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Expression of Pax2 and patterning of the chick inner ear

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Summary

Early regionalized gene expression patterns within the otocyst appear to correlate with and contribute to development of mature otic structures. In the chick, the transcription factor Pax2 becomes restricted to the dorsal and entire medial side of the otocyst by stage 16/17. The dorsal region of the otocyst forms the endolymphatic duct and sac (ED/ES), and the cochlear duct is derived from the ventromedial region. In the mouse, however, Pax2 expression is reported only in the ventromedial and not the dorsal otocyst. In Pax2 null mice, the cochlea is missing or truncated, but vestibular structures differentiate normally. Here we demonstrate that in the chick, the emerging ED/ES express high levels of Pax2 even when the position of the emerging ED is altered with respect to its environment, either by 180° otocyst rotations about the anterior/posterior axis or transplantation of the otocyst into the hindbrain cavity. However, the Pax2 expression pattern is plastic in the rest of the otic epithelium after 180° rotation of the otocyst. Pax2 is upregulated on the medial side (formerly lateral), and downregulated on the lateral side (formerly medial and expressing Pax2) indicating that Pax2 expression is influenced by the environment. Although Pax2 is upregulated in the epithelium after 180° rotations in the region that should form the cochlear duct, cochlear ducts are truncated or absent, and the ED/ES emerge in a new ventrolateral position. Ablation of the hindbrain at the placode or early otic pit stage alters the timing of regionalized Pax2 expression in the otocyst. The resulting otocysts and ears are generally smaller, vestibular structures are abnormal, ED/ES are missing but cochlear ducts are of normal length. The hindbrain and dorsal periotic mesenchyme provide unique trophic and patterning information to the dorsal otocyst. Our results demonstrate that the ED is the earliest structure patterned in the inner ear and that the hindbrain is important for its specification. We also show that, although normal Pax2 expression is required for cochlear duct development, it is downstream of ventral otocyst patterning events.

Introduction

The inner ear of vertebrates develops almost entirely from an epithelial placode, the otic placode. A complex labyrinth of fluid-filled cavities, in which sensory organs of three different types are localized, is molded from the placode (see reviews by Fekete (1996, 1999) and by Fritzsch *et al.* (1997)). With a few exceptions noted below, all of the cells of the mature inner are derived from this epithelium. Recent evidence from Mayordomo *et al.* (1998) suggests that part of the medial otic placode originates directly from the neural crest within the neural folds of the hindbrain (HB). Later in development, neural crest cells directly contribute to a number of inner ear structures. These include melanocytes of the stria vascularis, a small neuronal population in Scarpa's ganglion and glial cells of the otic ganglia (LeDouarin, 1982; Cable *et al.*, 1994, 1995; Meyer zum Gottesberge, 1995; Fritzsch *et al.*, 1997). Morphogenesis of the inner ear is complex and at very early stages also requires the influence of the unique environment surrounding the otocyst for normal development. The HB, the periotic mesenchyme (POM), notochord and neural crest (Chisaka *et al.*, 1992; Mark *et al.*, 1993) provide patterning and inductive information necessary for normal development (reviewed in Fritzsch *et al.* (1997)).

Numerous experiments have demonstrated that, in amphibians, the anterior/posterior (A/P) axes are fixed at early stages of otic pit formation (Yntema 1933, 1939; Harrison 1936). A recent study (Wu *et al.*, 1998) demonstrated that axial patterning occurs later in the chick

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than in amphibians and that patterning of sensory organs was specified first along the anterior/posterior (A/P) axis and then the dorsal/ventral (D/V) axis, as in amphibians. However, when otocyst axes were altered at embryonic day 2.5, the A/P axis of sensory organs was fixed but the A/P axis for most of the non-sensory structures was still plastic. These structures could be re-specified by the new axial information provided by the host (Wu *et al.*, 1998).

Determination of the axes and inner ear morphogenesis are dependent on specific gene expression patterns, some of which are established at least as early as the placode stage (see reviews in Fritzsch *et al.* (1997), Fekete (1996, 1999) and Torres and Giraldez (1998)). Several genes have asymmetric expression patterns at the stage of the otic pit or early otocyst, which may be among the first indications of early regional patterning. This study focuses on the protein expression pattern of the transcription factor Pax2 (Nornes *et al.*, 1990; Herbrand *et al.*, 1998; Rinkwitz-Brandt *et al.*, 1995, 1996).

Paired-box (*PAX*) genes are vertebrate homologues of *Drosophila* pair-rule genes that play a role in the segmentation of the *Drosophila* embryo. Pax proteins are developmentally regulated transcription factors (Walther *et al.*, 1991) with a highly conserved, 128–129 amino-acid DNA-binding domain, the paired domain. There are nine known members of the mammalian *PAX* gene family, *PAX1* to *PAX9* (Stapleton *et al.*, 1993). With the exception of *PAX1*, all of them appear to be important for development of the nervous system and brain (Kessel & Gruss, 1990; Noll, 1993; Stoykova & Gruss, 1994; Dahl *et al.*, 1997). PAX proteins are both activators and repressors of transcription (Chalepakis *et al.*, 1994), but Dahl *et al.* (1997) propose that Pax2 genes regulate embryonic pattern formation through their involvement in signal transduction cascades during tissue (e.g. epithelial-mesenchymal) interactions, leading to position-specific regulation of cell proliferation. In this paper, we report that portions of the otic epithelium in which Pax2 is upregulated after 180° rotation also increase in thickness.

PAX2 is expressed in the developing HB, eye, kidney, and ear (Dressler *et al.*, 1990; Nornes *et al.*, 1990; Dressler *et al.*, 1993). Pax2 protein has been found in the inner ears of avians (Goulding *et al.*, 1993; Barald *et al.*, 1997; Herbrand *et al.*, 1998), mice (Nornes *et al.*, 1990; Dressler & Gruss, 1988; Rinkwitz-Brandt *et al.*, 1995, 1996) and humans (Dressler & Gruss 1988; Burri *et al.*, 1989; Terzic *et al.*, 1998). In mice, Pax2 is reported to be restricted to the region of the otocyst from which the cochlea is derived. Torres *et al.* (1996) demonstrated that in most but not all (Dressler, personal communication) Pax2 null mutant mice, the cochleae are either missing, truncated or severely deformed, providing evidence for the importance of this gene in murine cochlear development. Therefore, investigating the role of the Pax2 in ear development may provide promising clues to the mechanisms by which a functional inner ear emerges.

Studies reported here were performed to investigate the unique influence of the localized environment, specifically rhombomeres (rh) 5 and 6 of the HB and the POM on Pax2 expression, and on subsequent morphogenesis of the chick inner ear. We retained the otocyst in its normal A/P position near rh 5 and 6, and used the A/P axis as a fulcrum to rotate it 180°, exchanging the dorsomedial side (where Pax2 was expressed) for the ventrolateral side (where Pax2 was not expressed) at sts. 15–18. To remove the HB influence, we either unilaterally or bilaterally ablated rh 5 and 6 at otic placode/early otic pit stages. We also examined Pax2 expression in otocysts that were transplanted into the HB space. These experiments demonstrated that the Pax2-expressing ED is the first otic structure specified and that upregulation of Pax2 in the ventromedial otocyst after rotation is not sufficient for subsequent normal cochlear duct development. We also report that the dorsal otic epithelium is patterned before the ventral epithelium and requires the unique dorsal environment for normal vestibular development.

Materials and methods

EMBRYO SURGERY

White Leghorn chick embryos (Bilbie Aviaries, Ann Arbor, MI) were incubated at 37.5°C in a Favorite Incubator (Sears) until they reached the desired stage according to Hamburger and Hamilton (HH) (1951). Eggs were windowed and embryos prepared for surgery according to previously published methods (Gardner & Barald, 1991).

In the rotation experiments, the right otocyst and overlying ectoderm of stage 15–18 chicks were dissected from the surrounding mesenchyme using a tungsten needle. The otocyst was then pivoted 180° about the A/P axis with respect to the developing HB ($n = 51$) (Fig. 1). In sham-operated embryos ($n = 13$), the otocyst was removed and immediately replaced in the original orientation. Control embryos were not windowed. Lateral positioning of the ED at the time of surgery and at subsequent stages served as an indicator of whether the surgery was successful. In all cases, the untouched left ear served as an internal control. Chicks were re-incubated for an additional 24 hr—14 days until the embryo reached the desired stage for Pax2 and/or hair cell antigen (HCA) immunohistochemistry or paint-fills to assess gross morphology.

In the ablation experiments, the dorsolateral fold(s) of HB at rh 5 and 6 in st. 11–13 chick embryos ($n = 31$) were ablated on the right side ($n = 21$) or bilaterally ($n = 10$) using a microcautery needle or excised with tungsten needles as previously described (Bockman *et al.*, 1987). No difference could be detected between the cautery or excision methods. Because of the regenerative capacity of the HB, some embryos were re-ablated ($n = 12$) 6 hrs. after the first ablation. The embryos were re-incubated until the desired stage and analyzed for either Pax2 expression or gross morphology.

In the transplant experiments, otocysts including the overlying epithelium from st. 15–18 embryos were excised and

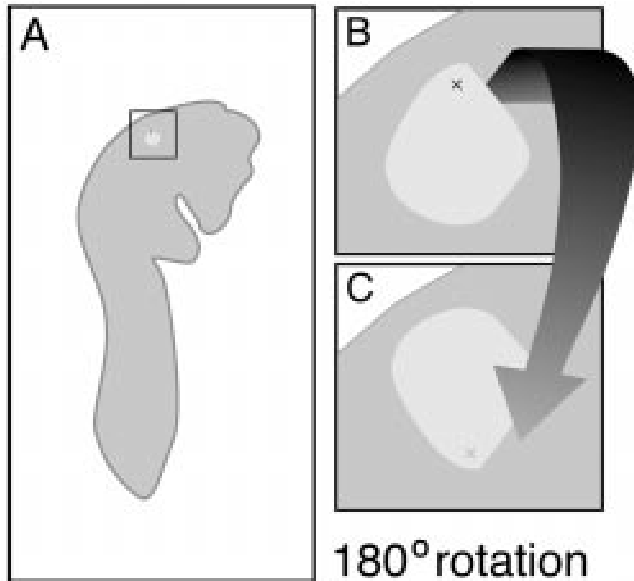

Fig. 1. Schematic showing 180° rotation of the right otocyst. Boxed area in (A) is magnified in (B) and (C). Arrow from (B) to (C) indicates 180° rotation of otocyst about the A/P axis. (B) Black "X" marks dorsolateral surface of the otocyst that becomes ventromedial (gray "X" in C).

transplanted into the HB space of stage-matched host embryos ($n = 19$) through a midline incision in the roof plate of the HB at the HB/spinal cord junction. The otocyst was then positioned internally at rh 5/6. The embryos were reincubated for 24–72 hrs. and analyzed for Pax2 expression and ED formation.

IMMUNOHISTOCHEMISTRY AND PAINT-FILLS

A rabbit polyclonal antibody recognizing chick Pax2 protein was kindly supplied by Dr. Greg Dressler, (University of Michigan) and diluted 1/100. The commercially available (BAbCO) version of the Dressler Pax2 polyclonal antibody was also used at a 1/100 or 1/200 dilution. A mouse monoclonal antibody recognizing a 275 kD hair-cell antigen (HCA) specifically associated with the apical surface of hair cells was kindly supplied by Dr. Guy Richardson, University of Sussex (Bartolami *et al.*, 1991; Goodyear *et al.*, 1994). Antibodies were used in single- and double-labelling experiments. Secondary antibodies included goat anti-rabbit FITC-conjugated IgG for labeling Pax2 (Jackson ImmunoResearch) and goat anti-mouse CY3 conjugate for HCA staining. Secondary antibodies were also selected for their minimal cross-reactivity to either mouse or rabbit.

Control and experimental embryos were fixed for no longer than 2 hrs. in 4% paraformaldehyde, equilibrated in 20% sucrose, and flash frozen in 1 : 1 (v/v) 20% sucrose and Tissue-Tek OCT embedding medium (Miles Inc.). Embryos were cryosectioned at 10 μm and mounted on gelatin coated slides. Sections were blocked in 10% normal goat serum (Gibco), incubated at 4°C overnight in primary antibody, washed 2× in PBS, incubated 2 hrs. in the secondary antibody, washed 2× in PBS and coverslipped with Permafluor (Lipshaw).

Embryos with HB ablations or otocyst rotations were analyzed at embryonic day 10 for morphological changes by injecting the inner ears with a 1% solution of opaque white house paint in methyl salicylate (Martin & Swanson, 1993; Bissonnette & Fekete, 1996). Images were scanned into Adobe Photoshop 4.0 and, in some cases, the background was blackened to better visualize paint-filled inner ear structure (as in Bissonnette and Fekete (1996)).

Results

PAX2 PROTEIN EXPRESSION BECOMES RESTRICTED TO THE RHOMBENCEPHALIC SIDE OF THE OTOCYST BY ST. 16/17

Pax2 expression patterns were followed from placodal stages to st. 22 embryos when the otocyst begins to lose its simple oval shape (Fig. 2A–J). In the placode (not shown) and at the very early otic pit stage (Fig. 2A), Pax2 was evenly distributed at high levels. As the pit invaginated, Pax2 was localized at the rim of the otic cup (Fig. 2B). As the pit closed at st. 15, Pax2 labelling was again evenly distributed throughout the otic epithelium (Fig. 2C). By st. 16 (Fig. 2D) and 17 (Fig. 2E–G), Pax2 protein became restricted medially to cells nearest the HB as well as in the ventral region of the otocyst that will extend to form the cochlear duct. In addition, Pax2 was expressed within the ventral HB (Figs. 2C–J and 5D) and extended above and below the otic forming region.

It was important to understand the complete regional distribution of Pax2 protein at sts. 17 and 22 in order to analyze Pax2 expression patterns after manipulation of the otocyst and/or its environment. Serial sections were made of the otocyst at these stages and stained for Pax2 protein. Representative sections from three different rostro-caudal levels of the otocyst are shown in Fig. 2. The anterior (Fig. 2E and H), middle (Fig. 2F and I) and posterior (Fig. 2G and J) regions of the otocyst are compared. At both sts. 17 and 22, the ED, closely associated with the HB, expressed high levels of Pax2 (arrows in Fig. 2E and I). This was especially notable at st. 22 (Fig. 2H and I). Pax2 staining was found on the medial side of the otocyst closest to the HB and was absent from the lateral epithelium in the middle of the otocyst (Fig. 2F, H and I). In more posterior sections (Fig. 2G and J) Pax2 protein was ubiquitous throughout the epithelium. The cells in this region give rise to the cochlear duct.

PAX2 EXPRESSION IN OTOCYSTS ROTATED 180° IS PLASTIC EXCEPT IN THE ED

To analyze the influence of otocyst position on Pax2 expression, the otocyst was rotated 180° (Fig. 1) at stages 15–18 effectively changing two of the three axes. What was once dorsal and lateral became ventral and medial and vice versa. The majority of the rotations were performed at sts. 16 and 17 because the otocyst was easily removed and it was at these stages that Pax2 became regionalized. As early as st. 16, the emerging ED can

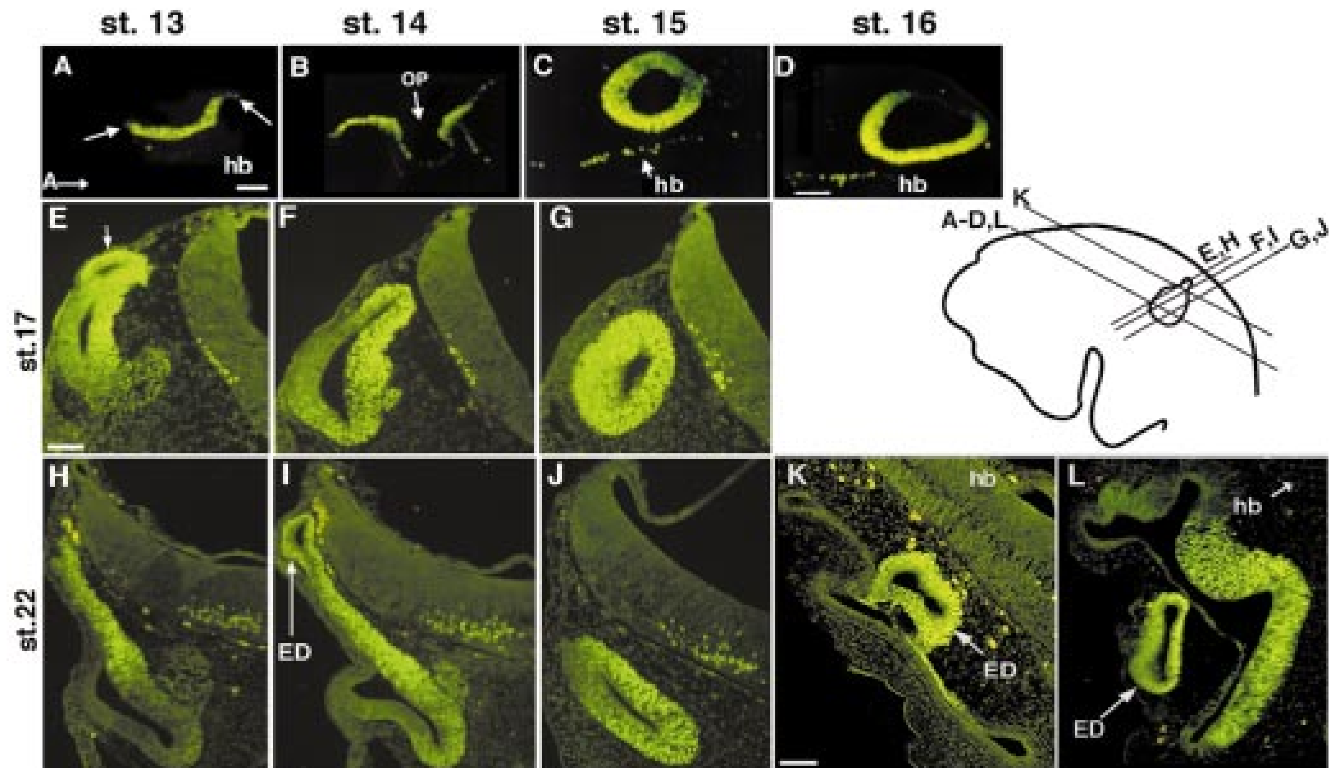

Fig. 2. (A–D) Immunolocalization of Pax2 protein during early stages of inner ear development. (A) Pax2 is distributed uniformly throughout the very early otic pit. Arrows indicate edges of the pit epithelium. (B) At st. 14, Pax2 becomes concentrated at the lateral rim of the otic pit. (C) At stage 15, as the pit begins to close to form the otocyst, Pax2 is redistributed throughout the otic epithelium. (D) At st.16, Pax2 is becoming localized to the entire medial side of the otocyst adjacent to the HB. See schematic for planes of section. Pax2 staining in transverse sections (rostral to caudal) through the otocyst at st. 17 (E)–(G) and 24 hrs. later at st. 22 (H)–(J). See schematic for planes of section. In the anterior-most sections (E and H), Pax2 is expressed dorsomedially in cells of the emerging ED and ES (arrow in E). Sections midway through the otocyst (F and I), show high Pax2 expression in the ED (arrow in I) and on the entire medial side of the otocyst, including the ventromedial portion. In the most caudal sections (G and J), Pax2 expression is uniformly distributed in the region that will give rise to the CD. (K and L) Pattern of Pax2 localization 24 hrs. after 180° rotation of the right otocyst in a st. 22 embryo (L). See schematic for planes of section. (K) Dorsal section through the normal left otocyst shows high Pax2 expression in the ED as well as the medial otic epithelium. Note the position of the ED, which forms between the HB (hb) and the rest of the otic epithelium. (L) Pax2 staining in the 180° rotated right ear. The photograph of this section has been transposed horizontally so the HB is oriented to the right (i.e. we have photographically created its mirror image). This facilitates a more direct comparison of Pax2 localization in the rotated right ear (L) with the control ear of the same embryo (K) as well as a comparably staged embryo in (H)–(J) (different planes of section). Note the once dorsomedial ED is now ventrolateral (L). In order to show the lateral position of the ED in the rotated ear, the plane of section in (L) is more ventral than the plane of section shown in the control ear in (K)(see schematic). Abbreviations: otic pit (OP); hindbrain (hb); endolymphatic duct (ED). All scale bars are 50 μm. The bar in (A) also applies to (B and C). The scale bar in (E) applies to (F)–(J). The scale bar in (K) applies to (L).

be detected morphologically as the tip of the teardrop-shaped otocyst. After the otocyst was rotated 180°, the once dorsomedial ED became re-oriented ventrolaterally (Fig. 2L and right ear in Fig. 3A). We found that the ED was specified at least as early as st. 15 since 75% of the rotations performed at this stage ($n = 20$) resulted in ventrolaterally placed ED/ES. Subsequently, we used the ED/ES at all stages of analysis as a marker for successful rotation of the otocyst.

Pax2 expression was analyzed 24 hrs. after 180° otocyst rotations ($n = 21$). In all embryos examined, Pax2 protein was re-distributed and matched the unrotated left otocyst in most areas except in the area of the emerging ED (compare Fig. 2K and L). The ventrolaterally positioned Pax2-expressing ED maintained high Pax2 expression. The other areas of the otocyst that were previously medial and expressing Pax2, down-regulated Pax2 when relocated laterally (Fig. 2L). The once laterally-positioned epithelium (now medial) up-regulated Pax2 expression (Fig. 2L). In order to show the lateral position of the ED in the rotated ear, the plane of section in Fig. 2L is more ventral than the plane of

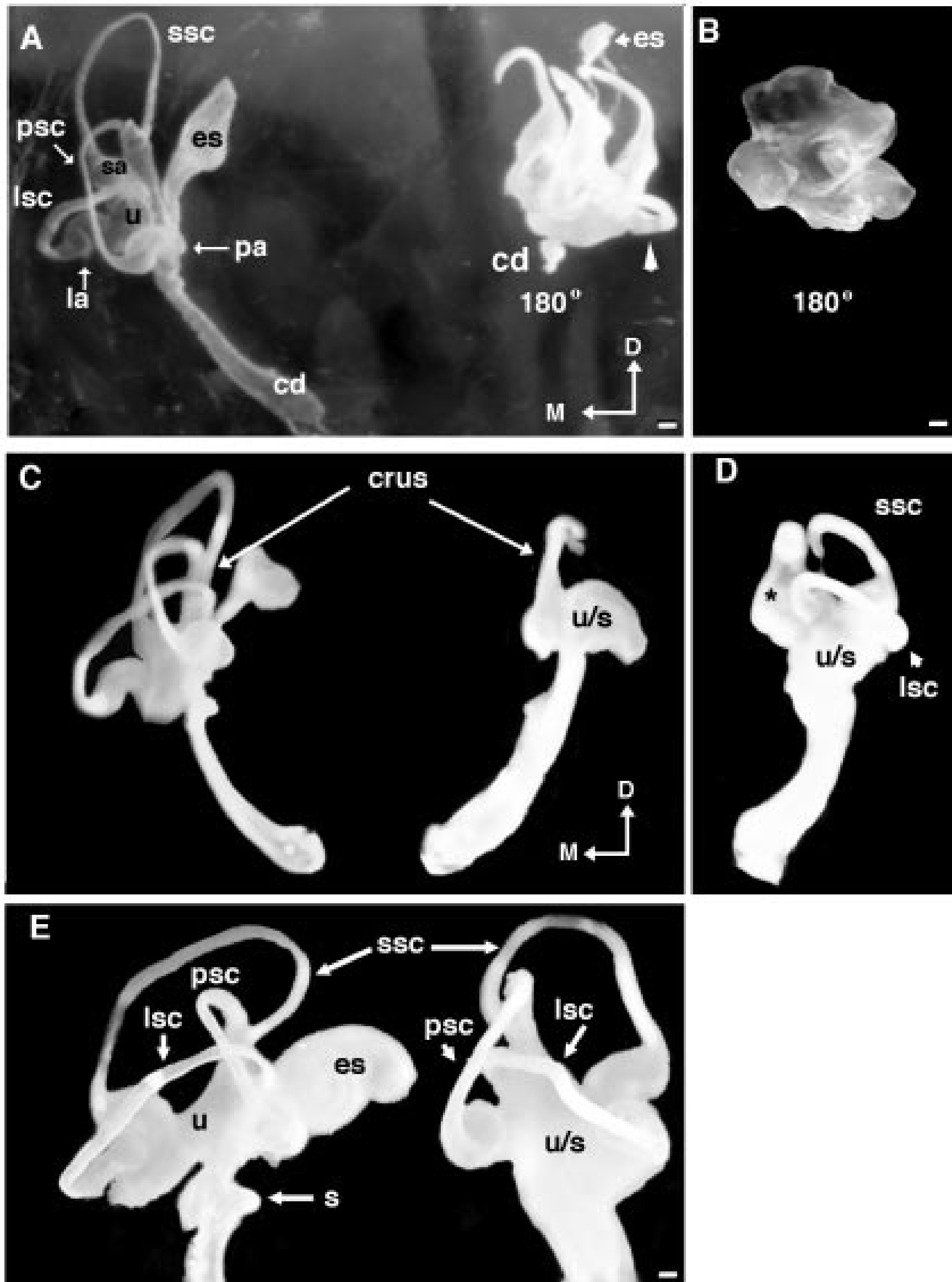

Fig. 3. Paint-filled right inner ears at day 10 after either 180° rotation (A and B) or HB ablation (C)–(E). (A) Paint-filled inner ear after 180° rotation of the right otocyst at st. 17. The right ear has a truncated CD (cd), only one completely formed semicircular canal (white arrowhead), and 2 partially formed canals. Note that the ES is positioned laterally (arrowhead, es), indicating that the 180° rotation was successful. (B) The most severe effect of a 180° rotation was a simple sac. (C) Paint-filled inner ear after right-sided HB ablation by cautery at stage 12 and again 6 hrs. later. The right ear has no ES, a common utriculosaccule space (u/s) with a partial canal structure that appears to be the common crus. (D) Lateral view of right ear after a double bilateral HB ablation at st. 11. Note the common u/s space, the absence of the ES/ED and the presence of only one fully formed canal (LSC). The PSC region is still pouch-like (asterisk). (E). Lateral views of right and left day 10 inner ears after a single right-sided ablation of the HB at stage 12. The right ear has no ED/ES and has a common u/s and a shortened superior and lateral semicircular canal compared to the left ear. The wider cochlear ducts of the right ears in (C)–(E) are artifacts: filling of the perilymphatic space has occurred. Abbreviations: posterior ampulla (pa); posterior semicircular canal (psc); lateral ampulla (la); lateral semicircular canal (lsc); superior ampulla (sa); superior semicircular canal (ssc); utricle (u); saccule (s). Scale bar = 100 μm in (A) also applies to (C and D). Scale Bars = 200 μm in (B and E).

section shown in the control ear in Fig. 2K (also see Fig. 2 schematic). Therefore the more extensive Pax2 expression in the medial otic epithelium of the rotated ear is more comparable to Pax2 distribution in ventral sections through the otocyst of control ears (compare with Fig. 2J, for example). Also note the increased thickness of the otic epithelium in which Pax2 is upregulated in Fig. 2L. In all 180° rotations examined, Pax2 protein was present in the cells on the rhombencephalic and ventral sides of the otocyst. This indicated that at this stage Pax2 expression was plastic (except in the ED) and responded to environmental cues to establish the Pax2 expression pattern seen in the normal control otocyst. Sham operations, in which the otocyst was removed and replaced in its original orientation, resulted in normal Pax2 expression patterns as well as normal inner ear structure (data not shown).

Pax2 redistributes in rotated otocysts 18 hrs. post-surgery

In order to determine the time course of redistribution of Pax2 protein expression, embryos were examined at 6, 12, or 18 hrs. post-surgery (data not shown). Pax2 expression at 6 and 12 hrs. showed a range of patterns with some Pax2 staining clearly seen on the lateral side of the otocyst. By 18 hrs., however, Pax2 expression was upregulated in the medial epithelium and downregulated laterally (data not shown). The redistribution of the Pax2 expression 12 to 18 hrs. after rotation confirmed that Pax2 responded to local patterning cues and was actively being downregulated in some areas of the otocyst and upregulated in others.

180° otocyst rotations between sts. 15–18 perturbs normal inner ear morphogenesis

By day 10 of development, the chick otocyst has formed a complex labyrinth containing sensory and supporting cells (Fig. 3A, left ear). The membranous labyrinth in rotated and sham-operated embryos was injected with paint to reveal the architecture of the vestibular and auditory systems in addition to the ED/ES (Fig. 3A and B, Table 1, $n = 31$). The sham-operated inner ears were normal (data not shown). Wu *et al.* (1998) have shown that altering one of the 3 axes in early inner ear development disrupts mature inner ear morphology. Therefore, we expected to see altered morphology after changing 2 of the 3 axes in our rotation experiments. A range of dismorphogenesis was found. The most severe result was a simple hollow sac (Fig. 3B, Table 1, $n = 2/31$). Defects in cochlear duct (CD) formation and elongation were the most consistent changes observed. The CDs were either truncated, never fully reaching the length of the CD in the normal left ear from the same embryo (Fig. 3A-right ear; Table 1, $n = 18/31$), or absent (Table 1, $n = 10/31$). Very few appeared normal (Table 1, $n = 3/31$). In every case in which a CD could be identified, including the 3 normal CDs (Table 1, $n = 21/31$), its projection was in the correct, ventromedial direction.

The morphological defects in the vestibular system varied. These included partial semicircular canals that ended blindly with either one (Fig. 3A, arrowhead in right ear) or two complete semicircular canals (Table 1). The identity of these canals was deduced according to the axial position in which they formed. For example in Fig. 3A (arrowhead), the one complete canal formed in the same plane as the lateral semicircular canal (LSC) of the left ear of the same embryo. None of the ears examined had all 3 canals completely formed (Fig. 3A, right ear and Table 1, $n = 17$). None of the partial canals emerged in the reversed orientation from that of a canal seen in a normal ear. Therefore, rotating the otocyst did not result in a normal ear in an abnormal orientation in the head (i.e. no "upside down ears" were found). The only structure that formed in the wrong axial orientation was the ED (Table 1 $n = 28/31$) further indicating the early specification of this structure. However, the ED maintains a lateral position but not a ventral orientation (Fig. 3A, right ear). After st. 22 it turns to grow dorsolaterally.

Table 1. Summary of day 10 right inner ear morphology after 180° rotation.

St. of 180° rotation	Total # analyzed (31)	*Endolymphatic sac*[a]			*Cochlear duct*[b]			*Semicircular canals*		
		Not detected	*Intermediate*	*Lateral*	*Absent*	*Truncated*	*Normal*	*All canals incomplete*	*Partial + 1 or more complete canal*	*Simple sac*
15	2	0	0	2	1	1	0	2	0	0
16	6	1	0	5	0	4	2	2	4	0
17	19	1	2	16	7	11	1	7	11	1
18	4	1	1	2	2	2	0	1	2	1

Analysis of ear structure after 180° rotation. It is important to note that the operations represented in this table are only those embryos that survived to day 10.

[a]"Not detected" = the ED and ES were not filled by paint after injection and could not be detected visually. "Intermediate" = ES/ED were located in a position intermediate between the normal medial orientation and the lateral position of a completely successful rotation.

[b]Length of CD was visually compared to the left ear (control) and only significantly shorter CDs were classified as truncated.

DESPITE ABNORMAL MORPHOLOGY, HAIR CELLS FORM IN ROTATED EARS

Auditory and vestibular hair cells are derived from cells of the otocyst. Onset of hair cell differentiation in the chick occurs in the saccule and the utricle as early as st. 27 and in all the sensory tissues by st. 29 (Goodyear *et al.*, 1994). Because the 180° rotations disrupted the overall inner ear morphology, we wanted to determine if hair cells differentiated within the resulting structures, and whether the hair cells also expressed Pax2. In double labeling experiments at st. 31 ($n = 4$), we examined the distribution of both Pax2 and HCA, an antibody that specifically recognizes developing hair cells in the chick (Fig. 4). St. 31 was selected for these analyses because, at this stage, hair cells have differentiated (Fig. 4A–C) and all of the major morphological features of the inner ear have formed. Strong HCA staining was seen in several areas in the rotated ears (Fig. 4D–F). HCA staining was detected in sections from utriculo/saccular regions (Fig. 4D) of the rotated ear as well as the truncated cochlear duct (Fig. 4F). This is similar to staining seen in the normal left ear, which shows hair cell development in the posterior ampulla and the extending cochlear duct (Fig. 4A–C) and also in sham operated ears ($n = 3$, data not shown). We examined the rotated ears for patches of ectopic hair cells and found none. For example, HCA staining was observed in the putative sensory regions and not detected in any partially formed canals. However, considering the severely altered gross morphology of the rotated ears, we cannot rule out the possibility of absolutely no ectopic HCA positive cells. These experiments did show that 180 rotation does not abolish hair cell differentiation and that the hair cells are located in sensory regions within the misshapen rotated ears. In addition, very few cells in the normal (Fig. 4A–C) or the rotated (Fig. 4D–F) ears had overlapping Pax2 and HCA expression patterns. Therefore, since so few HCA-positive cells also express Pax2, it remains unclear whether the plasticity of Pax2 expression in some cells of the otic epithelium is associated with the differentiation of hair cells in the rotated ears.

THE ED CONTINUES TO DIFFERENTIATE AND MAINTAIN HIGH Pax2 EXPRESSION WHEN TRANSPLANTED INTO THE RH 5/6 HB SPACE

To eliminate the effect of the POM environment on Pax2 expression, otocysts from sts. 15–20 were transplanted into the rh 5/6 HB space of stage-matched hosts and analyzed 24 (Fig. 5A–C), 48 (Fig. 5D–I) and 72 (Fig. 5J–L) hrs. after transplantation. Parts of the transplant had uniform Pax2 expression levels similar to those of the endogenous otocyst (compare Fig. 5A to C) at 24 hrs. Forty-eight hrs. after transplantation, Pax2 was regionally expressed at low levels in the otic epithelium (Fig. 5F) but at very high levels in what appears to be a differentiating ED/ES (Fig. 5G and H). The expression was comparable to the Pax2 expression in the host ED/ES (compare Fig. 5G to both H and I). The high levels of Pax2 expression in the ED/ES of transplanted otocysts were maintained up to 72 hrs. after transplantation (Fig. 5L), the latest time point examined. In addition, after 72 hrs., regionalized Pax2 expression was observed in areas not associated with the ED/ES (Fig. 5 J–L). This regionalized expression pattern was not as extensive as the regionalized Pax2 expression seen in normal otocysts. No correlation was found between the Pax2 expression pattern in the graft and the orientation of the graft within the HB. In summary, the transplanted otocyst showed (1) overall relatively complex three-dimensional organization, (2) the emergence of the SAG (Fig. 5K), (3) the elongation of the ED/ES and (4) persistence of Pax2 in the graft. These results suggested that early differentiation of the transplanted otocyst was supported by conditions in the HB space in the absence of the mesenchymal environment.

Pax2 EXPRESSION IN THE OTOCYST IS ALTERED AFTER HB ABLATION

To ascertain the effect of the HB on Pax2 expression in the otocyst, we ablated the HB (rh 5/6) adjacent to the developing otic placode or pit in st. 11–13 embryos. Because of the regenerative capacity of the HB at these early stages, some HBs were re-ablated 6 hrs. later (double ablations, $n = 12/31$). One to two days after either right-sided or bilateral ablations, the otocyst on the affected side was smaller (Fig. 6A and C) and had more uniform Pax2 expression patterns (Fig. 6B and D), similar to patterns seen in younger otocysts (Fig. 2C). The delayed Pax2 expression and the smaller otocyst are consistent with other studies (reviewed in Fritzsch *et al.* (1997)) indicating that, at least at early times, the HB provides a trophic influence on otocyst development.

HB ABLATIONS AT STS. 11–13 PERTURBS NORMAL INNER EAR MORPHOGENESIS

To determine the effect of the HB ablations on the gross morphology of the inner ear, we paint-filled inner ears at Day 10 as described above (Fig. 3C–E). As in the rotation experiments, a range of defects was seen. In all ears analyzed ($n = 14$), the vestibular structures and ED/ES were the most affected, with the most severe malformations observed after double HB ablations. In 13 out of 14 ears, the saccule could not be distinguished from the utricle (Fig. 3C and D). In the most severe cases ($n = 5/14$), there was very little differentiation of vestibular structures (Fig. 3C). The posterior and the superior canals were the most commonly affected canals. Some pouch-like structures were found (asterisk

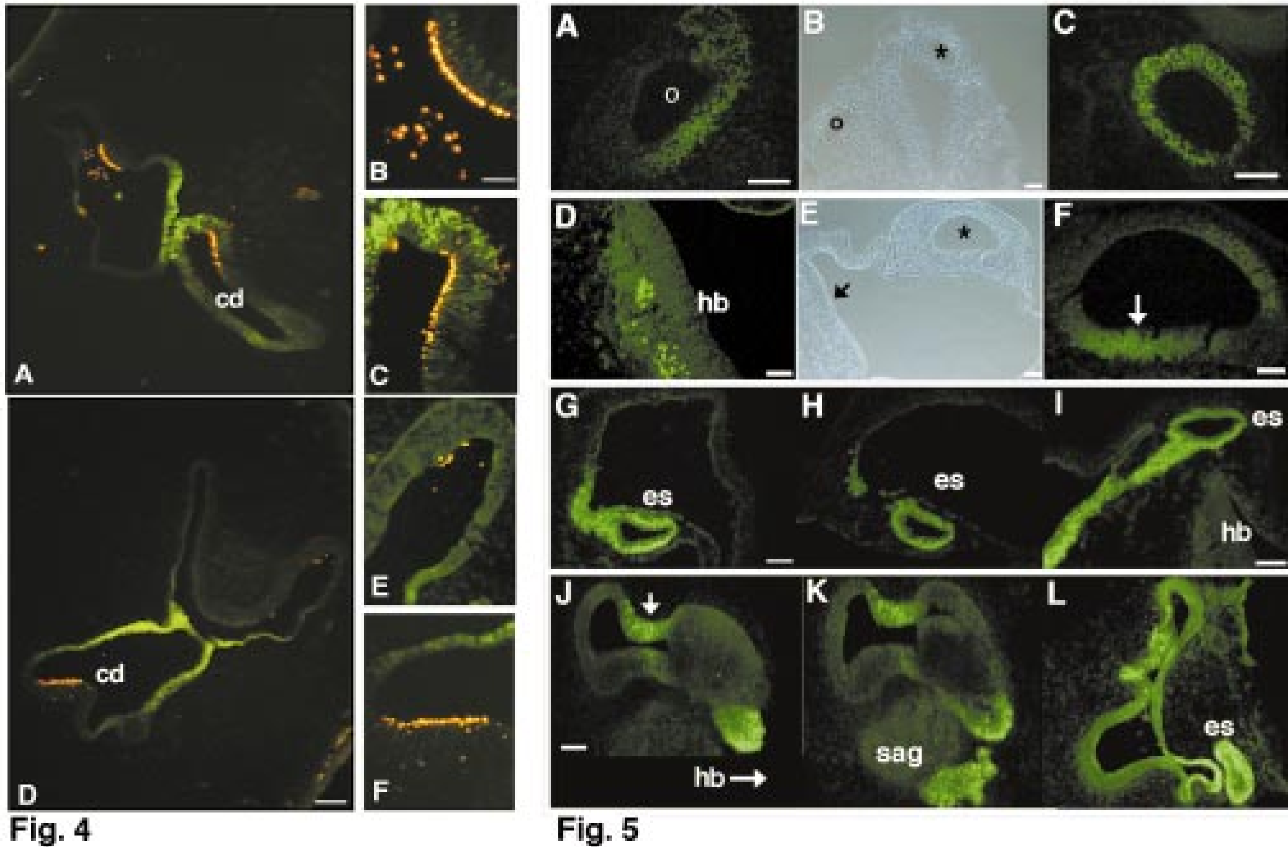

Fig. 4. Coronal sections showing immunolocalization of Pax2 (FITC, green) and HCA (CY3, orange) in a st. 31 left (A)–(C) and right (D)–(F) inner ear after 180° rotation of the right otocyst at st. 17. (A) Pax2 and HCA localization in a section through the posterior ampulla and cochlear duct (cd) in the control left ear. (B) Higher magnification of the HCA positive area in the posterior ampulla seen in panel (A). (C) Higher magnification of HCA and Pax2 positive areas in the cochlear duct seen in panel (A). (D) Pax2 and HCA localization in a section through the truncated cochlear duct and putative utriculo/saccule region in an ear rotated 180° at st. 17. (E) Higher magnification of utriculo/saccule region in panel (D) showing weak Pax2 and strong HCA staining. (F) Higher magnification of HCA positive cells in the cochlear duct in panel (D). Scale bar = 50 μm in (B) also applies to (C, E and F). Scale bar = 100 μm in (D) also applies to (A).

Fig. 5. Pax2 antibody labelling in otocysts transplanted into the HB space of rh5/6. (A)–(C) Transverse sections through a st. 20 embryo, 24 hrs after otocyst transplantation at st. 17. (B) Interference contrast image shows the transplant in the roof of the HB (asterisk). The left host otocyst is labelled "o". (A) and (C) are fluorescence photomicrographs at higher magnifications of the left otocyst ("o" in A) and the transplant (C) shown in panel (B). Pax2 staining in the left otocyst (A) is sided compared to the uniform Pax2 staining in the transplant (C). (D)–(I) are transverse sections through a st. 23 embryo 2 days after transplantation at st 17. (E). Black arrow indicates the HB region shown in (D). The otocyst transplant remains in the roof of the HB (asterisk and shown in F). (D) Compare high Pax2 expression in the HB to the low but sided Pax2 expression in the transplant (white arrow in F) from the same section. (G) and (H) are different sections through the same implant in (E) showing high Pax2 expression in what appears to be the emerging ED/ES (es). (I) The Pax2 expression in the normal ES of the left ear from the same embryo is comparable to transplant expression levels (G and H). (J)–(L) Transverse sections through 3 different cranio-caudal levels of a transplant 72 hrs. after implantation at st 17. (J)–(L) High levels of Pax2 expression in the ES (L) as well as areas of sided expression not associated with the ES (white arrow in J). (J)–(L) The HB is to the right. Abbreviations: statoacoustic ganglion (sag). All scale bars = 50 μm. The scale bar in G also applies to H. The scale bar in J applies to K and L.

in Fig. 3D) as if the canals were never modeled. In general, the vestibular area was considerably smaller than normal even when the canals were completely formed, although most canals were shorter (Fig. 3E). The ED/ES was missing or not detected ($n = 11/14$) in most of the ears. The cochlear duct appeared not to be affected, in that it was always present and of normal length. These results indicate that the greatest patterning and trophic influences of the HB are on the dorsally-derived vestibular system and the ED/ES.

Discussion

How the environment shapes the development of the inner ear has been the subject of study for many years (reviewed in Fritzsch *et al.* (1997) and see Fell (1928)).

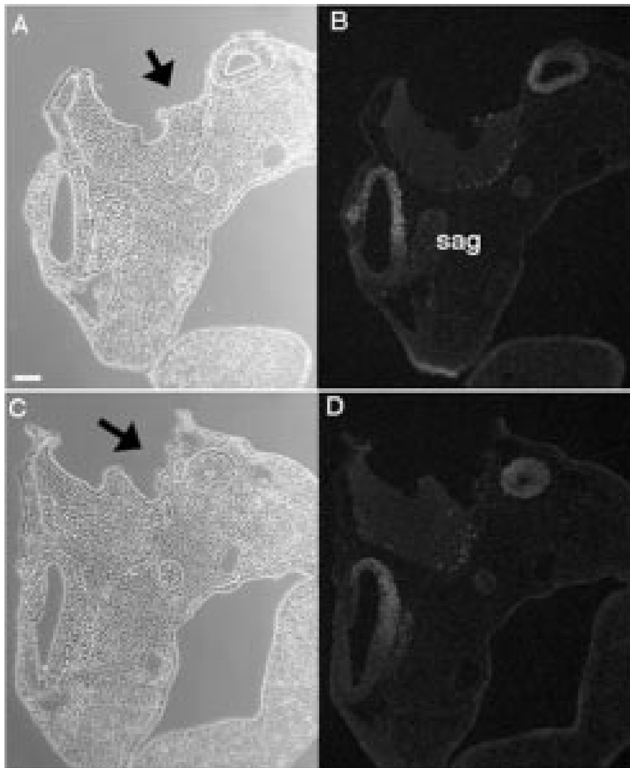

Fig. 6. Pax2 antibody labelling in transverse sections of a st. 20 embryo 2 days after a double right-sided HB ablation at st. 12. Matching interference contrast (A) and fluorescence (B) photomicrographs of a transverse section through the ablated-HB (black arrow in A). (B) Section through the anterior otic epithelium as indicated by the presence of the statoacoustic ganglion (sag). Compare Pax2 staining to the control left side. (C) More caudal section through the HB at the ablation site (black arrow in C). (D) Pax2 immunofluorescence is uniform throughout the smaller otocyst on the right side. Scale bar = 50 μm in A and also applies to (B–D).

Effects of the HB, POM, neural crest and notochord on the patterning of the complex three dimensional structure of the inner ear have been documented (Fekete, 1996, 1999; Fritzsch *et al.*, 1997; Torres & Giraldez, 1998). However, very little is known about these tissues' interactions with each other and with the otocyst. The two key issues are a) when and to what extent the otocyst is autonomous and b) how interactions between otic cells and the cells and/or cell products of its neighbors influence this process.

We examined the environmental influences on otic Pax2 expression and otic morphology in 3 different kinds of experiments. First, we changed the axial environment of the otocyst by rotating it 180° *in situ* without moving it from the vicinity of rh5/6. We then removed the effects of the HB by early HB ablations. Finally, we attempted to remove the effect of the POM environment by implanting the otocyst in the HB space. In addition, we performed these experiments at a variety of developmental stages to try to establish when specific structures became specified and were no longer subject to effects of the surrounding tissue. Our experiments demonstrate that the HB influences otic Pax2 expression patterns at early times in development, and that Pax2 expression is largely plastic in much of the otic epithelium with the notable exception of the ED/ES. In contrast, other studies from this laboratory have demonstrated that, for example, bone morphogenetic protein 4 (BMP4) expression patterns in the otocyst epithelium at stages 16–24 are not plastic (Lewis *et al.*, 1996). BMP4 is expressed in small anterior and posterior cell foci within the otic epithelium (Wu & Oh, 1996; Gerlach *et al.*, 2000). Rotation of the otocyst either 180° or 90° does not change BMP4 expression in these cell foci. Therefore, BMP4 expression does not change in response to alterations of the otocyst with respect to the otic environment, whereas Pax2 expression is sensitive to such cues at the same stages.

ENVIRONMENTAL INFLUENCE ON Pax2 EXPRESSION AND ON EARLY OTIC PATTERNING

Torres *et al.* (1996) demonstrated that Pax2 knockout mice did not develop normal cochleae. In the 180° rotation experiments reported here, the upregulation of Pax2 in ventromedial and ventrolateral regions that should form a cochlear duct was insufficient to produce a normally patterned cochlear duct (Table 1) because 90% of the rotations resulted in truncated (18/31) or missing (10/31) cochlear ducts. These demonstrate that the upregulation of Pax2 in the formerly dorsolateral epithelium is insufficient to instruct cochlear duct elongation. The inability of the dorsolateral epithelium to produce a cochlear duct of normal size and shape is most likely due to prior dorsal patterning influences that render the epithelium incompetent to interpret the environmental cues in its new position. This incompetence may be temporally and/or spatially based.

Studies of mutant and knockout mice have demonstrated the effect of the hindbrain on otic development. In *Kreisler* mutant mice (Deol, 1964; Frohman *et al.*, 1993; Manzanares *et al.*, 1999) and *Hoxa-1* (*Hox1.6*) knockouts (Lufkin *et al.*, 1991; Chisaka *et al.*, 1992), HB patterning, particularly in rh 5 and 6, is altered both anatomically and molecularly. In all of these mice the inner ear forms only as a simple epithelial sac. We found that elimination of the HB at early placode or otic pit stage initially produced smaller otocysts with developmentally delayed Pax2 expression patterns. In mature ears, dorsally-derived vestibular structures and the ED/ES were more affected than ventral auditory structures. Furthermore, in the rotation experiments, the HB appears to have a trophic effect on the once lateral epithelium when it is rotated to lie beside the HB, because the epithelium thickens markedly in its new location (Fig. 2L). Our results, combined with the studies cited above, show that the HB provides trophic and

patterning information critical for the correct formation of the inner ear. In addition, this information is supplied to the otic epithelium as early as the placode stage (and perhaps even prior to placode formation). The HB seems to be particularly important for ED specification as well as later in CD formation. It is possible, however, that the trophic and patterning effects we see are the result of removing neural crest cells that may emerge from this region of the hindbrain, contributing either to the placode itself (Mayordomo *et al.*, 1998) or to the dorsal POM.

Rh 5/6 appears to have some unique properties with respect to otic induction. Herbrand *et al.* (1998) examined Pax2 expression in chick otic placodes that were transplanted to more rostral locations along the HB. The levels and patterns of Pax2 expression varied considerably between transplants, showing no consistent pattern. Their results suggested that more rostral regions of the HB did not have the same ability to influence expression of Pax2 in the ectopic otocysts (Herbrand *et al.*, 1998). Although our rotations were performed at later stages of development, we retained the otocyst in the vicinity of rh5/6 and our experiments resulted in very consistent changes in Pax2 expression patterns. Two possible explanations for differences between our results and those of Herbrand *et al.* (1998) are:

1) the HB and/or POM can influence the Pax2 expression in the otic epithelium at st. 15–18, but not as early as st. 10–12,

2) rh 5/6 of the HB and/or the mesenchyme in the area of rh 5/6 are capable of influencing Pax2 expression patterns in the otocyst, but other regions of the HB do not exert a similar influence.

We believe the latter explanation to be the more likely. Otocysts explanted into tissue culture at stage 16 without the HB do not maintain a "sided" expression of Pax2 protein, and show a marked variability in the levels of Pax2 in culture (Lindberg *et al.*, 1995; data not shown).

The experiments in which we transplanted the otocysts into the HB at sts. 15–18 reflected our efforts to remove the influence of the periotic mesenchymal environment. We did not ensure that all mesenchyme was removed from the graft because we wished to preserve as much of the basal lamina as possible; however very few mesenchymal cells were transplanted. Furthermore, the mesenchymal environment with its gradients, cues and polarity are missing in the few cells associated with the transplanted otocyst especially after 72 hrs. The sided expression of Pax2 that we observed in the otic transplant (although somewhat reduced) was not correlated with the position of the graft within the HB space or the small number of mesenchymal cells included with the transplanted otocyst. Therefore it appears that regionalized Pax2 expression can occur in the absence of the normal periotic mesenchymal environment that is present in the embryo.

At first, we considered the possibility that these transplants might emphasize the effect of the HB. However, secondary considerations lead us to postulate that the environment of the HB space might, in fact, exert less "HB" influence than that to which the otocyst is normally exposed. For example, FGF3 is released by the HB in the vicinity of the otocyst (Tannahill *et al.*, 1992). It is not known whether FGF3 is released into the HB space from the apical surface of the HB epithelium. There is also evidence that the developing otocyst is gap-junction coupled to the developing HB on the basal side of the HB neuroepithelium (Fritzsch *et al.*, 1997). Removing the otocyst at st. 15 would certainly sever any such connections. Furthermore, the apical side of the HB neuroepithelium (the HB space) may present a completely different environment from that of the basal side.

HIGH Pax2 EXPRESSION IN THE ED OF ROTATED AND TRANSPLANTED EARS MAY REFLECT EARLY COMMITMENT OF THE DORSAL REGION TO ED FORMATION

Elongation of the ED dorsomedially is one of the earliest morphological events during inner ear development (Fekete, 1996). FGF3 is produced by rh 4/5 and later, rh 6 in the vicinity of the developing otocyst (Tannahill *et al.*, 1992; Mahmood *et al.*, 1992, 1995) and has been shown to be important for the formation of the ED. FGF3 was initially reported to be responsible for induction of the otic vesicle in chick embryos (Represa *et al.*, 1991). However, "knockout" mice carrying a null allele of *int2* (FGF3) have normal otocyst induction, but abnormal otocyst development (Mansour *et al.*, 1993) and the endolymphatic duct fails to form. Although Torres *et al.* (1996) do not say whether an ED and/or ES formed in Pax2 knockout mice, it is possible that, since the ED region may not express Pax2 in mice (Herbrand *et al.*, 1998), loss of Pax2 may not affect ED patterning in this species. Additional detailed analysis of mouse Pax2 expression patterns is necessary.

The ED is the only structure in the 180° rotation experiments that is not re-patterned by its new environment but continues to form in its new lateral location. The environments of the ventral POM and the HB interior were incapable of downregulating Pax2 in the ED of rotated or transplanted otocysts. Juxtaposition of the ED to the HB was also not necessary for continued ED Pax2 expression after st. 15. The HB may be a major early influence on the specification of the ED/ES, since few ED/ES (3/14) are seen at day 10 in inner ears after the HB was ablated at st. 11–13 (placode/early pit stage).

ROTATION OF THE OTOCYST DOES NOT INHIBIT HAIR CELL DEVELOPMENT

In our studies, hair cell development still occurred despite abnormal gross morphological development of the inner ear after 180° rotation. No ectopic hair cells were seen outside of putative sensory structures. These findings support the results of experiments performed by Wu *et al.* (1998) in which sensory cell patterning and axial patterning are not temporally linked.

Our results reflected the different stages at which a specific population of otocyst cells became committed to a given pathway of development, and, in turn, lost the ability to respond to environmental influences. We believe that the difference between the effect of otocyst rotation on development of the ED and the CD is an example of just such varying rates of structural commitment in the inner ear. Knowledge of these different rates of structural commitment and correlation with various gene expression patterns will help to decipher both the function and the specific environmental influences on genes important for inner ear development.

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