998; Su and Meng, 2002). In mice, the expression of Gbx2 in the otic placode is correlated with proper otocyst formation, but its specific role in inner ear development is not known (Wright and Mansour, 2003). In this study, we analyzed the inner ears of Gbx2 knockout mice and show that Gbx2 is a key molecule in patterning both vestibular and auditory components of the inner ear. Based on comparisons of inner ear phenotypes and gene expression analyses between Gbx2 and some of the hindbrain mutants, in particular, kreisler, we postulate that Gbx2 is an important downstream target of hindbrain signaling.

Materials and methods

Animals
Mice heterozygous for a deletion of the Gbx2 gene were kindly provided by Alexandra Joyner (New York University). Gbx2 heterozygous mice were maintained in a Swiss Webster background, and the offspring of these mice were genotyped using PCR as described (Wassarman et al., 1997).

Paint fill and in situ hybridization
Paint-fill analyses and in situ hybridization experiments were performed as described (Morsli et al., 1998). A total of 50 Gbx2 homozygous mutant embryos between 9.5 and 15.5 dpc were used for in situ hybridization analyses, and a total of 40 Gbx2+/- embryos between 8.5 and 10.5 dpc were processed for whole-mount in situ hybridization. Heterozygous and homozygous Gbx2 embryos from 8.5 to 9.0 dpc for whole-mount in situ hybridization were age matched based on the total number of somite pairs. RNA probes for bone morphogenetic protein 4 (Bmp4), lunatic fringe (Lfng), neurofilament protein 68 kDa (NF68; Nefl – Mouse Genome Informatics) and orthodenticle 2 (Otx2) were prepared as described (Morsli et al., 1999). RNA probes for Eya1 (Xu et al., 1997), Gata3 (Karis et al., 2001), Gbx2 (Bouillet et al., 1995), Myo15a (Anderson et al., 2000), Neurod1 (Ma et al., 1998) and Pax2 (Dressler et al., 1990) were prepared according to cited references.

Cell proliferation and apoptosis assays
Cell proliferation and apoptotic assays were performed as described (Burton et al., 2004). Apoptotic cells were identified using terminal dUTP nick-end labeling (TUNEL) method (ApopTag).

Results
Expression of Gbx2 in the developing inner ear
We first examined the spatial and temporal expression patterns of Gbx2 in the developing mouse inner ear between 8.5 and 15.5 dpc. Gbx2 mRNA is first detectable in the otic placode at 8.5 dpc (data not shown) (Wright and Mansour, 2003). In the newly formed otocyst, Gbx2 transcripts are present in the entire dorsomedial region (Fig. 1A,B; arrow). At 10.5 dpc, the endolymphatic duct forms in the dorsomedial region of the otocyst, and it is Gbx2 positive (Fig. 1C). In addition, the Gbx2 expression domain extends ventrally beyond the endolymphatic duct to the equator of the otic vesicle (Fig. 1C,D,F).

We correlated this Gbx2 expression domain at 10.5 dpc with the location of presumptive sensory patches, using Bmp4 and Lfng as markers. At this age, Bmp4 is expressed in the three presumptive cristae and Lfng is expressed in the other presumptive sensory tissues, the macula utriculi, macula sacculi and organ of Corti, as well as in the neurogenic region that delaminates to form the cochleovestibular ganglion (Morsli et al., 1998). Comparisons of adjacent cryosections probed for Gbx2 and Bmp4 transcripts (Fig. 1D,E) or Gbx2 and Lfng transcripts (Fig. 1F,G) show that the Gbx2 expression domain does not overlap significantly with the presumptive sensory regions. By 13 dpc, only the endolymphatic duct is positive for Gbx2 hybridization signals, and no Gbx2 expression within the inner ear is detectable by 15.5 dpc.

In addition, Gbx2 transcripts are detected in the mid-hindbrain junction at 9.5 dpc (Fig. 1A, arrowheads) (Bouillet et al., 1995), as well as longitudinal columns in the dorsal and intermediate regions of the hindbrain and spinal cord (Fig. 1A,C, asterisks).

Fig. 1. Expression of Gbx2 in the developing mouse inner ear. (A) Dorsal view of a mouse embryo at 9.5 dpc probed for Gbx2 transcripts. Arrows indicate Gbx2 expression in the otocyst. Arrowheads indicate Gbx2 expression in the mid-hindbrain junction. (B) An enlarged lateral view of the right otocyst in A, showing Gbx2 hybridization signals in the dorsomedial half of the otocyst. (C) Gbx2 expression in the endolymphatic duct (ed) at 10.5 dpc. (D-G) Pairs of 12 µm adjacent sections probed for Gbx2 (D,F) and Bmp4 (E) or Lfng (G) transcripts. Arrows in E indicate Bmp4 expression in the presumptive cristae. Asterisks indicate Gbx2 expression in the neural tube. Approximate levels of sections for C-G are shown in the schematic diagram. Orientation in D applies to D-G; A, anterior; D, dorsal; L, lateral. Scale bar in G applies to D-G.
Development

Phenotype (Fig. 2, Table 1). Overall, four categories (I, II, III and IV), based on the severity of phenotypes was observed. We divided the specimens into four categories (I, II, III and IV) at 15.5 dpc using the paint-fill technique (Fig. 2). A total of 19 specimens are more malformed than those of Type I and Type II, and have less than one coil. Inner ears categorized as Type III are the most severe; they are cystic without any discernible structure. Numbers within the brackets represent the cases (n=5/7), in addition to the absence of the endolymphatic duct (n=7/7). The utricle and saccule are not easily discernible, and the cochlear duct is shortened. Type III: the inner ear is missing the anterior and posterior canals, as well as the membranous swelling. Numbers within the brackets represent the cases of specific structures (n=3/5).

Developme

Approximately half of the specimens in Gbx2 mutant inner ears are missing the anterior and posterior canals, as well as the common crus (Table 1). The anterior and posterior canals form from a vertical outpouch in the dorsal region of the otocyst (Fig. 2B, VP). Over time, the opposing epithelia in the center region of each presumptive canal approach each other, fuse and reabsorb, leaving behind the two canals connected by the common crus. This morphogenetic process is completed by 13 dpc in mice (Fig. 2A) (Morsli et al., 1998). The absence of the canals and common crus in Gbx2 mutants could be due to a failure of canal pouch formation. Alternatively, excessive epithelial resorption could eliminate the epithelia that normally form the canals and common crus. To further investigate these two possibilities, we examined 11 paint-filled ears during canal pouch development between 11 and 12 dpc (seven embryos between 11 and 11.5 dpc; four embryos between 12 and 12.75 dpc). Figure 2C-E illustrate three paint-filled inner ears at 11.5 dpc with a normal (Fig. 2C), a small (Fig. 2D) or a non-existent (Fig. 2E) vertical canal pouch. Interestingly, 45% of these specimens between 11 and 12 dpc have either no or a small Vertical canal pouch formation in Gbx2 mutants

Next, we investigated the gross anatomy of Gbx2 mutant inner ears at 15.5 dpc using the paint-fill technique (Fig. 2). A total of 19 homozygous mutant embryos were analyzed, and a repertoire of phenotypes was observed. We divided the specimens into four categories (I, II, III and IV), based on the severity of the phenotype (Fig. 2, Table 1). Overall, Gbx2 mutant inner ears are usually missing the endolymphatic duct (Table 1, n=18/19), with an enlarged membranous labyrinth (Fig. 2A). By contrast, the lateral canal and ampulla are usually present (Table I, n=17/19). Type I, the mildest phenotype, shows an enlarged membranous labyrinth, and three out of the four specimens are missing the endolymphatic duct (Table 1). In the Type II category, most of the inner ears are missing the common crus (Fig. 2A, asterisk; n=5/7), in addition to the absence of the endolymphatic duct (n=7/7). The utricle and saccule are not easily discernible, and the saccule is often fused with the cochlear duct. In the Type III inner ears, the anterior and posterior semicircular canals are also missing but the lateral canal is present (n=3). The cochlear ducts of Type III specimens are more malformed than those of Type I and Type II, and have less than one coil. Inner ears categorized as Type IV are the most severe; they are cystic without any discernible structures except for the presence of the lateral canal in some cases (n=3/5). Taken together, the lack of Gbx2 function affects inner ear structures such as canals and cochlear duct that do not express Gbx2 (Fig. 1; Table 1), suggesting that Gbx2 has a non-cell autonomous role in inner ear development. Despite the variable phenotypes among the Gbx2 mutants, the two ears of a given specimen usually display similar phenotypes (n=8/10).

Paint-fill analysis of Gbx2 mutant inner ears

Table 1. Affected inner ear structures in Gbx2 homozygotes

<table>
<thead>
<tr>
<th>Structure</th>
<th>Number affected*</th>
<th>% of total mutants lacking specific structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED/ES</td>
<td>3(3)</td>
<td>95% (18/19)</td>
</tr>
<tr>
<td>ASC</td>
<td>3(0)</td>
<td>42% (8/19)</td>
</tr>
<tr>
<td>PSC</td>
<td>3(0)</td>
<td>42% (8/19)</td>
</tr>
<tr>
<td>LSC</td>
<td>3(0)</td>
<td>42% (8/19)</td>
</tr>
<tr>
<td>CC</td>
<td>4(0)</td>
<td>68% (13/19)</td>
</tr>
<tr>
<td>AA</td>
<td>1(0)</td>
<td>21% (4/19)</td>
</tr>
<tr>
<td>PA</td>
<td>0(0)</td>
<td>21% (4/19)</td>
</tr>
<tr>
<td>LA</td>
<td>1(0)</td>
<td>11% (2/19)</td>
</tr>
<tr>
<td>Utricle</td>
<td>4(0)</td>
<td>0% (0/19)</td>
</tr>
<tr>
<td>Saccule</td>
<td>4(1)</td>
<td>42% (8/19)</td>
</tr>
<tr>
<td>CD</td>
<td>4(0)</td>
<td>26% (5/19)</td>
</tr>
</tbody>
</table>

*Total number of specimens with malformation of specific structures, including membranous swelling. Numbers within the brackets represent the number of specimens missing specific structures.
†As the utricle is part of the inner ear proper, it is difficult to determine if the structure is missing or poorly developed.
vertical canal pouch. This percentage corresponds well to the 42% of the specimens missing anterior and posterior canals at 15.5 dpc (Table 1), suggesting that the canal defect originates during the canal pouch outgrowth stage. Whether misregulated epithelial resorption is also involved in the phenotype is not clear. In addition to the canal pouch defects, the lack of the endolymphatic duct and cochlear duct extension is also evident by 11.5 dpc (Fig. 2C-E).

**Loss of endolymphatic duct markers in Gbx2 mutant inner ears**

The most prevalent phenotype of the Gbx2 mutants, the absence of the endolymphatic duct, was examined in more detail using gene expression analyses. Wnt2b is normally expressed in the endolymphatic duct, and its expression is initiated in the dorsal pole of the otocyst starting at 9.5 dpc (Fig. 3A) (Riccomagno et al., 2002). Wnt2b expression is not detected in otocysts of Gbx2 mutants at 9.5 or 10 dpc, suggesting a failure of endolymphatic duct specification (Fig. 3B; n=4).

In a normal otocyst at 9.5 dpc, Dlx5 is expressed in the entire dorsal region of the otocyst (Fig. 3C), whereas Hmx3 is expressed in the lateral region only (Fig. 3E). Consistent with the expression patterns in the otocyst, in a more mature inner ear, Dlx5 is expressed in both dorsal structures including the endolymphatic duct and the semicircular canals (Acampora et al., 1999; Depew et al., 1999; Merlo et al., 2002). However, Hmx3 is only expressed in the canals, which are dorsolateral structures (Rinkwitz-Brandt et al., 1996; Rinkwitz-Brandt et al., 1995). Therefore, we extrapolated from these results that the dorsomedial domain of the otocyst that is Dlx5 positive and Hmx3 negative, gives rise to the endolymphatic duct; the dorsolateral domain that is positive for both Dlx5 and Hmx3, gives rise to the three semicircular canals. This idea is supported by results from knockout mouse studies showing that Dlx5 is important for both endolymphatic duct and canal development, whereas Hmx3 is primarily required for canal formation (Acampora et al., 1999; Depew et al., 1999; Hadrys et al., 1998; Wang et al., 1998). According to the proposed boundary model of cell fate specification in the inner ear (Fekete, 1996; Kiernan et al., 1997) the absence of Gbx2 could affect the normal boundaries of Dlx5 and Hmx3 expression domains, leading to malformations of both the endolymphatic duct and semicircular canals. In the Gbx2 mutants, there is a loss of Dlx5 expression in the medial region of the otocyst but its expression in the lateral region remains (Fig. 3D, arrows; n=4), suggesting Gbx2 is required to maintain Dlx5 expression only regionally. Despite the loss of Dlx5 in the medial otocyst, there is no expansion of the Hmx3 expression domain mediially (Fig. 3F, arrows; n=4). Therefore, these results suggest that the failure of endolymphatic duct specification in Gbx2 mutants is not due to a change in domain boundaries, but rather a change in the induction and/or maintenance of the expression of genes such as Wnt2b and Dlx5. In addition, these gene expression changes in the mutants are not associated with an obvious change in either cell proliferation or apoptosis in the dorsomedial region of the otocyst at this age (data not shown).

**Ganglion and sensory organ development in Gbx2 mutants**

Despite the fact that Gbx2 is not normally expressed in the Lfng-positive neurogenic and sensory competent region (Fig. 1F,G), the variable and sometimes severe phenotypes observed in Gbx2 mutants suggest that the development of this region is also affected. We examined ganglion and sensory organ formations in Gbx2 mutants at 15.5 dpc using in situ hybridization. Owing to the variability of phenotypes, cryosections from each Gbx2 specimen were partially reconstructed and categorized as Type I to Type IV. A total of seven Gbx2 mutant ears were analyzed for the presence of vestibular and spiral ganglia (Type I, n=2; Type II, n=3; and Type IV, n=2) using RNA probes for Nf68 and Gata3 transcripts. The vestibular ganglion is present in the mutant ears of all phenotypes examined (Fig. 4A,B). The spiral ganglion is present in most of the Type II specimens (Fig. 4C,D; n=2/3) but missing in both Type IV specimens.

To investigate the cause for the ganglion defect, we examined the delamination of the neuroblasts at E10.5 based on the expression pattern of Neurod1 (Fig. 4E,F; n=6). Neuroblasts appear to delaminate normally in the Gbx2 mutants, but there is an increased number of apoptotic cells in the delaminated neuroblasts between 9.5 and 10.5 dpc compared with normal (Fig. 4G,H; n=5). This increase in cell
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death could contribute to the ganglion phenotype observed at later stages.

The Lfng expression domain that encompasses the neurogenic/sensory region appears normal in the Gbx2 mutants at 10.5 dpc (Fig. 5A,B, area between the red lines; n=3). However, based on the 3-D reconstruction of serial sections, the otic region dorsal to the Lfng domain is smaller, suggesting that this region is underdeveloped in Gbx2 mutants (Fig. 5A,B).

Despite the normalcy of the Lfng domain at 10.5 dpc, sensory organ formation is variable, particularly among the ventral sensory organs (Table 2). At 15.5 dpc, the lateral crista is found in all the specimens examined, consistent with the paint-fill results (Fig. 5C,E; Table 2). In addition, a sensory patch that corresponds to the position of the macula utriculi is also present, even in the most severely affected ears (Fig. 5C,E). Both the lateral crista and macula utriculi express a sensory hair cell-specific gene, Myo15a (Fig. 5D,F). Anterior and posterior cristae are generally present, except in some of the Type IV ears. The ventral sensory patches, however, such as the macula sacculi and organ of Corti, are severely affected in most mutants (Table 2). No discernible sensory patches for the macula sacculi can be positively identified even in the Type

Fig. 4. Ganglion formation in Gbx2–/– inner ears. Heterozygous (A,C,E,G) and homozygous (B,D,F,H) Gbx2 inner ears probed for Nf68 (A,B) Gata3 (C,D) and Neurod1 (E,F) transcripts, and apoptotic cells (G,H) at (A-D) 15.5, (E,F) 10.5 and (G,H) 9.5 dpc. (G,H) TUNEL analyses show an increased in apoptotic cells in the cochleo-vestibular ganglion (G,H, red arrowheads) of a –/– otocyst (H) compared with a +/– otocyst (G, black arrowheads). Arrows indicate a region of cell death within the otic epithelium of +/– and –/– inner ears. cvg, cochleo-vestibular ganglion; gg, geniculate ganglion; sg, spiral ganglion; vg, vestibular ganglion. Schematics on the right indicate the levels of sections. Scale bars: in D, 200 µm for A-D; in F, 100 µm for E,F; in H, 100 µm for G,H.

Fig. 5. Sensory organ formation in Gbx2–/– inner ears. Sections of heterozygous (A,C,D,G,H) and homozygous (B,E,F,I,J) Gbx2 inner ears probed for Lfng or Myo15a transcripts at 10.5 (A,B) and 15.5 (C-J) dpc. (C-J) Pairs of 12 µm adjacent sections. Red lines indicate comparable Lfng expression domains between +/– and –/– ears. Lfng (E) and Myo15a (F) are expressed in the lateral crista (lc) and macula utriculi (mu) of a Type IV Gbx2–/– inner ear. Arrowheads in H indicate the sensory hair cells. Only one or two Lfng- (I) and Myo15a (J)-positive sensory patches are present in a Type II Gbx2–/– cochlear duct. Insert in J shows a higher power view of the Myo15a positive sensory region. Arrowheads indicate the region that is positive for both Lfng and Myo15a. Scale bars: in A, 100 µm for A,B; in C, 100 µm for C-F; in G, 100 µm for G,H.
II ears, and the cochlear ducts usually consist of one or two small sensory patches that express a hair cell-specific marker, Myo15a (Fig. 5G-J).

**Expansion of Otx2 expression domain in Gbx2–/– inner ears**

We further investigated the cause for the poorly developed ventral sensory structures. Eya1, Gata3 and Pax2 are all important for the normal development of the cochlear duct (Burton et al., 2004; Karis et al., 2001; Torres et al., 1996; Xu et al., 1999). Expression patterns of these three genes were examined in the cochlear duct of Gbx2 mutants, but none were changed significantly to provide any insight into the cause of the cochlear malformation. As there is good evidence that Gbx2 and Otx2 negatively regulate each other in the mid-hindbrain region, we examined the expression of Otx2 in Gbx2–/– inner ears.

At 10.5 dpc, Otx2 is normally expressed in the ventral posterolateral region of the otic vesicle, and its expression is complementary to the Lfng domain (Fig. 6A-D). In the Gbx2 mutants, the dorsal region of the Otx2 expression domain is fairly normal, but ventrally its expression expands medially into the Lfng domain (Fig. 6E-H, brackets; six otocysts from four embryos). The extent of the Otx2 expression domain expansion is variable among specimens. One ear from one embryo shows medial expansion of the entire Otx2 expression domain, while another specimen shows a normal expression domain. Both of these specimens show variability between left and right ears. This aberrant expression of Otx2 in the ventromedial region is also observed at later stages, even though the extent of Otx2 domain expansion varies between specimens (Fig. 6f, double arrows; Fig. 6J-L; arrows; n=8 between 11.5 to 15.5 dpc). Interestingly, unlike the situation in the mid-hindbrain region, the Gbx2 expression domain in a normal mouse inner ear does not abut the Otx2 domain (Fig. 6M-N). Similar expression patterns have been reported in the chicken inner ear (Hidalgo-Sanchez et al., 2000).

**Abnormalities in the caudal hindbrain of Gbx2 null mutants**

Next, we assessed between 9 and 9.5 dpc the integrity of the hindbrain region (r4-r6) that is closest to the developing inner ear. It has been shown that the anterior hindbrain rostral to r4 is missing in the Gbx2 mutants and is replaced by an ill-defined zone with aberrant gene expression patterns (Wassarman et al., 1997). Gene expression patterns caudal to r4 have not been reported in detail. At 9 to 9.5 dpc, Krox20 (Egr2 – Mouse Genome Informatics) and EphA4 are normally expressed in r3 and r5. In Gbx2 mutants, the levels of both Krox20 and EphA4 expression in r5 are reduced compared with heterozygotes (Fig. 7A-D; n=4). Note the smaller size of the otocysts in some Gbx2 mutants (Fig. 7D, arrows). However, Hoxb1, a marker for r4, is expressed in its normal position relative to the otocyst,
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except that the rostral boundary of its expression domain is not as well-defined as controls (Fig. 7E,F; n = 2).

As kreasler/Mafb and Fgf3 have been implicated in inner ear development, we examined in more detail the expression patterns of these two genes in Gbx2 mutants. kreasler/Mafb is expressed in the presumptive r5 and r6, starting at 8.0 dpc (Cordes and Barsh, 1994) (Fig. 8A), and its expression is downregulated in the hindbrain, with a weak expression in r6 by late 9.5 dpc (Fig. 8C, arrowhead). In Gbx2 mutants, there is no difference in kreasler/Mafb expression up to 9.5 dpc (Fig. 8B; n = 3). However, by late 9.5 dpc (27 somites), kreasler/Mafb is still expressed in r6, while it is downregulated in controls by this stage (Fig. 8C,D; n = 2).

Fgf3 is normally expressed in the hindbrain beginning at 8 dpc (four somites) (Mahmood et al., 1996; McKay et al., 1996). From 8.5 dpc to 9 dpc, the expression of Fgf3 in r5-r6 is high, and then subsides by 9.5 dpc (Fig. 8E,G). In the Gbx2 mutants, Fgf3 is strongly expressed in r5 and r6 (Fig. 8F). In addition, its expression domain extends into r4 and possibly to the undefined region rostral to r4 (n = 4). By late 9.5 dpc, Fgf3 expression in normal hindbrain is barely detectable (Fig. 8G), whereas in Gbx2 mutants, Fgf3 expression in r6 persists (Fig. 8H, arrowhead). Strong expression of Fgf3 is observed also in r4, and possibly the region rostral to r4 (Fig. 8H, asterisk; n = 3). Despite these aberrant gene expression patterns in the hindbrain, the Fgf3 expression in the anterolateral region of the otocyst in Gbx2 mutants is normal (Fig. 8G,H). Taken together, loss of Gbx2 function in the neural tube evidently affects the development of the caudal hindbrain, which in turn may affect inner ear development. However, hindbrain genes such as kreasler/Mafb and Fgf3 that have been implicated in inner ear development are upregulated in the hindbrain rather than downregulated.

Discussion

The role of Gbx2 in patterning dorsal inner ear structures

Our results demonstrate that Gbx2 is required for the formation of the endolymphatic duct, a dorsomedial structure. In the Gbx2 mutants, expression patterns of both Wnt2b and Dlx5 are affected in the presumptive endolymphatic duct region. As Gbx2 is normally expressed in this region, Gbx2 is probably required cell autonomously for endolymphatic duct formation. By contrast, Gbx2 has a non-cell autonomous role in canal
In between the two domains is the Lfng-positive sensory competent region that is negative for both Gbx2 and Otx2 (Fig. 9A). Here, we show that the Lfng domain appears to form normally in the Gbx2 mutants. However, the lack of Gbx2 results in an expansion of Otx2 domain medially and possibly affects the subsequent development of the Lfng positive, sensory region (Fig. 9A). This aberrant expression of Otx2 in the ventral region is not observed in other knockout mice with cochlear defects such as sonic hedgehog and Pax2 knockout mice (Burton et al., 2004; Riccomagno et al., 2002). Given the known functions of Otx2 in regional identity in other systems (Acampora et al., 1995; Ang et al., 1996; Rhinn et al., 1998), the ectopic Otx2 expression in the inner ear of Gbx2 mutants could be causal to the ventral defects observed. Interestingly, the lack of the endolympathic duct in mice is invariably associated with defects in other parts of the inner ear (Fekete, 1999; Kiernan et al., 2002), possibly owing to loss of Gbx2 expression. This suggests that a disrupted relationship between Gbx2 and Otx2 expression patterns could be a common molecular mechanism underlying other inner ear defects in mouse mutants lacking an endolympathic duct. This hypothesis needs to be tested directly in other experimental paradigms.

The cause for the increased apoptosis in the delaminated neuroblasts is unclear as Gbx2 expression is not detected in the cochleovestibular ganglion. Nevertheless, this observed cell death could attribute to the ganglion phenotype observed at later stages. In addition, the reduction or absence of the spiral ganglion in some specimens could also be caused by a reduction in the supply of neurotrophins from the poorly developed sensory tissues.

**The role of Gbx2 in the hindbrain**

In the hindbrain of Gbx2 mutants, we observed a change in gene expression patterns caudal to r3, in addition to defects described for the anterior hindbrain (Wassarman et al., 1997). We show that r5 is abnormal based on Krox20 and Epha4 expression patterns. The size of r4 appears relatively normal, but Fgf3 is ectopically expressed in this region. In addition, the temporal expression patterns of both kreisler/Mafb and Fgf3 are prolonged in r6. These results suggest that Gbx2 is required for normal development of the caudal hindbrain; it regulates the temporal expression of kreisler/Mafb and Fgf3 and maintains the expression of Krox20 and Epha4. However, many of these affected genes are thought to regulate each other in the hindbrain (Theil et al., 2002; Theil et al., 1998). For example, ectopic expression of kreisler/Mafb in r3 and r5 results in an upregulation of Fgf3 in these rhombomeres (Theil et al., 2002). However, in chicken, the loss of FGF signaling using an inhibitor of the FGF receptor abolishes kreisler/Mafb expression in the hindbrain (Marin and Charnay, 2000). These results suggest that kreisler/Mafb and FGF3 positively regulate each other. Furthermore, ectopic expression of Mafb also represses the expression of both Krox20 and Epha4 in the chicken hindbrain (Giudicelli et al., 2003). Taken together, these results suggest that the downregulation of Krox20 and Epha4 in the chicken hindbrain is mediated by the upregulation of kreisler/Mafb and/or Fgf3.

**Inner ear Gbx2 expression is downstream of hindbrain signaling**

Signaling from the hindbrain plays an important role in inner
ear development. As Gbx2 is expressed in both the hindbrain and inner ear itself, the inner ear phenotypes observed in the Gbx2 knockout might be due to disrupted hindbrain formation and signaling and/or the lack of Gbx2 activity within the inner ear.

We postulate that Gbx2 expression in the inner ear is a primary downstream target of hindbrain signaling (Fig. 9B). Several lines of evidence support this hypothesis. First, kreisler/Mafb expression in the hindbrain at 7.5 dpc precedes Gbx2 expression in the inner ear at 8.5 dpc. Second, the downregulation of Gbx2 expression in the inner ears of kreisler mice strongly supports the notion that expression of Gbx2 in the inner ear is regulated by the kreisler/Mafb pathway (D. Choo, personal communication). Third, hindbrain rotation experiments in chicken indicate that the maintenance of otic Gbx2 expression is dependent on signaling from the hindbrain (Bok et al., 2004). Fourth, there is a strong resemblance in the inner ear phenotypes described here for the Gbx2 mutants and those described for kreisler (D. Choo, personal communication). In kreisler, the endolymphatic duct fails to develop, and the lateral ampulla and canal are the least affected structures, similar to the Gbx2 mutants. Finally, similar molecular changes such as ectopic expression of Otx2 and downregulation of Dlx5 are also observed in kreisler mutants (D. Choo, personal communication). It remains to be determined, however, whether other hindbrain mutants such as Hoxa1 knockout mice show similar gene expression changes as we have observed for Gbx2 mutants.

Other evidence supports the idea that Gbx2 expression in the inner ear is a more important requirement for inner ear development than Gbx2 expression in the hindbrain. Fgf3 expression in the hindbrain is prolonged in the Gbx2 mutants. It has been shown that ectopic expression of Fgf3 in the hindbrain of chicken resulted in a distended endolymphatic duct (Vendrell et al., 2000). Therefore, the prolonged expression of Fgf3 in the hindbrain of Gbx2 mutants could have resulted in a larger endolymphatic duct, but this is not the case. The lack of an endolymphatic duct phenotype in Gbx2 mutants is more consistent with a loss rather than a gain of Fgf3 function. These results, however, could be reconciled if kreisler/Mafb and/or Fgf3 in the hindbrain normally induce or maintain Gbx2 expression in the inner ear. Then, the absence of Gbx2 within the inner ear would negate any phenotype caused by the upregulation of either kreisler/Mafb or Fgf3 and would result in a phenotype that resembles a loss of kreisler function.

Although we postulate that the majority of the inner ear phenotype is due to the loss of Gbx2 expression within the inner ear, any of the gene expression changes observed in the hindbrain of Gbx2 mutants could also contribute to the inner ear phenotype. No inner ear malformations due to the lack of Krox20 and Epha4 have been reported (Dottori et al., 1998; Sham et al., 1993; Swiatek and Gridley, 1993). Even though the role of Wnt2b in inner ear development is not known, the absence of Wnt2b expression in Gbx2 mutants could be due to gene expression changes at the level of the hindbrain because the expression patterns of Wnt2b and Gbx2 within the inner ear appear to be independently regulated (Riccomagno et al., 2002). A tissue-specific knockout of Gbx2 only in the inner ear or hindbrain should determine which source of Gbx2 is more important for inner ear development.

Under the assumption that Gbx2 in the inner ear is a major downstream target of signaling from the hindbrain, this Gbx2 expression domain probably mediates hindbrain signaling by maintaining expression of genes such as Dlx5 to promote formation of dorsal structures (Fig. 9). However, its role in ventral patterning is inhibitory and possibly mediated by restriction of Otx2 expression. This proposed inhibitory role of Gbx2 is different from the postulated inductive role of sonic hedgehog, another signaling molecule that emanates from the hindbrain and notochord, and induces or maintains expression of genes such as Otx2 and Pax2 (Riccomagno et al., 2002). Otx1, Otx2 and Pax2 are all required for the normal development of the saccule and cochlear duct (Burton et al., 2004; Cantos et al., 2000; Morsli et al., 1999). Multiple inductive and inhibitory molecules at the level of transcriptional control are likely to be involved in achieving the formation of an intricate organ such as the inner ear, and this paper presents insight into such a mechanism.

We are indebted to Dr Daniel Choo for discussion and sharing experimental results prior to publication, to Dr Alex Joyner for the Gbx2/– mice, and to Drs Susan Sullivan and Thomas Friedman for critical reading of the manuscript.

References


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