**Otx1** and **Otx2** activities are required for the normal development of the mouse inner ear

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**SUMMARY**

The Otx1 and Otx2 genes are two murine orthologues of the Orthodenticle (Otd) gene in *Drosophila*. In the developing mouse embryo, both Otx genes are expressed in the rostral head region and in certain sense organs such as the inner ear. Previous studies have shown that mice lacking Otx1 display abnormal patterning of the brain, whereas embryos lacking Otx2 develop without heads. In this study, we examined, at different developmental stages, the inner ears of mice lacking both Otx1 and Otx2 genes. In wild-type inner ears, Otx1, but not Otx2, was expressed in the lateral canal and ampulla, as well as part of the utricle. Ventral to the mid-level of the presumptive utricle, Otx1 and Otx2 were co-expressed, in regions such as the saccule and cochlea. Paint-filled membranous labyrinths of Otx1−/− mutants showed an absence of the lateral semicircular canal, lateral ampulla, utriculosaccular duct and cochleosaccular duct, and a poorly defined hook (the proximal part) of the cochlea. Defects in the shape of the saccule and cochlea were variable in Otx1−/− mice and were much more severe in an Otx1−/−;Otx2−/− background. Histological and in situ hybridization experiments of both Otx1−/− and Otx1−/−;Otx2−/− mutants revealed that the lateral cristata was absent. In addition, the maculae of the utricle and saccule were partially fused. In mutant mice in which both copies of the Otx1 gene were replaced with a human Otx2 cDNA (hOtx2¹/hOtx2¹), most of the defects associated with Otx1−/− mutants were rescued. However, within the inner ear, hOtx2 expression failed to rescue the lateral canal and ampulla phenotypes, and only variable rescues were observed in regions where both Otx1 and Otx2 are normally expressed. These results suggest that both Otx genes play important and differing roles in the morphogenesis of the mouse inner ear and the development of its sensory organs.

Key words: Inner ear development, Gene expression, Sensory organ, Mouse, Otx1, Otx2

**INTRODUCTION**

Otx1 and Otx2 are murine orthologues of the *Drosophila* orthodenticle (otd) gene (Simeone et al., 1992). These genes are transcription factors containing a bicoid-like homeobox (Simeone et al., 1992). In *Drosophila*, otd is important for head formation and different levels of otd protein are required for the formation of specific subdomains of the adult head (Hirth et al., 1995; Royet and Finkelstein, 1995). *otd* and its orthologues share sequence homology and have been shown to substitute for each other functionally (Acampora et al., 1998a,b, 1999; Leuzinger et al., 1998). In mouse, both Otx genes are involved in regional specification and proper morphogenesis of brain structures. Otx1 is expressed in the presumptive forebrain and midbrain regions of the neural tube of mice starting at 8 dpc (days post coitum) and the absence of this gene product causes malformations of the telencephalon, mesencephalon and cerebellum (Simeone et al., 1993; Acampora et al., 1996). Otx1−/− mutant mice also display spontaneous epileptic behavior (Acampora et al., 1996). Otx2 gene expression is activated in the entire ectoderm and visceral endoderm of the mouse before gastrulation, and is restricted to the anterior part of the embryo by 7.5 dpc (Simeone et al., 1993; Ang et al., 1994). Mice homozygous for Otx2 are early embryonic lethal and lack anterior neural tissues (Ang et al., 1996; Matsuo et al., 1995; Acampora et al., 1995). Otx1−/−;Otx2−/− mice also display a more severe brain phenotype than Otx1−/− alone suggesting that the dosage of Otx gene products is important for normal development (Acampora et al., 1997).

Both Otx1 and Otx2 are expressed in sense organs such as the inner ear, eye and olfactory epithelium (Simeone et al., 1993). The absence of these gene products affects the formation of these sense organs (Acampora et al., 1996; Matsuo et al., 1995). Otx1−/− mutant mice showed a rapid turning behavior and their inner ears lacked the lateral semicircular canal (Acampora et al., 1996). This defect of Otx1−/− mutant mouse was not rescued with the otd or hOtx2
cDNA, suggesting a unique role of *Otx1* in inner ear development (Acampora et al., 1998, 1999).

In this study, we examined the normal expression of the two *Otx* genes in the mouse inner ear and the characterization of *Otx1*+/−, *Otx1*+/−;*Otx2*+/− as well as *hOtx2*† mutant ears. The mouse inner ear is a complex organ that is derived embryonically from a rudimentary structure known as the otocyst. There are six major sensory organs, three cristae, two maculae and a cochlea, that are responsible for vestibular and auditory functions. Each sensory organ requires a precise pattern and position within the inner ear in order to properly mediate its function. The molecular mechanisms that underlie these features of sensory organ formation are largely unknown. Here, we demonstrate that the lack of *Otx1* and *Otx2* gene products affected the development and final positioning of some of the sensory organs within the mouse inner ear as well as the shaping of its cochlea. The difference in the phenotype of *Otx1*+/− and *Otx1*+/−;*Otx2*+/− inner ears and the inability of *hOtx2* cDNA to rescue defects of *Otx1*+/− inner ears suggest that these two genes play separate roles in inner ear development. These data are also discussed in light of the recently proposed boundary model for sensory organ specification in the inner ear.

**MATERIALS AND METHODS**

**Embryos**

Mice were generated and genotyped as described (Acampora et al., 1996, 1997, 1999). Mutant mice have the same mixed genetic background as 129Sv, BL6 and DBA2. All embryos were individually staged according to the method of Theiler (1989).

**Probes**

The RNA probes for *Fng*, *BMP4* and *Brain3.1* (*Brn3.1*) were prepared as previously described (Morsli et al., 1998). The probe for *Otx1* was a 396 nucleotide SacI-PstI fragment (Simeone et al., 1993). Approximately 1 kb of each respective cDNA was used to generate probes for *Otx2* (Ang et al., 1994) and *TRP-2* (Steel et al., 1992). The probe for the 68 kDa neurofilament protein was a PCR product generated between nucleotides 851 and 1350 (gift of XianJie Yang, UCLA).

**Paint injection**

All embryos were harvested and injected as previously described (Morsli et al., 1998). Inner ears injected were as follows: for wild type, five 12 dpc and five 15 dpc; for *Otx1*+/−, four 15.5 dpc; for *Otx1*+/−;*Otx2*+/−, two 15 dpc; for *Otx1*+/−;*Otx2*+/−, four 12 dpc, two 15 dpc and seven 15.5 dpc and two 16 dpc; for *Otx1*+/−;*Otx2*+/−, four 12 dpc, two 15 dpc and seven 15.5 dpc; for *hOtx2*†, seven 16 dpc and two postnatal day 1 (P1).

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed as previously described (Riddle et al., 1993) with the following modifications. All embryos were permeabilized with proteinase K (Boehringer Mannheim, Indianapolis, IN) using concentrations from 1 to 15 μg/ml. Hybridization, washings and detection procedures were performed as described by Riddle et al. (1993).

**In situ hybridization of frozen section**

Frozen 12 μm sections for both wild-type and mutant embryos were processed for in situ hybridization as previously described (Morsli et al., 1998). The mutants analyzed were as follows: for *Otx1*+/−, two 10.5 dpc, five 11.5 dpc, three 12 dpc, three 13 dpc, three 14 dpc and four 18 dpc; for *Otx1*+/−;*Otx2*+/−, two 12 dpc, one 14 dpc and five 18 dpc. Each specimen was serially sectioned at the level of the inner ear and adjacent sections were processed for two or more probes.

**Hematoxylin and Eosin staining**

After analysis of paint-injected inner ears, specimens were transferred and embedded in paraffin. All embryos were sectioned at 10 μm.

**RESULTS**

**Otx1 and Otx2 expression in the developing inner ear**

The histology of the mouse inner ear becomes distinct at 14 dpc. In order to characterize the domains of *Otx1* and *Otx2* expression in earlier developmental stages, we have used previously established markers for the presumptive sensory organs. *Bone morphogenetic protein 4* (*BMP4*) was used as a marker for the three presumptive cristae, whereas *lunatic fringe* (*Fng*) served as a marker for the cochlea, maculae utriculi and sacculi at early stages, and for all sensory organs including the cristae for embryos 13 dpc and older.

Both *Otx1* and *Otx2* were expressed in mostly nonsensory regions of the developing inner ear. *Otx1* was expressed in a region destined to form the lateral canal, lateral ampulla, utricle and pars inferior. *Otx2* was expressed mostly in the region destined to form the pars inferior.

**10-11.5 dpc**

At 10.25 dpc, *Otx1* mRNA was detected in the posteroventral quadrant of the lateral part of the otic vesicle (arrow in Fig. 1A). *Otx2* transcripts were localized at the most ventral tip of the vesicle, where it was coexpressed with *Otx1* (arrow in Fig. 1B). At 11.5 dpc, *BMP4* was expressed in the lateral part of the otocyst as an anterior streak which encompassed the presumptive anterior and lateral cristae (Morsli et al., 1998). Fig. 1C illustrates the most posterior portion of the anterior streak. *Otx1* expression domain, at 11.5 dpc, also remained in the most ventrolateral part of the otocyst (blue stripes in 11.5 dpc drawing of Fig. 1). Its dorsal boundary most likely corresponded to the presumptive lateral canal plate region judging by the position of the anterior streak of *BMP4* domain at the same level (compare Fig. 1C and 1C¢). It is interesting that the anterior portion of the *Otx1* boundary overlapped with the posterior portion of the anterior streak of *BMP4* expression (compare Fig. 1C and 1C¢). This region most likely gives rise to the lateral crista (Morsli et al., 1998). The *Otx1* expression domain extended ventrally to the tip of the cochlear anlage. *Otx2* transcripts also remained in the ventrolateral part of the otocyst, thus being coexpressed with *Otx1* (red stripes in 11.5 dpc drawing of Fig. 1) starting in the middle of the presumptive utricle (Fig. 1G) and extending ventrally to the tip of the cochlear anlage.

**12 dpc to P5**

At 12 dpc, the *Otx1* expression domain remained in the most ventrolateral part of the developing inner ear (blue stripes in 12 dpc drawing of Fig. 1). Its dorsal boundary was at the level of the lateral canal plate which, at this stage, was fully developed (Ip in Fig. 1D). The anterior portion of the boundary overlapped with the presumptive lateral crista, as highlighted...
by BMP4 expression (lc in Fig. 1D, compare to Fig. 1D). At the level of the presumptive macula utriculi, as highlighted by the Fng expression pattern, the Otx1 expression domain was lateral but not overlapping with the Fng domain (mu in Fig. 1E, compare to Fig. 1E).

At the cochlear level, Otx1 was also expressed in the lateral wall, not overlapping with the Fng-positive sensory tissue of the cochlea (Fig. 1F and F'). At 13 dpc, the pattern of Otx1 expression remained the same. From 14 dpc and thereafter, no Otx1 transcripts were detected in the inner ear.

At 12 dpc, Otx2 transcripts remained co-expressed with Otx1 in the ventrolateral part of the developing inner ear (red stripes in 12 dpc drawing of Fig. 1). The dorsal boundary of its expression domain remained in the middle of the utricular anlage (Fig. 1H), extending ventrally to the tip of the coiling cochlea. By 13 dpc, Otx2 transcripts were localized ventral to the macula utriculi, and restricted to the lateral wall of both the saccule and cochlea. This expression pattern remained the same at least until P5, where Otx2 transcripts localized to the lateral wall of the saccule (arrowhead, Fig. 1I) and Reissner’s membrane of the cochlea (arrowhead, Fig. 1J). Otx1 and Otx2 transcripts did not overlap with BMP4 or Fng in the developing cochlea.

**Gross morphology of Otx1−/− and Otx1−/−;Otx2−/− mutants**

**Anatomy of a wild-type inner ear at 15 dpc**

By 15 dpc, the mouse inner ear had nearly attained its mature shape (Fig. 2A). The pars superior consisted of all three canals and ampullae, the endolymphatic duct and sac, and the utricle. The pars inferior consisted of the saccule and the coiled cochlea. The saccule connected to the utricle and the cochlea via the utriculosaccular (usd in Fig. 2A) and cochleosaccular (csd in Fig. 2A) ducts, respectively. The dorsal tip of the cochlea is defined as the hook. The cochlea consisted of 1.5 turns at this age and reached its mature 1.75 turns by 16 dpc (data not shown). Membranous labyrinths of all Otx1−/−;Otx2−/− mutants showed normal morphology at this stage (data not shown).

**Consistent phenotypes**

Both Otx1−/− and Otx1−/−;Otx2−/− mutants exhibited common consistent phenotypes (Fig. 2B-F). In the pars superior, the lateral semicircular canal and ampulla were absent. In the pars inferior, the utriculosaccular and cochleosaccular ducts were absent. As a result, the utricle, saccule, and cochlea were not separated from one another. In addition, the hook of the cochlea was poorly defined.

**Fig. 1.** Gene expression patterns of Otx1, Otx2, BMP4 and Fng in developing mouse inner ears ranging from 10.25 to 12 dpc. At 10.25 dpc, (A) Otx1 expression was detected in the posteroventral quadrant of the lateral part of the otocyst (arrow), whereas (B) Otx2 transcripts were localized at the most ventral tip of the lateral part of the otocyst (arrow), thus being coexpressed with Otx1. At 11.5 dpc, (C) Otx1 expression domain remained localized in the lateral part of the otocyst, with its dorsal boundary at the level of the anterior streak of (C’) BMP4 transcripts. The anterior portion of the dorsal boundary overlapped with the posterior portion of the anterior streak. This region most likely gives rise to the lateral crista. Otx2 is coexpressed with Otx1 in the lateral portion of the inner ear starting in the middle of the utricle (G). At 12 dpc, (D) Otx1 transcripts were restricted in the developing lateral canal plate overlapping with the developing lateral crista, as highlighted by (D’) BMP4 gene expression. Otx1 transcripts were also restricted in the lateral wall of the utricle (E) and cochlea (F), juxtaposed to the macula utriculi (E’) and the sensory tissue of the cochlea (F’), as highlighted by Fng transcripts. Also, at 12 dpc, Otx2 remained coexpressed with Otx1 in the lateral part of the otocyst, starting at the mid-level of the utricular anlage, and extending ventral to the tip of the coiling cochlea (compare E and H). Blue stripes, Otx1 expression; red stripes, Otx2 expression; as, anterior streak; lp, lateral crista; lp, lateral canal plate; mu, macula utriculi; s, saccule; sm, scala media; st, scala tympani; sv, scala vestibuli. Orientation: A, anterior; D, dorsal; L, lateral. Scale bar, 100 μm. Scale bar and orientation shown in A apply to B. Orientation shown in C applies to C’-J. Scale bar shown in C applies to C’-H and J.
Variable phenotypes

In addition to the consistent phenotypes, both Otx1<sup>−/−</sup> and Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mutants exhibited variable phenotypes in the pars inferior, which we classified according to severity (Table 1). Both Otx1<sup>−/−</sup> and Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mutants exhibited mild to severe phenotypes. The mildest of phenotypes displayed a nearly normal saccule and cochlea (Fig. 2B,D). More severe phenotypes showed a saccule, either abnormally small or mis-shaped, and a coiled but abnormal cochlea (Fig. 2C,E). The most severe phenotypes consisted of an abnormally shaped saccule and an aberrant cochlea that failed to coil or coiled in the opposite direction (Fig. 2F). Overall, Otx1<sup>−/−</sup> mutants exhibited milder phenotypes than Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mutants (Fig. 3). The mixed genetic background of these mutant mice most likely contributed to the variability of the phenotype observed. In support of this, the severity of the phenotypes between the right and left ears within the same animal was comparable (asterisk in Table 1).

Since the inner ear phenotypes of Otx1<sup>−/−</sup> and Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mutants were scored between 15 and 16 dpc (Table 1), a cochlea of less than 1.5 turns could have represented a delay in development rather than a true malformation. Therefore, 10 Otx1<sup>−/−</sup> mice and 3 Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mice were analyzed at P1, focusing on the shape of the cochlea. Results of the dissected membranous labyrinths showed that most of the cochleae in Otx1<sup>−/−</sup> mice were fairly normal in shape, whereas all three cochleae in Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mice were abnormal. These results suggest that, while some of the Otx1<sup>−/−</sup> cochlear phenotypes at 15.5 dpc may represent a developmental delay, most of the more severe cochlear phenotypes in Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mice were indeed permanent defects. In addition, the hook of the cochlea of the inner ears analyzed at P1 remained poorly defined (data not shown).

**Inner ear sensory organs of Otx1<sup>−/−</sup> and Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> mutants**

Abnormal development of the lateral canal, ampulla and crista

The lateral semicircular canal originated as an outpouch or

**Table 1. Quantitation of the variable phenotypes in the inner ears of Otx1<sup>−/−</sup>, Otx1<sup>−/−</sup>;Otx2<sup>−/−</sup> and hOtx2<sup>+/−</sup;/hOtx2<sup>+/−</sup> mutants**

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<th>Aberrant saccule</th>
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The severity of phenotype was scored according to aberrant formation or lack of inner ear structures. For the cochlea, the anatomical features evaluated were the presence of a hook region, the number of turns and its shape. For example, the inner ears shown in Fig. 2C,E,F were scored as aberrantly shaped. Specimens displaying a phenotype in a given region were assigned a value of 1. The total score for each specimen represents the summation of the values and provides an index of the severity of phenotypes with 1 being mildest and 9 being most severe. The scoring system is skewed towards cochlear phenotypes.

*Both ears of an embryo were successfully paint-filled and each showed identical phenotypes for right and left ears.

**Postnatal day 1.**

++The ages of the mutant mice scored were between 15 dpc and P1. At these stages, the cochlea should coil from 1.5 to a mature 1.75 turns. In order to accommodate for variability in staging and slight delay in development of mutant mice, we considered any cochlea of mutants that had 1.5 turns to be normal.

The lateral semicircular canal originated as an outpouch or
“lateral plate” from the utricle. Opposing epithelium in the center of the canal plate soon came together, fused and reabsorbed, leaving a mature canal-like structure. Since both Otx1+/− and Otx1+/−;Otx2+/− mutants did not possess a lateral semicircular canal and ampulla at 15 dpc, we analyzed inner ears at 12 dpc, a stage when the lateral canal plate was fully developed (Fig. 4A,B). Both Otx1+/− and Otx1+/−;Otx2+/− mutants exhibited a rudimentary lateral canal plate at 12 dpc, which failed to develop normally (Fig. 4E,F). We then analyzed the generation of both anterior and lateral cristae using BMP4 gene expression as a marker. Fig. 4C illustrates BMP4 expression in a normal inner ear, showing the two presumptive cristae (anterior and lateral), which were still connected but distinguishable from each other (arrows in Fig. 4C). Otx1+/− inner ears showed a comparable BMP4 expression pattern at 11.5 dpc (arrows in Fig. 4G). However, by 12 dpc, when the anterior and lateral cristae were separate entities in wild-type inner ears (Fig. 4D), BMP4 was expressed as a single domain in both Otx1+/− (Fig. 4H) and Otx1+/−;Otx2+/− mutants (data not shown). Tracing this expression pattern in subsequent stages showed that this domain developed into the anterior crista and the lateral crista was not detected in these mutants.

Abnormal development of the macula utriculi and the macula sacculi

In wild-type mice, by 15 dpc, the utriculosaccular duct is well formed, thus leading to the simultaneous compartmentalization and separation of the utricle and saccule (Figs 2A, 5A). Fig. 5C illustrates Fng gene expression in the supporting cells of the lateral crista, the macula utriculi and the macula sacculi in a wild-type embryo. Both Otx1+/− and Otx1+/−;Otx2+/− mutants did not form utriculosaccular ducts (see Fig. 2B-F). As a consequence, the utricle and saccule failed to separate, and thus were housed in a common chamber (Fig. 5B). The macula utriculi was also displaced laterally (compare mu in Fig. 5A,B). In addition, the two sensory organs, macula utriculi and the macula sacculi were partially fused, as highlighted by Fng gene expression (Fig. 5D). The crista shown in Fig. 5D (asterisk) is a part of the anterior crista, which was often found in sections of mutant mice at the level of the utricle.

Cell types in the cochlea of Otx1+/−;Otx2+/− mutants

The histology of most of the Otx1+/− cochlea were fairly normal and indistinguishable from wild type (n=5). Preliminary results showed that three out of four Otx1+/− mice

![Image](https://example.com/image1.png)
tested positive for Preyer reflex indicating that these mice are sound-responsive (data not shown). Because the Otx1<sup>-/-</sup>;Otx2<sup>+/</sup>- mice die at birth, their ability to respond to sound cannot be evaluated. Since the cochlea of Otx1<sup>-/-</sup>;Otx2<sup>+/</sup>- mice were mostly abnormal in shape, we examined the relationship of the spiral ganglion and the cochlea, using neurofilament gene expression as a ganglion marker. Despite the abnormal shape of the cochlea, the spiral ganglion was always present and located underneath the cochlear duct (compare Fig. 6A and B). The sensory regions in the cochlea of Otx1<sup>-/-</sup>;Otx2<sup>+/</sup>- appeared normal, as indicated by the expression of Brn3.1 in the inner and outer hair cells (Fig. 6C), and Fng in the underlying supporting cells (Fig. 6D). Other specialized regions in the cochlea of mutants also appeared normal. The relative location of Hensen’s and Claudius’ cells in relation to the sensory hair cells also appeared normal as indicated bybmp4 expression (Fig. 6E). To investigate the early development of the stria vascularis, we examined the expression of tyrosinase related protein 2 (TRP-2). TRP-2 is expressed in all melanocytes and serves as an early marker for the neural crest cells that migrated from the neural tube to form part of the stria vascularis (Steel et al., 1992). Our results show that TRP-2 was expressed in an appropriate location within the cochlea that was destined to form the stria vascularis (Fig. 6F). Taken together, these results suggest that, while the shapes of the cochlea in Otx1<sup>-/-</sup>;Otx2<sup>+/</sup>- mice were aberrant, the relative locations of various sensory and non-sensory components within the cochlea were normal.

**Rescue of Otx1<sup>-/-</sup> phenotype with hOtx2 cDNA**

The predicted murine OTX1 and OTX2 proteins show extensive homology between the N-terminal and the end of the homeodomain (Simeone et al., 1993). Beyond the homeodomain, OTX1 protein contains blocks of amino acid insertions that are absent in OTX2. The similarity between the murine and human OTX2 proteins is even more extensive, with a single amino acid substitution of a serine to a threonine, out of 289 amino acids. To test if Otx2 can functionally rescue the absence of Otx1, a full-coding region of hOtx2 cDNA was introduced into a disrupted Otx1 locus (Acampora et al., 1999). The inner ear of hOtx2<sup>+/</sup>/hOtx2<sup>+/</sup> mice did not show rescue of the lateral canal and ampulla defect (Table 1; Fig. 7; Acampora et al., 1999). In regions where Otx1 and Otx2 are co-expressed, a spectrum of partial rescues was observed (Table 1, Fig. 3). Fig. 7A illustrates a hOtx2<sup>+/</sup>/hOtx2<sup>+/</sup> inner ear with the most extensive rescue. It consisted of a fairly normal utriculosaccular duct (long arrow in Fig. 7A), cochleosaccular duct (short arrow in Fig. 7A), and the hook of the cochlea (compare to Fig. 2A). In contrast, the inner ear in Fig. 7B showed no rescue in any of these structures. In addition, the cochlea remained shortened, suggesting the inability of hOtx2 to rescue the cochlear phenotype in this case. However, the shape of the sacculus was normal in all hOtx2<sup>+/</sup>/hOtx2<sup>+/</sup> inner ears examined (Table 1).

**DISCUSSION**

**Functions of Otx genes in the inner ear**

**Lateral crista and canal**

When Otx genes were activated in the developing otocyst, the domain of Otx1 expression was broader than that of Otx2. Only Otx1 was expressed in the presumptive lateral crista and canal area, and these structures were consistently affected in the Otx1 knock-out mice. Otx1 most likely mediates the normal development of these structures in a cell autonomous fashion. Previously, using BMP4 as a presumptive sensory marker for cristae, we proposed that the anterior and lateral cristae originated from a common streak of BMP4-positive area in the
anterior portion of the otocyst, which later split to form two separate sensory organs (Morsli et al., 1998). The presumptive lateral crista most likely existed in the Otx1 mutants initially, based on the early pattern of BMP4 expression. However, the lateral crista either remained fused to the anterior crista, or it split from the common streak but failed to continue to develop into a normal dome-shaped crista.

Utricle and saccule
Starting from the mid-level of the presumptive utricle to the ventral tip of the inner ear, the expression of Otx1 overlapped with that of Otx2 at 11.5 dpc. In Otx1 mutants, the sensory organs of the utricle and saccule were partially fused, and the fusion was also more severe in Otx2 heterozygous background (data not shown). Both Otx genes were acting cell non-autonomously to specify the final position of the two maculae within the inner ear since the presumptive maculae were not within the Otx expression domain. The partial fusion of the two maculae in the Otx mutant mice also supports our previous hypothesis that these two sensory organs share a common origin (Morsli et al., 1998).

Cochlea
The cochlear phenotype in the Otx1−/− mice was variable and was much more consistent in Otx1−/−;Otx2+/− mice. Otx1 expression was no longer detectable in the inner ear by 14 dpc, which was 2 days earlier than the completion of the cochlear coiling. However, Otx2 expression remained at least until P5, and was expressed in the lateral wall of the saccule and Reissner’s membrane of the cochlea. Based on the gene expression pattern and the severity of the phenotype, Otx2 may play a more significant role than Otx1 in the development of the cochlear region as well as the saccule. Unfortunately, the loss of Otx2 functions alone cannot be evaluated easily since Otx2 mutant mice die around 10 dpc, well before inner ear morphogenesis.

Fig. 5. Comparison of the utricle and saccule of wild-type (A,C), and Otx1−/− mutants (B,D). (A) An H&E staining of a wild-type embryo at 18 dpc in which the utricle and saccule are compartmentalized and separated. (C) Fng transcripts were localized in the supporting cells of the macula utriculi, the macula sacculi, and the lateral crista at P5. (B) An H&E staining of an Otx1−/− mutant in which the utricle and saccule are located in a common chamber. (D) Fng transcripts were localized in the supporting cells of the anterior cristae (*), and the fused macula utriculi and macula sacculi. lc, lateral crista; ms, macula sacculi; mu, macula utriculi; s saccule; u, utricle; *, anterior crista. The region between the arrowheads indicates torn tissue between the lateral crista and the macula utriculi. Orientation: A, anterior; L, lateral. Scale bar, 100 μm. Scale bar and orientation shown in A apply to all panels.

Fig. 6. Gene expression patterns of neurofilament, Brn3.1, Fng, BMP4 and TRP-2 in the cochlea of Otx1−/−;Otx2+/− mice at 18 dpc (B-F). Neurofilament transcripts were localized in the nuclei of the spiral ganglion in both wild type at P5 (A) and mutants at 18 dpc (B). In mutants, (C) Brn3.1 was expressed in the inner and outer hair cells of the cochlea, whereas (D) Fng transcripts localized underneath in the supporting cells. (E) BMP4 transcripts were localized in the Hensen’s and Claudius’ cells, juxtaposed to the supporting cells. (F) TRP-2 transcripts were localized in a region destined to form the stria vascularis. Ce, Claudius’ cells; He, Hensen’s cells; ihc, inner hair cells; ohc, outer hair cells; sc, supporting cells; sm, scala media; sp, spiral vessel; sv, scala vestibuli; *, presumptive stria vascularis. Orientation: A, anterior; L, lateral. Scale bar, 100 μm. Orientation shown in A applies to all panels. Scale bar shown in A applies to B. Scale bar in C applies to D, E, and F.
Otx genes are expressed in a similar ventral region of the developing inner ear among zebrafish (Li et al., 1994), frogs (Kablar et al., 1996), chicken (Wu et al., 1998) and mouse (Simeone et al., 1993). However, zebrafish and frog inner ears do not contain a cochlea, and the cochlea in chicken, the basilar papilla, is not a coiled structure. Therefore, although the lack of proper coiling of the cochlea was one of the manifestations in Otx mutant mice, the role of Otx genes in inner ear development may be more general, involving specification of the ventral compartment of the inner ear.

In Otx1+/− mutants, the smaller size of the telencephalon compared to wild type was attributed to a reduction in cell proliferation (Acampora et al., 1998, 1999). It remains to be tested whether cell proliferation is affected in the inner ears of Otx1+/− mutants. However, a reduction in growth can easily account for all the phenotypes observed. The presence of primordial structures such as the lateral canal plate and sensory patches which did not develop properly in mutants are consistent with this hypothesis.

Otx1 and Otx2

It is interesting that the Drosophila otd shares sequence homology with Otx1 and Otx2, and these genes have been shown to substitute functionally for each other (Acampora et al., 1998a,b, 1999; Leuzinger et al., 1998). While most of the defects in brain and other organs of Otx1 mutant mice are rescued by otd and hOtx2, these genes fail to rescue the lateral canal and ampulla phenotypes (Acampora et al., 1998, 1999). Also, only partial rescue was observed in regions of the inner ear where Otx1 and Otx2 are normally co-expressed. These results suggest that Otx1 serves a unique role in the patterning of the inner ear. At this point, we cannot rule out the possibility that the lack of rescue by hOtx2 in Otx1+/− inner ears is due to insufficient levels of HOTX2 protein in these mice (approx. 35% less than the normal OTX1 levels, Acampora et al., 1999). However, it is noteworthy that when both copies of the Otx2 gene were replaced by human Otx1 cDNA, the specification of anterior neural plate and proper gastrulation were recovered, but the forebrain and midbrain identities failed to be maintained (Acampora et al., 1998b). Taken together, these data suggest that Otx1 and Otx2 have overlapping as well as separate roles during embryogenesis.

The lateral canal and ampulla first appeared in gnathostomes and were absent in agnatha (for review, see Wersall and Bagger-Sjöback, 1974). It would also be interesting to see if the appearance of the lateral canal and ampulla structures in gnathostomes is also correlated with the initiation of Otx1 expression in inner ears.

Patterning of the inner ear – the boundary model

Recently, a boundary model was proposed for the specification of sensory organs in the inner ear (Fekete, 1996; Kiernan et al., 1997). This model proposes that broad domains of gene expression (selector genes) divide the otocyst into sectors. Depending on the combination of selector genes at a particular position, the location and possibly the identity of a particular sensory organ are specified, with various sensory organs forming at boundaries of interacting genes. In the case of Otx1, its expression was restricted in the posterolateral portion of the otocyst and several sensory organs were located at its boundary. For example, the two maculae and the cochlea were located outside the Otx1 domain but abutting its boundary. However, the lateral crista was located within, but at the outer limits of the Otx1 expression domain. Therefore, the expression pattern of Otx1 and its relationship to the locations of these presumptive sensory organs qualifies Otx1 as a candidate for a selector gene. In support of this role for Otx1, the absence of the Otx1 gene product affected the development of the lateral crista and the normal positioning of the two maculae. However, the identity of these sensory organs including the presumptive lateral crista, did not appear to be affected based on the early expression patterns of BMP4 and Fng in Otx1+/− mutants (Fig. 4 and data not shown). Furthermore, in wild-type inner ears, these two sensory markers were differentially expressed in the otocyst prior to the onset of Otx1 gene expression (compare Fig. 2 in Morsli et al., 1998 to Fig. 1 presented here). Assuming the differential expression of the two sensory organ markers reflect a committed state, the fate of the sensory organs may be specified independently of Otx1 and, possibly, by other selector genes. Taken together, the phenotype of Otx1+/− mutants supports the boundary model as it relates to positioning of the sensory organs, and further suggests that the identity and final positioning of a sensory organ are conferred by separate developmental events.

A similar scenario can be applied to the role of Otx2 in inner ear development, except the Otx2 domain of expression was more restricted than that of Otx1. Both Otx genes are bicomoid-like transcription factors. So far, there is no evidence that either of these genes directly activates a secreted ligand or other downstream targets within the inner ear that formalize their role as selector genes in this model. However, several genes such as engrailed and wnt are thought to be regulated by Otx in Drosophila head formation and vertebrate brain development (Royet and Finkelstein, 1995, 1996; Ang and
Rossant, 1993; Ang et al., 1994; Rhinn et al., 1998; Acampora et al., 1997, 1998b). Therefore, Otx genes could mediate their role as a selector gene through the regulation of 
tobiled and wnt in the inner ear, and the expression of these genes during inner ear development warrant further examination.

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