Hmx2 homeobox gene control of murine vestibular morphogenesis

Weidong Wang¹, Edwin K. Chan², Shira Baron², Thomas Van De Water² and Thomas Lufkin¹,*

¹Brookdale Center for Developmental and Molecular Biology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574, USA
²Departments of Otolaryngology and Neuroscience, Albert Einstein College of Medicine, 1410 Morris Park Avenue, Bronx, NY 10461, USA
*Author for correspondence (e-mail: thomas.lufkin@mssm.edu)

Accepted 18 September 2001

SUMMARY

Development of the vertebrate inner ear is characterized by a series of genetically programmed events involving induction of surface ectoderm, preliminary morphogenesis, specification and commitment of sensory, nonsensory and neuronal cells, as well as outgrowth and restructuring of the otocyst to form a complex labyrinth. Hmx2, a member of the Hmx homeobox gene family, is coexpressed with Hmx3 in the dorsolateral otic epithelium. Targeted disruption of Hmx2 in mice demonstrates the temporal and spatial involvement of Hmx2 in the embryonic transition of the dorsal portion (pars superior) of the otocyst to a fully developed vestibular system. In Hmx2 null embryos, a perturbation in cell fate determination in the lateral aspect of the otic epithelium results in reduced cell proliferation in epithelial cells, which includes the vestibular sensory patches and semicircular duct fusion plates, as well as in the adjacent mesenchyme. Consequently, enlargement and morphogenesis of the pars superior of the otocyst to form a complex labyrinth of cavities and ducts is blocked, as indicated by the lack of any distinguishable semicircular ducts, persistence of the primordial vestibular diverticula, significant loss in the three cristae and the macula utriculus, and a fused utriculosaccular chamber. The developmental regulators Bmp4, Dlx5 and Pax2 all play a critical role in inner ear ontogeny, and the expression of each of these genes is affected in the Hmx2 null otocyst suggesting a complex regulatory role for Hmx2 in this genetic cascade. Both Hmx2 and Hmx3 transcripts are coexpressed in the developing central nervous system including the neural tube and hypothalamus. A lack of defects in the CNS, coupled with the fact that not all of the Hmx2-positive regions in developing inner ear are impaired in the Hmx2 null mice, suggest that Hmx2 and Hmx3 have both unique and overlapping functions during embryogenesis.

Key words: Homeobox gene, Hmx, Inner ear development, Semicircular ducts, Gene knockout, Mouse, Cell proliferation

INTRODUCTION

The vertebrate inner ear is derived from one of three pairs of sensory placodes located in the anterior embryonic ectoderm. The other two placodes located more rostrally will contribute to the development of the sensory organs for olfaction and vision. Induction of the otic placode takes place at the 3- to 6-somite stage in vertebrates, as characterized by the acquisition of competence by the surface ectoderm adjacent to the hindbrain, and subsequent thickening of the placodal ectoderm (Torres and Giraldez, 1998).

During development, the otic placode invaginates and pinches off from the surface ectoderm to form an ellipsoid-shaped vesicle termed the otic vesicle (Hilfer et al., 1989). During the closure of the otic vesicle, positional information is gradually acquired by different regions of the otic epithelium, as indicated by the regionally restricted expression of different combinations of inner ear marker genes. Axial specification in the inner ear for sensory organs happens earlier than that for nonsensory organs. Transplantation experiments performed in chick demonstrated that patterning of sensory organs along the anterior-posterior (AP) axis is fixed first, followed by specification of the dorsal-ventral (DV) axis. At the same time, nonsensory epithelial cells maintain a certain degree of plasticity, since they can be reprogrammed when placed in a new environment (Wu et al., 1998). Different regions of the otocyst possess distinct positional information and express unique combinations of inner ear marker genes, which in turn ultimately determines their specific identities (Fekete, 1996). Regions with different identities display distinct capabilities in cell differentiation, proliferation and programmed cell death, a prerequisite for the transformation from an otocyst to an elaborate three-dimensional labyrinth. Precise outgrowth and fusion of the otic epithelium are morphogenetic milestones in the maturation of the inner ear. In the mouse, around E9.5 the anlagen of the endolymphatic duct bulges from the dorsal portion of the otic vesicle and elongates dorsally to form a hollow tube, which enlarges at its distal end to form a sac. Meanwhile, the otic epithelium destined to give rise to the cochlea enlarges ventrally and finally develops into a coiled duct. Morphogenesis of the otocyst pars superior into a vestibule requires very sophisticated shape changes. Formation
of the semicircular ducts initiates from paired outpocketings of otic epithelium. In the central regions of these pockets, the two epithelial layers on the opposing walls first become thinner and then detach from the underlying mesenchyme. Afterwards, these two walls approach one another to form an extensive region termed the fusion plate. Epithelial cells meeting at the fusion plate will mold into a single layer, and subsequently disappear via a possible mechanism involving either programmed cell death or epithelium cell retraction, or both. The semicircular ducts and associated canals are formed by the interactions between the remaining otic epithelium and its adjacent periotic mesenchyme (Fekete et al., 1997; Frenz and Van De Water, 1991; Lang et al., 2000; Martin and Swanson, 1993). Undoubtedly, a balance between cell proliferation and programmed cell death plays a critical role in this process. Previous work has confirmed the existence of several regions of programmed cell death in the developing inner ear, including the fusion plates of the semicircular canals, the ventromedial otic vesicle and the base of the endolympathic sac. In the chick, the overexpression of inhibitors of normal programmed cell death, such as bcl2, block semicircular canal fusion (Fekete et al., 1997). It has been proposed that the driving force pushing the apposing walls inward to form the fusion plate is generated by the interaction between the otic epithelium and the surrounding mesenchyme. The analysis of the inner ears of netrin 1 null mice support this mechanism (Salminen et al., 2000). netrin 1 appears to be involved in a signaling pathway regulating cell proliferation in the periotic mesenchyme. When netrin 1 production by the presumptive fusion plate was disrupted, reduced cell proliferation in the underlying mesenchyme resulted in severe defects in fusion plate formation and therefore semicircular duct morphogenesis.

In the mouse, by E14.5, the basic architecture of the membranous labyrinth of the inner ear has been fully established. From E14.5 through adult stages, maturation occurs and a functional inner ear emerges, with the vestibule and cochlea being responsible for the senses of balance and hearing, respectively. The identification of developmentally important genes expressed in the inner ear has been steadily increasing, and the function of certain of these genes has been determined (Represa et al., 2000). Antagonizing the normal Bmp4 signaling pathway with noggin disrupts inner ear development and demonstrates that local signals in the otic epithelium are essential for the correct formation of the inner ear (Chang et al., 1999; Gerlach et al., 2000). Different members of the homeobox gene superfamily are involved in all steps of inner ear development ranging from otic placode induction to maturation of a fully functional inner ear. Recent progress in mouse molecular genetics has allowed us to acquire specific information regarding the developmental contribution of individual genes to inner ear development. Certain homeobox genes are involved in regional specification of the inner ear, and some of these genes determine the fate of cells in their restricted expression domains. For example, Pax2 is predominately expressed in the ventral otic epithelium, and Pax2 null inner ears lack an identifiable cochlea (Represa et al., 2000; Torres et al., 1996). In addition, expression of a functional Dlx5 gene in the dorsal portion of the otic vesicle is required for proper morphogenesis of the semicircular canals (Acampora et al., 1999; Depew et al., 1999). Murine Otx1, an orthologue of the Drosophila orthodenticle gene, is expressed in the ventrolateral wall of the otocyst with a dorsal limit in the presumptive lateral semicircular canal. Otx1/null inner ears show an absence of the lateral semicircular canals and a malformed cochlea (Acampora et al., 1996; Morsli et al., 1999). However, it is common for a homeobox gene family that has several members to exhibit similar or even identical expression profiles and to be functionally interchangeable in certain aspects (Greer et al., 2000). The Hmx homeobox genes belong to a distinct family, which is of ancient origin, and their existence has been reported in many species (Bober et al., 1994; Stadler et al., 1995; Stadler et al., 1992; Stadler and Solursh, 1994; Wang et al., 1990; Wang et al., 2000). Three members of the Hmx homeobox gene family, designated Hmx1, Hmx2 and Hmx3 have been identified in mouse (Wang et al., 2000; Yoshiura et al., 1998). Regions where Hmx1 likely exerts its developmental function are neural crest derivatives including the dorsal root ganglion, cranial ganglia, branchial arches and in the developing neural retina. Unlike Hmx1, Hmx2 and Hmx3 are clustered together on the same chromosome and have nearly identical expression domains that include the inner ear, CNS and uterus. Defects caused by the inactivation of Hmx3 have been reported for the inner ear and in female fertility (Hadrys et al., 1998; Wang et al., 1998). An absence of horizontal cristae, a fused utriculosaccular chamber, a significant loss of vestibular hair cells and perimplantation infertility have been observed, indicating unique functions for Hmx3. However, analysis of these defects also suggests that some aspects of Hmx3 function might have been compensated for by its sibling Hmx2, since not all cells expressing Hmx3 are affected in the Hmx3 null mice. In this paper, we will present the defects exhibited by Hmx2 null mice to reveal its unique developmental function.

**MATERIALS AND METHODS**

**Targeting of the Hmx2 locus and generation of the Hmx2 heterozygous and homozygous null animals**

Genomic cloning of the Hmx2 gene has been reported previously (Wang et al., 2000). Plasmid pW64b was generated by inserting an 11 kb EcoRI genomic fragment spanning the Hmx2 gene into the vector pTZ18R (US Biochemicals). The 5' EcoRI site is located in the intragenic region between the Hmx2 and Hmx3 genes. The 3' EcoRI site is derived from the polylinker of the lambda DashII phage vector. In this EcoRI fragment, there are two XhoI sites. One is located in the Hmx2 homeobox and the other is 1.2 kb 3' to the homeobox. pW64b was double digested with Sall and XhoI and the 1.2 kb XhoI fragment was re-inserted into the Sall and XhoI site of pW64b, leaving an unique XhoI site in the Hmx2 homeobox. Finally, the ires.lacZ.neo cassette purified from p1099 (Wang et al., 1998) was inserted into the unique XhoI site in the Hmx2 homeobox, resulting in the targeting vector pW78a (Fig. 1A). The transcriptional orientation of the ires.lacZ.neo cassette was verified by DNA sequencing. The targeting construct was linearized by EcoRI digestion for electroporation and ES cell transfection, screening for recombinant R1 ES clones, production of chimeric animals and testing of germline transmission were performed as previously described (Wang and Lufkin, 2000; Wang et al., 1998). Insertion of the ires.lacZ.neo cassette into the Hmx2 locus introduces new CflI and XhoI sites, and Southern blot analysis was performed on tissue samples to genotype embryos and pups carrying the inactivated
Hmx2 allele designated Hmx2lacZ. RNAse protection was carried out essentially as previously described (Wang et al., 1998). 100 μg total RNA extracted from Hmx2lacZ+/+, Hmx2lacZ+/– and Hmx2lacZ–/– was used for RNAse protection assays. A 242 bp DNA fragment was PCR-amplified using primers TL245 (5’ AGCGCCCAAGCAGGAGG 3’) and TL246 (5’ CGACGCGTGTTGCCATGT 3’). This fragment contains the homeobox of Hmx2 and spans the restriction site used for insertion of the ires.lacZ.neo cassette and was used to make 32P-labeled anti-sense riboprobe. A 271 bp cDNA fragment from the mouse β-actin gene was used as the internal control for the quantity and quality of total RNA.

**β-galactosidase staining of whole-mount embryos, preparation of inner ears for histology, RNA in situ hybridization and analysis of cell proliferation (BrdU labeling) and apoptosis (TUNEL)**

Embryos between the ages of embryonic day (E) 8.5 and E18.5 were collected from mating between viable Hmx2lacZ–/– males and Hmx2lacZ–/– females. Staining for β-galactosidase was performed as previously described with a minor modification in that 1% sodium deoxycholate was added to the staining solution (Frasch et al., 1995). Embryos older than E16.5 were decalcified in PBS containing 10% EDTA and 2.5% polyvinylpyrrolidone before being embedded in paraffin wax, and then sectioned at 8 μm. For standard histology, mice
were prepared by cardiac-perfusion with 4% paraformaldehyde, and inner ears were dissected, trimmed and fixed as previously described (Wang et al., 1998). Specimens were decalcified in 10% EDTA at 4°C for 5 days, dehydrated in ethanol, and treated in histoclear in preparation for embedding in paraffin wax. 7 μm microtome sections were cut, stained with Toluidine Blue and mounted onto glass microscope slides in Permount for histological examination. Inner ears were obtained from embryos of E11.5, E14.5, E16.5 and E18.5. For each embryonic stage, three specimens were prepared from the Hmx2lacZ embryos (used as the controls) and three from the Hmx2lacZ/- embryos. In the E14.5-E18.5 specimens vestibular hair cells were identified and counted in the cristae of the semicircular ducts and the maculae of the utricle and saccule under a magnification factor of 400×. The total number of hair cells was counted in all three cristae, as well as in the superior and posterior cristae individually. Neurons of the spiral and vestibular ganglia were identified and counted under a magnification factor of 400×. Histological variables were analyzed for statistically significant differences between Hmx2lacZ/− and Hmx2lacZ/− at E14.5, E16.5 and E18.5, using the unpaired Student’s t-test and the SigmaPlot computer program.

The function of Hmx2 during early mouse embryonic development was examined by generating an Hmx2 loss-of-function allele in mice (Fig. 1). Homologous recombination of the targeting construct into the chromosome leads to the insertion of the ires.lacZ.neo cassette into the DNA sequence encoding the third helix of the Hmx2 homeodomain. Owing to the presence of translation stop codons in all three open reading frame in the ires.lacZ.neo cassette, this Hmx2lacZnull allele will produce three protein products: (i) a truncated Hmx2 protein lacking the C-terminal portion of Hmx2 along with the third helix, the most critical DNA-binding portion of the homeodomain, (ii) β-galactosidase, and (iii) neomycin phosphotransferase. Five out of 96 G418-resistant ES clones tested had undergone a correct homologous recombination event. Two of the five clones, 4H5 and 4D3 were injected into blastocysts and the mutant allele was successfully transmitted through the germline.

Since no Hmx2-transcription control elements were deleted in the targeting strategy, integration of the ires.lacZ.neo reporter gene into Hmx2 exonic sequences enables us to follow Hmx2 gene expression by examining β-galactosidase activity. β-galactosidase expression of the Hmx2lacZ heterozygotes faithfully reproduced Hmx2 expression patterns as revealed in situ hybridization on paraffin sections and in whole-mount embryos (Wang et al., 2000). Unlike Hmx3, Hmx2 is still negative in the otic placode at E8.5 (Fig. 2A). Hmx2 expression is first detectable at E9.0 and its expression becomes prominent from E9.5 onward in the anteriodorsal portion of the otic vesicle, as well as the cleft between the first and second branchial arches (Fig. 2B,C). In addition to its expression in the vestibular portion of the otic vesicle, from E12.0, Hmx2 transcripts are strongly present in the central nervous system, including the developing neural tube, pons and hypothalamus. Its expression in the CNS is maintained at later stages (Fig. 2D and Fig. 3A,C). After E13.5, β-galactosidase activity from the Hmx2lacZ/− allele is detected in both the sensory and nonsensory epithelia of all three well-formed semicircular canals, endolymphatic sac, utricle and saccule (Fig. 3A,C,E). From E14.5, the stria vasularis of the cochlea begins to show β-galactosidase activity (Fig. 3C,E). Hmx2lacZ/− and wild-type mice are indistinguishable in their viability, behavior and fertility. Approximately 65% of the Hmx2lacZ/− mice in the outbred genetic background C57BL/6J×129/SvJ (previously 129/SvJ; Jackson Laboratory #000691) show classic vestibular defects as indicated by hyperactivity, head tilting and circling activity. The remaining Hmx2lacZ/− mice do not display any abnormalities in their behavior or fertility. Despite its strong expression in the central nervous system, loss of Hmx2 was insufficient to cause any observable defect in the CNS as determined by the behavior, β-galactosidase activity and histological analysis of Hmx2lacZ/− mice and embryos (data not shown). Furthermore, RNA in situ hybridization with numerous probes corresponding to neurogenic developmental regulators from the paired, forkhead and homeodomain families (e.g. Pax, Fox, Dlx), as well as cell-cell signaling molecules of the TGFβ superfamily, failed to reveal any alterations in expression between wild-type and Hmx2lacZ/− null embryos in the CNS (data not shown).

**RESULTS**

**Hmx2 null mice display hyperactivity, head tilting and circling behavior**

The function of Hmx2 during early mouse embryonic development was examined by generating an Hmx2 loss-of-function allele in mice (Fig. 1). Homologous recombination of the targeting construct into the chromosome leads to the insertion of the ires.lacZ.neo cassette into the DNA sequence encoding the third helix of the Hmx2 homeodomain. Owing to the presence of translation stop codons in all three open reading frame in the ires.lacZ.neo cassette, this Hmx2lacZnull allele will produce three protein products: (i) a truncated Hmx2 protein lacking the C-terminal portion of Hmx2 along with the third helix, the most critical DNA-binding portion of the homeodomain, (ii) β-galactosidase, and (iii) neomycin phosphotransferase. Five out of 96 G418-resistant ES clones tested had undergone a correct homologous recombination event. Two of the five clones, 4H5 and 4D3 were injected into blastocysts and the mutant allele was successfully transmitted through the germline.

Since no Hmx2-transcription control elements were deleted in the targeting strategy, integration of the ires.lacZ.neo reporter gene into Hmx2 exonic sequences enables us to follow Hmx2 gene expression by examining β-galactosidase activity. β-galactosidase expression of the Hmx2lacZ heterozygotes faithfully reproduced Hmx2 expression patterns as revealed in situ hybridization on paraffin sections and in whole-mount embryos (Wang et al., 2000). Unlike Hmx3, Hmx2 is still negative in the otic placode at E8.5 (Fig. 2A). Hmx2 expression is first detectable at E9.0 and its expression becomes prominent from E9.5 onward in the anteriodorsal portion of the otic vesicle, as well as the cleft between the first and second branchial arches (Fig. 2B,C). In addition to its expression in the vestibular portion of the otic vesicle, from E12.0, Hmx2 transcripts are strongly present in the central nervous system, including the developing neural tube, pons and hypothalamus. Its expression in the CNS is maintained at later stages (Fig. 2D and Fig. 3A,C). After E13.5, β-galactosidase activity from the Hmx2lacZ/− allele is detected in both the sensory and nonsensory epithelia of all three well-formed semicircular canals, endolymphatic sac, utricle and saccule (Fig. 3A,C,E). From E14.5, the stria vasularis of the cochlea begins to show β-galactosidase activity (Fig. 3C,E). Hmx2lacZ/− and wild-type mice are indistinguishable in their viability, behavior and fertility. Approximately 65% of the Hmx2lacZ/− mice in the outbred genetic background C57BL/6J×129/SvJ (previously 129/SvJ; Jackson Laboratory #000691) show classic vestibular defects as indicated by hyperactivity, head tilting and circling activity. The remaining Hmx2lacZ/− mice do not display any abnormalities in their behavior or fertility. Despite its strong expression in the central nervous system, loss of Hmx2 was insufficient to cause any observable defect in the CNS as determined by the behavior, β-galactosidase activity and histological analysis of Hmx2lacZ/− mice and embryos (data not shown). Furthermore, RNA in situ hybridization with numerous probes corresponding to neurogenic developmental regulators from the paired, forkhead and homeodomain families (e.g. Pax, Fox, Dlx), as well as cell-cell signaling molecules of the TGFβ superfamily, failed to reveal any alterations in expression between wild-type and Hmx2lacZ/− null embryos in the CNS (data not shown).

**Structural abnormalities in the morphology and histology of Hmx2 null inner ears**

Multiple inner ear defects were evident in the Hmx2lacZ/− embryos following examination of whole-mount embryos and tissue sections stained for β-galactosidase activity. The most significant finding was a gross dysgenesis of all three semicircular ducts seen as early as E13.5 days in approximately 70% of the Hmx2lacZ/− embryos examined (compare Fig. 3B and 3A). The remaining 30% of the Hmx2lacZ/− embryos displayed variable defects in the vestibule between different individuals, and even between the membranous labyrinths of the same homozygous mouse embryos. At E18.5, in the most severe cases of malformation
of the vestibule, the \( Hmx2^{lacZ/-} \) inner ears had only a primordial vestibular diverticula in place of well-developed semicircular ducts (compare Fig. 3D,F with C,E). The primordial vestibular diverticula persisted without the development of absorption foci (fusion plates) at the center of the diverticula until birth. Fig. 4 shows inner ears from E13.5 to E18.5 embryos clearly demonstrating the vestibular diverticula without any sign of apposition or fusion of the canal plate epithelia. At E18.5, the severity of the dysgenesis of the semicircular ducts increased, with less distinct folds representing the vestibular diverticula in these older specimens. In contrast, the development of the endolympathic duct/sac and cochlear duct, appeared normal from the analysis of the both \( \beta \)-galactosidase stained specimens and tissue sections (Fig. 3 and Fig. 4, respectively).

Examination of the stained tissue sections also revealed the absence of semicircular duct formation as early as E13.5. As seen in Fig. 4A, control embryos at E13.5 show normal development of semicircular ducts as evident by two transected semicircular duct loops dorsal lateral to the utricle and saccule. At the same level, the \( Hmx2^{lacZ/-} \) embryo has no semicircular ducts with only a rudimentary attempt at their formation as evident by the presence of two vestibular diverticulae and an absence of any transected loops (Fig. 4B). Fig. 4 also illustrates the fused utriculosaccular chamber in the \( Hmx2^{lacZ/-} \) embryo, which was evident as early as E16.5. At E18.5 the same defects are still apparent with an increased severity in semicircular duct dysgenesis as well as enlargement of the fused common utriculosaccular chambers (Fig. 4E,F).
Fig. 4. Absence of semicircular duct formation and formation of a common macula in a fused utriculosaccular chamber in Hmx2lacZ–/– null mutants. (A) Section illustrating normal development of the semicircular ducts in the control embryo at E13.5. (B) Hmx2lacZ–/– inner ear at E13.5 illustrating the formation of two vestibular diverticulae that are dorsal and lateral to a fused utriculosaccular chamber. (C) Control inner ear at E16.5 illustrating normally developed utricle and saccule chambers with associated maculae and horizontal ampulla. (D) Hmx2lacZ–/– inner ear at E16.5 illustrating a common macula within a fused utriculosaccular chamber, lacking any distinction between the utricle and saccule other than the location of the maculae, as both appear to be combined ventrally. (E) Section illustrating normal development of the horizontal crista and ampulla coming off the utricular chamber in a control embryo at E18.5. (F) Hmx2lacZ–/– inner ear at E18.5 demonstrating a more severe dysgenic vestibular system and increased fusion and enlargement of the common utriculosaccular chamber relative to earlier stages. D, dorsal; HC, horizontal crista; L, lateral; M, medial; MS, macula of saccule; MU, macula of utricle; S, footplate of the stapes; SD, semicircular duct; V, ventral; VD, vestibular diverticulum.

In addition to a fused utriculosaccular chamber in the Hmx2lacZ–/– embryos, a portion of this chamber had an area of a common macula, which was identifiable as early as E14.5 in the vestibule of the Hmx2lacZ–/– embryos (data not shown). In other areas of the common cavity, sensory epithelium corresponding to the macula of the utricle was located on the lateral wall of the caudal utriculosaccular chamber where the utricle normally would be located (see Fig. 4). Accordingly, sensory epithelium corresponding to the macula of the saccule was also present in the Hmx2lacZ–/– embryo, located on the medial wall of the rostral utriculosaccular chamber, which is where the saccule would normally be located (Fig. 4B,D,F). In contrast, the thickened sensory epithelia of the maculae fuse on the caudal aspect of the utriculosaccular chamber in the Hmx2lacZ–/– embryos at E16.5 and persist until later stages (Fig. 4C,D). The posterior ampulla is present in Hmx2lacZ–/– embryos along with the posterior crista ampullaris. A distinctly formed superior ampulla, however, is not present, although a patch of sensory epithelium that corresponds to the superior crista ampullaris can be identified in the area where the superior ampulla would normally be located (data not shown). Furthermore, the absence of the lateral crista and lack of a distinct lateral ampulla become evident as early as E14.5. In the Hmx2lacZ–/– inner ear, the lateral crista ampullaris never forms and only a rudimentary attempt at formation of a lateral ampulla is evident off the vestibular diverticulum, with a lack of any transected loops to provide evidence of developed semicircular ducts. The attempt at formation of a lateral ampulla may also simply be a transection through the lateral vestibular diverticulum, which is melding with the superior/posterior diverticulum as the Hmx2lacZ–/– embryo develops in utero, further illustrating the increasingly severe lack of distinction in the vestibular diverticula as the mutants age.

**Quantitative analysis of histological variables between control and Hmx2lacZ–/– embryos**

Progressive impairment of the sensory system of the inner ear was carefully examined during development. Statistically significant deficits in the total number of ampullary hair cells were found in the Hmx2lacZ–/– embryos, with an 86% loss at E14.5, 64% at E16.5, and 68% at E18.5 (P-value=0.02, 0.0017 and 0.00056 respectively; Fig. 5A). Hair cell counts revealed statistically significant deficits in the number of utricular hair cells in the Hmx2lacZ–/– embryos of 63% at E16.5 and 69% at E18.5 of 63% (P-value=0.006 and 0.0005 respectively; Fig. 5B). In contrast, saccular hair cell counts revealed no statistically significant differences between the controls and mutants at all embryonic ages examined (P-value=0.14, 0.25 and 0.58 at E14.5, E16.5 and E18.5 respectively; Fig. 5C).

Although the differences in the number of neurons at E14.5 and E16.5 were not significant, there was a progressive increase in the percentage loss in the number of vestibular ganglion neurons among the Hmx2lacZ–/– embryos of 16% at E14.5, 31% at E16.5, and 51% at E18.5 (P-value=0.001; Fig. 5D). In contrast, there were no statistically significant differences between the controls and Hmx2lacZ–/– embryos in the number of spiral ganglion neurons (P-value=0.1 at E14.5, 0.82 at E16.5, 0.38 at E18.5; Fig. 5E). Analysis of the number of hair cells in the superior ampulla showed statistically significant losses of 73% at E14.5, 52% at E16.5, and 61% at E18.5 in the Hmx2lacZ–/– embryos (P-value=0.003, 0.01 and 0.0094; data not shown). The number of hair cells in the posterior ampulla also showed statistically significant deficits in the Hmx2lacZ–/– embryos.
embryos of 69% at E14.5, 43% at E16.5, and 54% at E18.5 (P-value=0.00058, 0.00064, 0.013; data not shown). Histological measurements showed significant differences in the area of the utricular macula. Again, an increase in the percentage loss in the area of the utricular macula was evident: 50% at E14.5 (P-value=0.14), 75% at E16.5 (P-value=0.0014), and 54% at E18.5 (P-value=0.0005). In contrast, the area of the saccular macula did not show statistically significant deficits in the Hmx2lacZ–/– embryos at E14.5 and E16.5, but did show a statistically significant loss at E18.5 of 32% (P-value=0.015). The total area of the cristae ampullaris showed statistically significant losses at all ages: 87% at E14.5, 43% at E16.5, and 30% at E18.5 (P-value=0.000076, 0.034, 0.0077 respectively). The measurement of the volume of the chambers constituting the Hmx2lacZ–/– embryo inner ears revealed significant findings in the utricle, saccule, and the ampullae. The volume of the Hmx2lacZ–/– utricle showed a significant deficit at E16.5 of 82% (P-value=0.0077). The measurement of the Hmx2lacZ–/– saccular volume showed significant deficits of 82% at E14.5 (P-value=0.044), 53% at E16.5 (P-value=0.0024), and 59% at E18.5 (P-value=0.0051). Interestingly, the total volume measurement of the ampullae only showed a statistically significant deficit in the Hmx2lacZ–/– embryos at E16.5 of 70% (P-value=0.015).

**Reduced cell proliferation in the otic epithelium and the periotic mesenchymal cells in the Hmx2lacZ–/– inner ears**

Since gross morphogenetic abnormalities in the developing vestibular system can be observed as early as E13.5 (Fig. 3), molecular events mediating these defects must have occurred before this stage. Even though no gross morphological alterations can be detected between control and Hmx2lacZ–/– whole-mount inner ears at E11.5, transverse sections through inner ears stained for β-galactosidase revealed clear differences in the epithelial invaginations between the Hmx2lacZ+/– and Hmx2lacZ–/– embryos (Fig. 6A and 6B). At this stage, Hmx2 expression visualized by β-galactosidase activity is present in the entire dorsal otic vesicle excluding the otic epithelium cells destined to form the endolymphatic duct and sac (Fig. 6A). A small region of the lateral epithelium becomes thinner and the entire lateral aspect of the otic epithelium begins moving medially (arrow in Fig. 6A). In the Hmx2lacZ–/– otic vesicle, neither the thinning nor the invagination of the lateral epithelium takes place (Fig. 6B). In wild-type embryos...
at E12.0, a thinned otic epithelial layer delaminates from the underlying mesenchyme and further moves toward the medial face, forming a structure termed the ‘fusion plate’ (arrows in Fig. 6C,E). However, in the Hmx2lacZ–/– inner ears, regional thinning and invagination of the lateral face of the otic epithelium towards the medial layer is not observed at this stage (Fig. 6D,F). Interestingly, at E13.5 when the 3-dimensional structure of the otic labyrinth has been well formed in the wild-type inner ear (Fig. 3A), the lateral epithelial layer of the Hmx2lacZ–/– vestibular diverticulum begins to become thin and eventually detaches from the underlying mesenchyme (data not shown). However, the subsequent fusion plate process never occurs in the Hmx2lacZ–/– inner ear, and this appears to be a principal reason for the failed vestibular morphogenesis in mice lacking Hmx2.

Our histological analysis has revealed that, in addition to gross morphological defects, Hmx2lacZ–/– inner ears also display severe cell loss in both the sensory and nonsensory epithelia in the vestibular system. Cell proliferation and cell death both play critical roles in inner ear morphogenesis, and the spatial and temporal patterns of cell proliferation and cell death in the developing inner ear have been elaborately mapped (Fekete et al., 1997; Lang et al., 2000). The analysis of programmed cell death using the TUNEL assay was performed to examine cell death during inner ear morphogenesis on serial sections of wild-type and Hmx2lacZ–/– embryos from E10.5 to E13.5. During the morphogenesis of the wild-type inner ear, the developing fusion plate is one of the ‘hot spots’ showing elevated apoptotic activities, which have been suggested to be critical in removing epithelial cells from the center of each canal and

Fig. 6. Cell proliferation in the developing inner ear is affected by the loss of Hmx2. A-F show the morphological alteration of the Hmx2lacZ–/– inner ear at E11.5 and E12.0 by examining β-galactosidase activity. Arrows indicate the presumptive fusion plate that is undergoing thinning and invagination. E and F are sections dorsal to C and D, respectively. The approximate level of sectioning is also indicated in Fig. 2. G-L are the comparable anti-BrdU-labeled sections showing the reduced rate of cell proliferation in the developing Hmx2lacZ–/– inner ears. Genotypes and embryonic stages are indicated at the top of each column. Control corresponds to either Hmx2lacZ+/+ or Hmx2lacZ+/– genotypes. Overt morphological differences can be seen at E11.5 when the invagination of epithelial cells around the posterolateral boundary of the otic vesicle is delayed in the homozygotes (A and B). At E12.0, the close apposition of epithelial walls to form the fusion plates was not present in the otocyst of the Hmx2lacZ–/– embryo (C,D,E,F). Both the epithelial cells and underlying periotic mesenchymal cells of the corresponding regions are undergoing reduced cell proliferation (J and L). M-P show the apoptotic activities of the otic epithelial cells in the control and Hmx2lacZ–/– inner ears at E11.5 and E12.0. Arrows indicate the regions of the fusion plates. A higher percentage of cells in the fusion plates are undergoing programmed cell death relative to other regions in both control and Hmx2lacZ–/– embryos. However, the Hmx2lacZ–/– otic vesicles do not show an altered rate of cell death relative to the corresponding regions in the control embryos. L, lateral; R, rostral.
A higher percentage of cells in the prospective fusion plate do show apoptotic activities relative to other regions in the wild-type mouse otic vesicle (arrows in Fig. 6M, O). However, regions comparable with the presumptive fusion plate of the wild-type inner ear showed no alteration in apoptotic activities in the Hmx2lacZ–/– otocyst (Fig. 6M and 6N). Similarly, in the other regions of the inner ear including the ventromedial otic vesicle, the base of the endolymphatic sac and the sensory organs, no significant difference in the rate of programmed cell death was observed in the Hmx2lacZ–/– inner ears relative to wild-type embryos of the same stage. Thus changes in programmed cell death do not appear to play a prominent role in the mechanisms of Hmx2-mediated dysmorphogenesis.

However, when the BrdU incorporation rate was examined, reduced cell proliferation was observed in the Hmx2lacZ–/– inner ears in both the otic epithelium and the periotic mesenchymal cells (Fig. 6G-L). In the wild-type otic vesicle at E11.5, epithelial cells, including the sensory and nonsensory epithelial cells show active cell proliferation (Fig. 6G and 6H), which was dramatically reduced in the Hmx2lacZ–/– otic vesicle (Fig. 6G and 6H). In wild-type embryos at E12.0, when the fusion plate is being pushed toward the medial epithelial face, mesenchymal cells underlying the presumptive fusion plate as well as the recipient face of the medial epithelial layer display
elevated cell proliferation activity as indicated by the many BrdU-labeled cells (Fig. 6L,K). But in the Hmx2lacZ−/− otic vesicle, less BrdU-positive cells were present in the corresponding regions and the distribution of proliferating cells was quite uniform throughout the periotic mesenchyme (Fig. 6J,L). Previous work has demonstrated that the periotic mesenchyme may provide the major driving force pushing the neighboring epithelial cells to undergo morphological changes (Salminen et al., 2000; Van De Water, 1983). Therefore, slowed cell proliferation exhibited by the otic epithelium and the surrounding periotic mesenchyme in the Hmx2lacZ−/− inner ear may account, in part, for disruption of the morphogenesis of the vestibular system.

**Inactivation of Hmx2 affects the expression profile of developmental regulators that control inner ear ontogeny**

Expression of genes with a demonstrated role in inner ear morphogenesis and differentiation was examined on paraffin sections and whole mounts of wild-type and Hmx2lacZ−/− inner ears at embryonic stages preceding and coincident with the disruption in vestibular morphogenesis. At E10.5, all genes examined, including BF1, Bmp4, Dlx5, Hmx3, netrin 1, Pax2, Otx1 and Sek1, showed no overt alteration in their expression in the Hmx2lacZ−/− inner ear (Fig. 7K and 7L; data not shown). Homeobox genes Hmx3 and Otx1, as well as the receptor tyrosine kinase gene Sek1, which all play a critical role in inner ear morphogenesis, do not show any altered expression patterns at any of the embryonic stages examined (Fig. 7A,B; and data not shown). Either the lack of a gene regulatory relationship between Hmx2 and the above mentioned genes or the inability of Hmx2 alone to alter the regulatory cascade may account for these findings. Owing to the severe morphological discrepancies between wild-type and Hmx2lacZ−/− inner ears older than E13.5, altered expression of certain markers at later stages may be caused by indirect structural changes rather than direct regulatory effects. Thus in situ hybridization results on sections of embryos immediately preceding and coinciding with Hmx2lacZ−/− inner ear dysmorphism (i.e. E11.5) are presented.

In wild-type embryos, the homeobox gene Dlx5 is preferentially expressed in three different regions including the developing endolymphatic sac and epithelial patches which are located between the two Bmp4-positive spots (presumptive anterior and lateral cristae) in the anterior portion of the lateral aspect of the otic vesicle, as well as in the region anterior to Bmp4-positive sensory patch, in the posterior otic vesicle (arrows in Fig. 7C,P). Loss of Dlx5 results in failed morphogenesis of the semicircular canals and failed cytodifferentiation within the vestibular epithelium (Acampora et al., 1999; Depew et al., 1999). In the Hmx2lacZ−/− otic vesicle, the lateral gap between the rostral and caudal Dlx5 expression domains was now filled with Dlx5-positive cells (arrowheads in Fig. 7C,D) suggesting either the derepression of Dlx5 expression or the migration of Dlx5-positive cells into this region. Pax2 is a paired-box containing transcription factor with a demonstrated role in patterning the inner ear. In addition to its expression in the medial epithelium of the dorsal otic vesicle, Pax2 is also seen in restricted otic epithelial cells slightly anterior to the Bmp4-positive sensory patch in the posterior portion of the lateral layer (arrow in Fig. 7E). In the Hmx2−/− lateral otic epithelium, Pax2 expression is diminished (Fig. 7E and 7F) indicating a requirement for Hmx2 to maintain Pax2 expression in these cells. At E10.5, Bmp4 transcripts were present in a stripe of epithelial cells in the rostro-dorsal otic vesicle (arrow in Fig. 7K). At this stage, Bmp4 mRNA can be detected in a correct spatial manner in the Hmx2lacZ−/− inner ear (Fig. 7L). At E11.5, Bmp4 transcripts mark presumptive sensory organs in the developing inner ear and were present primarily in two cell clusters at the anterior and posterior edges of the otic epithelium (arrows in Fig. 7M,P). These two discrete Bmp4-positive domains, which normally are separated by the region destined to form the fusion plate, will develop into cristae at later stages (Oh et al., 1996). Inactivation of Hmx2 results in the disappearance of sensory patch-restricted Bmp4 expression in the otic vesicle (Fig. 7N). In addition, Bmp4 is upregulated and expressed uniformly in the lateral aspect of the otic epithelium including the presumptive fusion plates which are normally negative for Bmp4 in wild-type inner ears (Fig. 7PR), suggesting a role for Hmx2 in repression of Bmp4 in the cells constituting the future fusion plate. At E12.0 during fusion plate formation, Bmp4 expression is strictly confined to the sensory organs (arrow in Fig. 7T) and is completely absent from the fusion plate in mouse (arrowheads in Fig. 7T). At this stage, ectopic presence of BMP4 in the fusion plate persists in the Hmx2lacZ−/− otic vesicles (arrowheads in Fig. 7V).

In summary, loss of functional Hmx2 leads to an alteration of the molecular identities of the cells in the dorsolateral aspect of the otic vesicle including the sensory cells and the prospective fusion plate. Taken together with the results from the cell proliferation assay, Hmx2lacZ−/− fate-altered lateral epithelium acquired a reduced cell proliferation capability, which in turn impacted on the adjacent periotic mesenchyme, which normally does not express Hmx2. Hence changes in cell fate in restricted regions of the otic epithelium subsequently affects global morphogenesis of the entire vestibular system. In addition, the above RNA in situ data also indicate the existence of a genetic regulatory interaction between Hmx2 and other inner ear developmental regulatory genes such as Bmp4, Dlx5 and Pax2. Other factors showing restricted expression pattern in the inner ear were also investigated in the Hmx2lacZ−/− embryos. Brain factor 1 (BF1), a winged helix transcription factor is confined to the epithelium on the medial aspect of the otic vesicle (Hebert and McConnell, 2000), however its absence in the dorsolateral face was maintained (Fig. 7G and 7H) in the Hmx2lacZ−/− embryos. Likewise netrin 1, a member of the laminin-related secreted proteins, is expressed in the nonsensory epithelium known to form the fusion plate. Expression of netrin 1 remains unchanged in the Hmx2lacZ−/− embryos (Fig. 7I and 7J), indicating that inactivation of Hmx2 alone is insufficient to affect netrin 1 expression even though loss-of-function alleles for these two genes present a similar phenotypic mechanism affecting inner ear morphogenesis.

**DISCUSSION**

The role of Hmx2 in murine inner ear development

Temporally, Hmx2 expression exhibits a multi-phasic pattern in the developing inner ear. At the early otic vesicle stage (E9.5) Hmx2 is strongly expressed in the anterior aspect of the vesicle. By E10.5 the strongest expression is in the
anterodorsal portion of the otocyst and at E12.5 the entire dorsal portion (pars superior) of the otocyst strongly expresses this gene. The areas of otic epithelium that express Hmx2 include areas that will form both sensory and nonsensory epithelium and exclude those cells that form the future endolympathic duct and sac. Its expression in the dorsal endolympathic duct becomes prominent at E12.5 after the overall structure has already been established. Its late onset of expression, together with the unaffected expression of early inner ear markers in the \textit{Hmx2}\textsuperscript{lacZ–/–} otocyst, indicate that Hmx2 is unlikely to be involved in the induction of the otic placode or the initial morphogenesis of the otic vesicle. An in vitro fate map study of the murine otocyst (E11-E12.5) has determined that the pars superior portion of the otocyst participates in the formation of all the vestibular sensory receptors and generates all three of the semicircular ducts (Li et al., 1978). The functional data presented in this paper clearly demonstrate that Hmx2 participates in the transformation of the pars superior of the otic vesicle into a complex mature vestibular labyrinth. Defects resulting from the absence of Hmx2 includes the lack of any distinguishable semicircular ducts, persistence of the primordial vestibular diverticula, a fused utriculosaccular space in which a common macula is formed, as well as a severe loss of epithelial cells (both sensory and nonsensory) in the developing vestibule, clearly indicating a severe disruption of inner ear development. During the morphogenesis of complex organs, the production of cells and the fusion of tissue layers at specific regions are critical events. Within the inner ear, cell proliferation provides both material and a mechanical driving force for epithelial fusion initiated at the fusion plate, the elaboration of which will ultimately finalize the basic architecture of the vestibular system. At E11.5 in the \textit{Hmx2}\textsuperscript{lacZ–/–} otocyst, a reduced rate of cell division was observed in the otic epithelium where Hmx2 is normally expressed, as well as in the adjacent periotic mesenchyme. At the same stage, thinning of the epithelial layer in specific regions of the otic vesicle fails to take place, suggesting that at this stage no region in the \textit{Hmx2}\textsuperscript{lacZ–/–} otocyst has become competent to form a fusion plate. However, the finding of a thinned epithelial layer and its detachment from the underlying basement membrane at E13.5 suggest a potential delayed initiation of fusion plate formation. However, the eventual fusion of the apposing walls of the otocyst to form a semicircular duct never takes place. During the transformation from an otic vesicle to an otic labyrinth, morphogenetic milestones such as the thinning of the epithelial layer, loss of epithelial morphology, detachment from the basement membrane and fusion of the apposing epithelial walls, are strictly coordinated in a temporal and spatial manner (Martin and Swanson, 1993). The delayed initiation of fusion plate formation in the \textit{Hmx2}\textsuperscript{lacZ–/–} inner ear may miss the time window when the otic vesicle and surrounding environment are competent to carry out the fusion process. Molecules from different gene families have been shown to play a critical role in epithelial fusion. One well-characterized molecule is the laminin-like protein, netrin 1. In addition to its function in axon guidance and cell migration in the central nervous system, netrin 1 is also required for fusion plate formation during inner ear morphogenesis (Salminen et al., 2000). Laminins are a major component of the ECM and previous work has shown that remodeling of the extracellular matrix (ECM) in the basement inner development is a key event in axonal guidance, cell migration, angiogenesis and morphogenesis of complex organs. It had been proposed that the robust presence of netrin 1 in the fusion plate might either compete with other laminin molecules to disrupt the laminin network in the basement membrane, or upregulate the production of matrix metalloproteinases to remodel the ECM network. In the netrin 1 null otocyst, the epithelial wall of the presumptive fusion plate became thinner. However, these thinned layers failed to change their epithelial morphology and subsequently detach from the underlying basement membrane. The strong expression of netrin 1 in the \textit{Hmx2}\textsuperscript{lacZ–/–} inner ears at all stages suggests that netrin 1 alone is insufficient for the formation of the fusion plate. It is possible that the prospective epithelium has to first become competent so that it can be responsive to netrin 1. In this respect, Hmx2 may be needed to determine the fate of epithelial cells in specific regions of the otocyst. The reduced cell proliferation rate observed in the \textit{Hmx2}\textsuperscript{lacZ–/–} periotic mesenchymal cells further suggests that Hmx2 and netrin 1 may work cooperatively to control fusion plate formation by affecting cell proliferation of the neighboring mesenchyme. Also, the entry points when the two genes exert their functions are different since Hmx2 clearly functions at an earlier stage than netrin 1. Based upon our in situ hybridization data, although these two genes share many overlapping expression domains, a clear regulatory interaction cannot be established between Hmx2 and netrin 1. There are two possible mechanisms to account for this. First, both genes may use independent pathways to regulate cell proliferation. Second, there is a regulatory interaction existing between these two genes, however, inactivation of Hmx2 alone is insufficient to alter netrin 1 expression, as other genes might compensate for the function of Hmx2. One such candidate is Hmx3. However, the persistence of netrin 1 in the developing Hmx3 null inner ear suggests either a possible functional redundancy between Hmx2 and Hmx3 in regulating netrin 1 (Salminen et al., 2000), or no regulatory interaction. The assessment of an overlapping role of Hmx2 and Hmx3 in controlling netrin 1 (and other inner ear-specific genes) will be obtained from the analysis of embryos carrying combined loss-of-function alleles for Hmx2 and Hmx3. Interestingly, certain phenotypic aspects of a previously generated Hmx3-PGK\textit{neo} null allele share some overlap with the Hmx2 phenotype described here (Hadrys et al., 1998). However, this overlap was not observed in mice carrying either of two independent Hmx3 null alleles that lacked PGK\textit{neo} and which were additionally shown to have no affect on the expression of the neighboring Hmx2 gene (Wang et al., 1998). As PGK\textit{neo} is notorious for affecting adjacent gene expression within tens of kilobases (Olson et al., 1996), a plausible explanation for the partial phenotypic overlap is that the previously described Hmx3-PGK\textit{neo} allele (Hadrys et al., 1998) is also affecting, in a negative manner, the expression of the closely linked Hmx2 gene. Differential expression of varied combinations of developmental patterning genes gives distinct molecular identities to different cell populations, which consequently display unique capabilities in cell proliferation, apoptotic activity and responsiveness to the environment (Fekete, 1996). In the \textit{Hmx2}\textsuperscript{lacZ–/–} otocyst, cells in a subset of the \textit{Hmx2}-expressing domains have changed their fate as indicated by the altered expression of specific inner ear markers. The loss of
expression in the presumptive sensory epithelium of Bmp4, Dlx5 and Fox2, together with ectopic activation of Bmp4 and Dlx5 in the prospective fusion plate, indicate a substantial alteration in cell fate in the otic epithelium of the pars superior. As a result of this cell fate alteration, the otic epithelium fails to communicate properly with the periotic mesenchyme, and in return, proper inner ear morphogenesis is severely impaired (Van De Water, 1983). Here we show that Hmx2, a homeodomain transcription factor affects the rate of cell proliferation of the otic epithelium, as well as the neighboring mesenchymal tissue. The identification of cell-cell signaling factors bridging the gap between the different tissues will be important. In this study, it is interesting that BMP4, a putative crista marker was present at E10.5, but disappeared by E11.5, suggesting that the cristae might be specified initially but fail to develop properly in the absence of Hmx2.

In summary, a normal function of Hmx2 is to govern the specification and commitment of epithelial cells in the pars superior portion of the otocyst to undergo the proliferative growth and fusion processes to form a mature and functional vestibular system.

Functional relationship between Hmx2 and Hmx3 in mouse development

Hmx2 and Hmx3 are an ideal pair of homeobox genes to investigate functional redundancy existing between members of the same gene family. The highly similar expression patterns and close linkage on chromosome 7 suggest that these two genes may share certain of the same transcriptional regulatory elements during mouse development and the similarity in the DNA-binding homeodomain suggest that these two genes may share many downstream regulatory targets. In the central nervous system and uterus, Hmx2 and Hmx3 show identical expression profiles (Wang et al., 2000; Wang et al., 1998), but in the Hmx2lacZ-/- animals, no obvious defect was detected in either of these tissues. By comparing the defects in the individual Hmx2 and Hmx3 null inner ears, the unique developmental function of each of these genes can be identified. In the Hmx3 null inner ear, the gross structure of the three semicircular canals forms except the horizontal ampulla and its associated crista are absent. In the ventral part of the vestibule, the utricle and saccule are fused into one chamber in which a significant cell loss occurs in both sensory maculae. This indicates that Hmx3 alone uses a different mechanism to effect cell fate determination than by facilitating fusion plate formation and subsequent closure of the semicircular ducts. Disruption of Hmx2, despite its later onset of expression, results in a more severe inner ear phenotype than loss of Hmx3. In the Hmx2lacZ-/- inner ear, anterior and dorsal regions along the corresponding AP and DV axes of the developing vestibule are more severely affected than in Hmx3 null mice. Even though Hmx2 and Hmx3 are both involved in cell fate determination in specific regions of the otic epithelium, the consequences are different. Loss of Hmx3 primarily influences the development of a subset of sensory receptor cells, but not the overall morphogenesis of the inner ear. In contrast, Hmx2 is not only involved in cell fate determination of vestibular sensory areas, but also the morphological transformation mediated by the nonsensory epithelial cells. Even though Hmx3 is transcriptionally activated about 8 hours earlier in the otic placode, the time point when Hmx3 exerts its function seems to be later than that of Hmx2. Moreover, inactivation of Hmx3 generates a milder phenotype, as fewer structures and tissue types are affected. It is also notable that some regions coexpressing Hmx2 and Hmx3, such as the saccule, posterior ampulla and endolymphatic duct are not severely affected in either mutant. One explanation is that Hmx2 and Hmx3 may share redundant functions during inner ear and CNS development or they may be expressed in tissues where they exert no developmental function, possibly owing to a lack of a necessary cofactor(s). The unique and overlapping functions of each gene will be more clearly understood by comparing the inner ear phenotypes of mice carrying individual as well as a combined mutation in Hmx2 and Hmx3.

The authors gratefully acknowledge Yan-Jie Chang, Jingxian Liu, Wei Liu, Dilip Madhani, Steve Raft and Cynthia Shoemaker for their technical assistance. We would like to thank Peter Gruss and Brigid Hogan for providing RNA in situ probes.

REFERENCES


Hmx2 in inner ear development


