Origins of Inner Ear Sensory Organs Revealed by Fate Map and Time-Lapse Analyses

Sung-Hee Kil and Andres Collazo
Leslie and Susan Gonda (Goldschmied) Department of Cell and Molecular Biology, House Ear Institute, 2100 West Third Street, Los Angeles, California 90057

The inner ear develops from a simple ectodermal thickening called the otic placode into a labyrinth of chambers which house sensory organs that sense sound and are used to maintain balance. Although the morphology and function of the sensory organs are well characterized, their origins and lineage relationships are virtually unknown. In this study, we generated a fate map of *Xenopus laevis* inner ear at otic placode and otocyst stages to determine the developmental origins of the sensory organs. Our lineage analysis shows that all regions of the otic placode and otocyst can give rise to the sensory organs of the inner ear, though there were differences between labeled quadrants in the range of derivatives formed. A given region often gives rise to cells in multiple sensory organs, including cells that apparently dispersed from anterior to posterior poles and vice versa. These results suggest that a single sensory organ arises from cells in different parts of the placode or otocyst and that cell mixing plays a large role in ear development. Time-lapse videomicroscopy provides further evidence that cells from opposite regions of the inner ear mix during the development of the inner ear, and this mixing begins at placode stages. Lastly, bone morphogenetic protein 4 (BMP-4), a member of the transforming growth factor β (TGF-β) family, is expressed in all sensory organs of the frog inner ear, as it is in the developing chicken ear. Inner ear fate maps provide a context for interpreting gene expression patterns and embryological manipulations.© 2001 Academic Press

INTRODUCTION

The inner ear is a morphologically complex structure for hearing and maintaining balance (Hudspeth, 1989). It develops from a simple ectodermal thickening of the embryonic head called the otic placode (Fritzsch et al., 1998; Torres and Giraldez, 1998; Yntema, 1955). The placode invaginates to form the spherical otic vesicle (or otocyst), which, during subsequent development, grows extensively and becomes subdivided into several distinct connected chambers. Unlike other vertebrate sensory structures, the ear is unique in that a relatively small percentage of the structure actually consists of sensory cells (Hudspeth, 1989). The mechanoreceptive sensory hair cells occur in several varying-sized sensory patches (or organs), spread across different chambers of the inner ear (Fekete, 1996). These sensory organs have precise geometric arrangements within the inner ear chambers.

The number of sensory organs contained in the inner ear varies between vertebrates, but all have at least six: five for the vestibular system plus one for the auditory system (Romer and Parsons, 1977). Three sensory organs called cristae detect angular acceleration and are located in chambers called ampullae, one at the base of each of the three semicircular canals (Goldberg and Hudspeth, 2000). The two largest sensory organs, the maculae, mainly sense linear acceleration and gravity and are arranged perpendicular to each other (Goldberg and Hudspeth, 2000). The auditory sensory organ in mammals and birds (cochlea and basal papilla, respectively) has an uncertain homology to the main auditory sense organ in frogs (Lewis and Narins, 1999). In birds, amphibians, and fish, several small maculae of uncertain function are also present (Romer and Parsons, 1977). Figure 1B illustrates the arrangement of sensory organs in an adult *Xenopus laevis* inner ear, and, while this species has a less elaborate auditory sensory organ than mammals, the shared vestibular sensory organs are highly conserved in position and structure.

The developmental origins and lineage relationships of inner ear sensory organs are unknown (Fekete, 1996). Morphological studies in embryonic birds, salamanders, and frogs suggested that all sensory organs arise from a single region in the ventro-medial wall of the otocyst (Fritzsch et al., 1998).
FIG. 1. Stage 47 Xenopus tadpole and anatomical view of an adult ear. (A) Stage 47 tadpole is shown (dorsal view). Blue oval represents location of the ear. (B) An anatomical illustration of an adult frog ear is shown (lateral view). Green areas represent sensory organs in (A) and (B). (C) The sensory organs of the inner ear can be labeled with a vital dye, 4-Di-2-ASP. This image is the projection of three different focal planes. (D) Bright-field image of inner ear in (C) showing two otooliths. (E) Diagram of the quadrants injected for the fate map experiments at placode and otocyst stages. The anterior (a), dorsal (d), and/or medial (m) directions are indicated. ac, Anterior cristae; lc, lateral cristae; pc, posterior cristae; mu, maculae utriculi; and ms, maculae saccule. Otolith (ot) is secreted by maculae utriculi and maculae saccule. Scale bar equals 200 μm.

FIG. 2. Fate-map injection of otic placode and otocyst. A small amount of vital dye, DiD, was injected into one of four quadrants of the otic placode or otocyst: anterior (A; stage 26), posterior (D; stage 26), dorsal (G; stage 26), and ventral (K; stage 26). A and G are medium-sized injections; D and J are small injections. After the initial injection, the embryos were grown until tadpole stage (stage 47-49) when most of the sensory organs have differentiated (panels B, C, E, F, H, I, K, L). The four sets of panels A–C, D–F, G–I, and J–L each represent one embryo. For example, A–C is the same embryo. Positive DiD (in red) labeling were found in both anterior (B and E) and posterior cristae (C and F) when anterior (A) and posterior (D) regions were labeled. In addition, dorsal and ventral (BMP-4 negative) regions gave rise to

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.
sensory organs (H–I and K–L). DiD is shown in red, and 4-Di-2-ASP is shown in green. Arrows indicate colabeling with DiD and 4-Di-2-ASP (which labels hair cells). All arrows but in (C) and (I) point to the apical edge of the hair cells. Arrows in (C) and (I) point to the basal and lateral edges, respectively, of hair cells. Blue circle represents otic placode or otocyst. The anterior (a), dorsal (d), and/or lateral (l) directions are indicated (orientation in A applies to D, G, and J; that in B applies to all remaining panels). Scale bar equals 100 μm.
In zebrafish, the maculae are thought to arise from a single ventral region, but the cristae arise separately and nearer to their final positions (Haddon and Lewis, 1996). More recently it has been shown that bone morphogenetic protein 4 (BMP-4), a member of the transforming growth factor-β (TGF-β) superfamily, marks all sensory organs in developing chicken inner ears before they can be morphologically distinguished (Wu and Oh, 1996). BMP-4 expression suggests that the anterior and posterior cristae are the first generated and that they are generated separately, at early otocyst stages, near their final positions. Over the next three days, the remaining six sensory organs begin to express BMP-4 (Wu and Oh, 1996). Molecular markers provide the potential for distinguishing prospective sensory organs earlier than with morphological criteria (Oh et al., 1996; Wu and Oh, 1996). Nevertheless, to directly determine the origin of sensory organs, a fate map of the inner ear showing which region of the placode and otocyst can contribute to sensory organs is needed. In this study, we generated a fate map of the Xenopus laevis inner ear at placode and otocyst stages to determine the origins of inner ear sensory organs. Vital dye was injected into one of four quadrants: anterior, posterior, dorsal, and ventral of the placode and otocyst, and these embryos were analyzed at stages when most sensory organs have developed. Results indicate that every region of the placode and otocyst gave rise to sensory organs (usually multiple ones), but that the range of sensory organs formed varied by region. For example, the posterior quadrant was least likely to give rise to sensory organs, while ventral injections most often gave rise to labeling in two sensory organs, the anterior and lateral cristae. Anterior or posterior injections resulted in labeled cells in both anterior and posterior sensory structures, demonstrating that there was no antero-posterior cell restrictions, even at otocyst stages. Time-lapse videomicroscopy experiments provide direct proof that cells from different regions of the inner ear intermingle during early inner ear development and that this mixing occurs during placode and otocyst stages. Taken together, these results suggest that a single sensory organ arises from cells in different parts of the placode or otocyst and that cell mixing plays a large role in ear development. In addition, we examined the expression of BMP-4 in the developing frog inner ear by in situ hybridization. Results show that BMP-4 mRNA is observed in all the sensory organs, similar to what has been described for avian inner ears. The inner ear fate map illustrates the origins of the sensory organs and provides a context for interpreting gene expression patterns.

**MATERIALS AND METHODS**

**Vital Dye Labeling of Cells**

Xenopus laevis eggs were fertilized in vitro and staged according to the normal table of Nieuwkoop and Faber (1967). Embryos were dejellied for 10 minutes in a 2% Cysteine-HCl solution in a rearing solution (20 mM Instant Ocean: a commercial aquarium salt) at pH 8.0. Living embryos were anesthetized with a 1:2500 solution of tricaine methanosulfonate (Finquel), in rearing solution. To better visualize the otic placode (stages 23–27) and otocyst (stages 28–31), the pigmented epidermis was peeled away by using sharpened tungsten needles. Removing this epidermis does not disturb ear development (unpublished observation), it heals back quickly and this most superficial epidermis does not contribute any tissue to the inner ear at placode and otocyst stages (Hausen and Riebesel, 1991). In fact, injections into the inner ear often resulted in epidermal labeling, suggesting that there was still prospective epidermis over the placode or otocyst. Following exposure, the placode or otocyst was injected with small amounts of vital dye solution in one of four quadrants: anterior, posterior, dorsal, or ventral (Fig. 1E) with a back-filled Quartz glass micropipette made on a P-2000 laser pipet puller (Sutter Instruments). A 0.25% stock solution of the lipophilic dye 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiD oil; Molecular Probes) was dissolved in 100% ethanol, and the stock solution was further diluted 1:200 in 0.3 M sucrose solution, immediately before injection. For time-lapse experiments, two fluorescently distinguishable dyes were injected to label opposite regions of the otic placode, DiD, as described above, and a 1:10 dilution of DII-CM (CellTracker; Molecular Probes, dissolved in 100% ethanol to form 0.25% stock) in 0.3 M sucrose. All injections were immediately checked under a fluorescence compound microscope (see Imaging below) to determine injection size and position. Ten to hundreds of cells were labeled per injection, and these labeled cells were within a single quadrant. A small subset of injections (n = 10) was made larger, marking cells in over half of the placode. These were included in the data set used to generate Fig. 4 and Table 1, but were only done for anterior or posterior injections. Omitting these embryos from our data set does not change our results. Embryos were then grown and analyzed between stages 47 and 49. Lipophilic dyes, such as DiI and DiD, become tightly integrated into cell membranes and do not spread via cell–cell contact in living embryos. These dyes provide a lineage label since they are only passed to other cells by cell division.

**Labeling Hair Cells in Vivo**

Sensory organs consist of at least two cell types: hair cells, specialized postmitotic mechanosensory cells, and supporting cells, which surround the hair cells and whose function is not well understood (Fekete, 1996). Inner ears of stage 47–49 tadpoles were injected with a solution of the fluorescent dye 4-Di-2-ASP (Molecular Probes) to visualize hair cells (Fig. 1C). 4-Di-2-ASP is a cationic mitochondrial dye that preferentially labels the mitochondria-rich hair cells. At these stages, many of the sensory hair cells have differentiated and can be labeled with the dye. Observations were concentrated on the five sensory organs (anterior, lateral, and posterior cristae and maculae of the utricle and saccus) visible at these stages (Fig. 1). All but the posterior cristae are in the anterior half of the ear. In some embryos, the macula lagena was formed at these stages, but the sample size was insufficient to include in this paper. Other sensory organs, like the amphibian papilla and the basal papilla, develop later (stage 50) (Nieuwkoop and Faber, 1967). Tadpoles were anesthetized with a 1:10,000 solution of tricaine methanosulfonate (Finquel) in rearing solution.
**Imaging**

Living embryos or tadpoles were prepared for imaging just after injection or at stages 47-49 as described above for lineage and hair-cell labeling. Labeled cells were visualized on a Zeiss Axio phot2 M ot epifluorescence microscope with long working distance air and Achromplan water objectives ranging up to a magnification of 40×. Data were recorded digitally from a light-intensifying camera (Hamamatsu SIT) by using MetaMorph 3.5 (Universal Imaging). Black and white fluorescent images were pseudo-colored in Adobe Photoshop (versions 4.0–5.5) as specified in the figure legends.

**Time-Lapse Analyses**

Two quadrants (anterior and posterior or dorsal and ventral) of the otic placode of stage 23–25 embryos were labeled simultaneously with two vital dye solutions: DiD and DiI-CM. The DiI-CM was typically diluted more than DiD to tritrate the brightness down to that observed for DiD. Immediately after the injections, images of the labeled embryos were recorded digitally as described above. A z-series stack of images (10 planes of focus) were collected every 5 minutes over a 10-h period at two different fluorescent wavelengths, cy3 and cy5 (Chroma filters), sequentially using the same microscope, camera, and software described above. A total of 11 time-lapse experiments were analyzed (n = 7 for anterior and posterior injections, n = 4 for dorsal and ventral injections).

**Histology**

Embryos were dehydrated with a series of ethanol washes, transferred through two Histosol washes, and embedded with Paraplast Plus (Oxford) in a vacuum oven. The blocks were sectioned at a thickness of 10 μm with a microtome (Leica). Sections were placed on slides (Fisher) and used for in situ hybridizations.

**Whole-Mount in Situ Hybridization and in Situ Hybridization in Sections**

The method of Knecht and workers (Harland, 1991; Knecht et al., 1995) was used to perform whole-mount in situ hybridization. For in situ hybridization on paraffin sections, a modified version of Strähle et al., (1994) and Henrique et al. (1995) was used (protocol obtained from Dr. H. Etchevers). Briefly, after paraffin was removed through a series of xylene washes, sections were rehydrated with ethanol/phosphate-buffered saline (PBS) solutions. Sections were then hybridized with 0.1 μg/μl of labeled digUTP under a coverslip, placed in a humidified chamber, and incubated at 70°C overnight. Sections were rinsed with 50% formamide, 1× SSC, 0.1% Tween-20 at 65°C for 15 and 30 minutes, respectively. After two 10-minute washes with 1× maleic acid buffer with 0.1% Tween-20 (MABT) solution, sections were blocked with 2% Boehringer blocking reagent (Roche), 20% sheep serum in maleic acid buffer (MAB). Sections were then incubated with anti-DIG–AP antibody (1:1000 dilution) overnight. After five MABT washes, the color reaction was performed using NBT and BCIP substrate. BMP-4 RNA probes were synthesized as previously described (Wilkinson, 1992).

Pictures of the in situ were taken with a ProgRes 3012 digital color CCD camera using the Roche Diagnostic software running under Windows for Workgroup 3.11. Images were collected through either a Zeiss SV11 dissecting scope (whole-mounts) or an Axioplan microscope (sections).

**RESULTS**

**Fate Map of Otic Placode**

Several genes that specifically mark prospective sensory organs (such as BMP-4) are expressed in two distinct regions (anterior and posterior) at early otocyst stages (Fekete, 1996; Morstli et al., 1998; Wu and Oh, 1996). These two initial expression domains do not necessarily represent all the sensory organs, but this separation is thought to represent the separation of certain anterior sensory organs (e.g., anterior crista) from posterior sensory organs (Fekete, 1996; Wu and Oh, 1996) (Fig. 1). The best experimental approach to address lineage questions in vivo is to mark specific regions of the otic placode and follow the marked cells through development. In our study, we have generated fate maps of the inner ear of Xenopus laevis at two different developmental stages to directly determine where in the otic placode (stages 24–27) and otocyst (28–31) cells of the sensory organs arise.

Our lineage marker, DiD, was injected into anterior, posterior, dorsal, or ventral regions of otic placodes (stage 25–27) and otocysts (stage 28–31) (Figs. 1E, 2, and 3). Restricting injections to a single quadrant minimized overlap. In a typical injection, approximately 10–100 cells were marked, and these numbers represent less than half the cells in a quadrant. Injected embryos were allowed to develop to stage 47–49, when most sensory organs have developed. Hair cells were then counter-stained in these living tadpoles with another vital dye, 4-Di-2-ASP (Fig. 1C). This dye has been used to label hair cells in the lateral line neuromast, another mechanosensory structure (Collazo and Fraser, 1998; Collazo et al., 1994), and its fluorescent wavelength is clearly distinguishable from DiD. Cells co-labeling with DiD and 4-Di-2-ASP were scored as sensory hair cells, and their positions allowed us to assign them to specific sensory organs in vivo (Figs. 2 and 3). Thus, we could determine which sensory organs contained labeled cells from the initial injection. Sensory organs consist of hair cells and adjacent supporting cells that do not label with 4-Di-2-ASP. Lineage-labeled cells directly adjacent to and contacting the basal side of hair cells were presumed to be supporting cells and scored to be in the sensory organs. Only DiD-labeled cells that were not contiguous with the basal side of hair cells were scored as nonsensory structures. This conservative approach meant that labeled cells residing in nonsensory structure directly adjacent to sensory organs might not be scored appropriately but ensured labeled cells in the sensory organ were not under-represented. Approximately 400 embryos (50 for each quadrant at two different times) were injected with DiD and their cell fates determined.
FIG. 3. Fate-map injection of otic placode and otocyst showing DiD-positive cells in both nonsensory regions as well as in sensory organs. In both embryos (A, stage 25, and E, stage 30), a small amount of vital dye, DiD, was injected into the posterior quadrant of the otic placode or otocyst. Panels A–D and panels E–H each represent one embryo at two different time points. After initial injection (A and E), the embryos were grown to stage 47+ (B–D and F–H). Panel’s B–D represent a dorsal view of the entire left inner ear at stage 47 of the same embryo shown in (A). (B) DiD-positive cells are shown. (C) Sensory organs labeled with 4-Di-2ASP. Only the anterior cristae is in focus in this view. Underneath it and to the right is the maculae utriculi. (D) Overlap of panels B (red) and C (green) is shown. A box in the left corner of panel D shows a magnified view of the posterior cristae. Panels F–H represent higher magnification dorsal views of a portion of the inner ear at stage 47, of the embryo shown in E. In panel F (DiD) and G (overlap of DiD, red, and 4-Di-2-ASP, green), the same posterior cristae is shown. (H) Macula utriculi is shown. Arrowheads indicate labeled cells in nonsensory regions, while the arrows show lineage labeled cells in sensory organs. Blue circle represents otic placode or otocyst. The anterior (a), dorsal (d), and/or lateral (l) directions are indicated (orientation in A applies to E, that in B applies to all remaining panels). ac, Anterior cristae; lc, lateral cristae; pc, posterior cristae; mu, maculae utriculi; and ms, maculae saccule. Scale bar equals 100 μm.

FIG. 4. Bar graph representing DiD labeling in the sensory organs of the otic vesicle. A small amount of vital dye DiD was injected into one of four quadrants of the otic placode or otocyst: anterior (black solid bar), posterior (bar with dots), dorsal (white hatched bars), and ventral (black hatched bars). Two time points were studied, otic placode (graph A) and otocyst (graph B) stages. Embryos were grown to stages 47–49 and analyzed for positive labeling in the various sensory organs. Data were then grouped into four categories: nonsensory organ region of the ear, anterior only (anterior and lateral cristae, maculae utriculi, and maculae saccular), posterior only (posterior cristae), and anterior and posterior sensory organs. Graph plots percentage of embryos (y axis) with positive labeling in the five groups (x axis).
FIG. 5. Time lapse of early otic placode development demonstrating cell mixing. To verify that cells from different region of the otic placode can indeed mix, we injected dorsal and ventral quadrants of the otic placode of stage 23 embryo (A) with two different vital dyes (DiI-CM in green and DiD in red). Labeled cells (lateral view) were followed for at least 10 h. This amount of time was sufficient to go from a placode to an advanced otocyst stage that is just beginning to undergo sensory organ differentiation. The white box in A indicates the region magnified in frames B–T. (B-H) Green cells are moving ventrally and intermingling with red cells (white arrows). Red cells are splitting into two groups, dorsal and ventral, within the first 3 h (H). (I) At least one green cell has moved ventral to the more dorsal group of red cells and is mixed with the ventral group of red cells, appearing yellow when colocalized (yellow arrow). (I–L) Green cells move from dorsal to the posterior ventral edge of the dorsal group of red cells (white arrows), mixing these two cell populations (yellow arrows indicate colocalized labeling). (M–P) Green and red cells remain colocalized for almost two h. The ventral group of red cells and its colocalized green cells (I) disappear from this plane of focus (O). (O–T) Green and red colocalized cells in P separate within 25 minutes. Green cells (arrowheads) remain separated and dorsal but within 2 h most move back in, colocalizing with the red cells (yellow arrow). Time elapsed in minutes (min) is indicated in each panel. Blue circle represents otic placode. The dorsal (d) and posterior (p) directions are indicated. Scale bar equals 100 μm. Time-lapse movies can be downloaded from http://www.hei.org/htm/cmbeard.htm.
Fate Map Results Demonstrated That Every Region of the Placode and Otocyst Can Give Rise to Sensory Organs

In most cases, we observed label in only one to six hair or supporting cells within a given sensory organ (Figs. 2 and 3). In more than half of the embryos, each quadrant except the posterior contained labeled cells in two or more sensory organs (Table 1). The posterior quadrant still had significant numbers of embryos with label in two or more sensory organs (42% at placode stages and 36% at otocyst stages). All quadrants at placode stage had at least one embryo in which labeled cells were found in all five sensory organs scored (Table 1). A sampling of injections was made large enough to label a region encompassing over half of the placode or otocyst, and these injections never resulted in an entire sensory organ being labeled. In every embryo examined, in addition to DiD label in the sensory organs, we observed labeled cells in nonsensory structures (Fig. 3).

To address the question of whether anterior and posterior sensory organs arise from different regions of the placode and otocyst, the data were grouped into four categories: 1) Nonsensory structures only; 2) anterior sensory organs only (anterior cristae, lateral cristae, maculae utriculi, maculae of sacculus); 3) posterior sensory organs only (posterior cristae); or 4) both anterior and posterior sensory organs (see Fig. 4). Each quadrant had significant numbers of embryos in which lineage-labeled cells were in both anterior and posterior sensory organs (Fig. 4). While anterior and ventral quadrants mostly gave rise to anterior sensory organs, the second most frequent category for both consisted of embryos in which both anterior and posterior sensory organs were labeled. For example, labeled cells of the anterior

<table>
<thead>
<tr>
<th>Sensory organs</th>
<th>Anterior</th>
<th>Posterior</th>
<th>Dorsal</th>
<th>Ventral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placode (n = 50)</td>
<td>Otocyst (n = 48)</td>
<td>Placode (n = 48)</td>
<td>Otocyst (n = 46)</td>
</tr>
<tr>
<td>Nonsensory</td>
<td>2</td>
<td>10</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Anterior cristae (ac)</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Lateral cristae (lc)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Posterior cristae (pc)</td>
<td></td>
<td></td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Maculae utriculi (mu)</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Maculae sacculus (ms)</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + lc</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ac + pc</td>
<td>1</td>
<td>9</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>ac + mu</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ac + ms</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lc + mu</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lc + ms</td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>pc + mu</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pc + ms</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mu + ms</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + lc + pc</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>ac + lc + mu</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + lc + ms</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + pc + mu</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + pc + ms</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + mu + ms</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pc + mu + ms</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + pc + mu + ms</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + lc + pc + ms</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + lc + pc + mu</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + lc + pc + mu + ms</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac + lc + pc + mu + ms</td>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Note. All combinations of sensory organs and non-sensory tissues that contained labeled cells. 27 different categories (out of 32 possible combinations) encompassed all the results from 400 injected embryos. It is important to remember that every injection gave rise to labeled cells in non-sensory structures and that the category “non-sensory” represents only tadpoles with label exclusively in non-sensory tissues. The categories are separated into three groups: (1) labeled cells in only one derivative, (2) labeled cells in two derivatives, and (3) labeled cell in three or more derivatives. That our fate map resulted in almost every possible combination of sensory organ labeling suggests that the sample size was nearly sufficient to reach saturation.
quadrant of the otic placode (Figs. 2A–2C) gave rise to cells in sensory organs that are located in anterior (Fig. 2B) as well as posterior (Fig. 2C) regions of stage 47+ Xenopus ears. More embryos labeled in the posterior region gave rise to labeled cells in both anterior and posterior regions than any other category of sensory organ labeling (Figs. 3A–3D). One example of an embryo with a posterior injection (Figs. 2D–2F) had labeled cells in both anterior (Fig. 2E) and posterior cristae (Fig. 2F). There was little difference in the results between the two sets of time points (otic placode and otocyst stages) (Fig. 4). There does not appear to be an anterior-posterior restriction of sensory organs even at early otocyst stages.

**Injected Quadrants Revealed Differences in the Range of Derivatives Formed**

Although the fate map demonstrated that every quadrant of the otic placode could give rise to sensory organs, the range of derivatives formed by a given region varied greatly across the four regions. The anterior quadrant was least likely to give rise to sensory organs of any region (36% at placode, 40% at otocyst stages; see Fig. 4 and Table 1). Anterior and ventral regions are the most likely to give rise to anterior sensory organs (58% and 76%, respectively) and never give rise to just posterior sensory organs. Injections into the dorsal quadrant are the most likely to give rise to anterior and posterior sensory structures in the same embryo. Again, there was little difference in the results between otic placode and otocyst stages.

Table 1 presents all the combinations of sensory organs and nonsensory tissues (this last category consisted of embryos with no label in sensory organs as all embryos had labeled cells in nonsensory structures) that contained labeled cells within a single tadpole. Twenty-seven different categories (which represent almost all of the 32 possible combinations) encompassed all the results from 400 injected embryos. Ventral regions of the otic placode were most likely to give rise to embryos with labeled cells in anterior and lateral cristae (33% at placode, 29% at otocyst stages). Anterior and posterior cristae combinations were most likely to be derived from the posterior region (18% at placode, 12% at otocyst stages). The anterior region was most likely to give rise to anterior crista (18%) with macula utriculi a close second (16%) at placode stages. At otocyst stages, the anterior quadrant is most likely to give rise to nonsensory structures alone (20%) with the combination of anterior and lateral cristae a close second (16%). The dorsal quadrant is most likely to give rise to nonsensory structures alone (17%) with anterior crista a close second (13%) at placode stages, while, at otocyst stages, the most common result was labeling in all three cristae (33%). Interestingly, neither the posterior quadrant at otocyst stages nor the dorsal and ventral quadrants at any stage injected ever gave rise to more than one sensory organ if the anterior crista was not labeled.

**Time-Lapse Videomicroscopy**

The fate-map data are consistent with a developmental pattern that involves a great deal of cell mixing even from otocyst stages. Most views of the developing inner ear have assumed that the molecules that pattern the ear impose their pattern on a relatively static field of cells. To verify that cells from different regions of the otic placode can indeed mix, we injected two regions of the otic placode of stage 23–25 embryos with two different vital dyes (DiI-CM and DiD) and followed the labeled cells for at least 10 h. This amount of time was sufficient to go from a placode to an advanced otocyst stage that is just beginning to undergo sensory organ differentiation. Anterior and posterior (n = 7) or dorsal and ventral (n = 4) regions of the otic placode were labeled in an individual embryo. Time-lapse videomicroscopy results demonstrated that cells from dorsal and ventral regions (Fig. 5) mix during otic placode development. The dynamic nature of these cell behaviors is particularly obvious in the time lapses. In the example shown, within the first 200 minutes, green cells move ventrally and colocalize with the red cells that are splitting into two groups (white and yellow arrows in Figs. 5C–5I). Green and red cells remain colocalized for almost 4 h (Figs. 5K–5P), separate (Figs. 5O–5S), and then become colocalized again (yellow arrow in Fig. 5T). Anterior and posterior injections also resulted in cells mixing (data not shown). Cell mixing was seen in all time-lapse experiments (n = 11).

Since images at 10 planes of focus were collected at every time point, we could determine if the mixing cells were in the same focal plane. Mixing cells were in the same plane of focus (Fig. 5) or within one focal plane, demonstrating that these cells were at similar depths and not just in lateral and medial walls of the otocyst. All time lapses had cells that moved into and out of a given plane of focus as well (Figs. 5N and 5O). Some labeled cells were seen to move away from the central region of the otocyst, and these cells were frequently many focal planes away, often outside the 10 focal planes collected. These cells may represent cells populating the extending endolymphatic duct and/or the auditory ganglion.

Time-lapse data also provide some insight into how the flat two-dimensional placode undergoes morphogenesis to form a three-dimensional sphere. The placode invaginates to form a pit in which the edges fuse to form an enclosed sphere (Hausen and Riebesell, 1991). It remains unknown whether the edges or the placode fold up equally or if there is asymmetry in this folding to form a sphere. Recent data in the chick otocyst shows that the ventral region moves dorsally during closure (Brigande et al., 2000). Our data suggest that cells from the ventral and dorsal edges appear to fold up equally with both rolling up to meet near the center of the otocyst’s lateral wall. Anterior and posterior edges also seem to fold up equally and meet in the center of the lateral wall. However, since our time lapse did not include the bright field image and, therefore, show the
extent of the otocyst at later stages, we cannot exclude the possibility that the ventral edge moved more dorsally.

**BMP-4 mRNA Expression during Otic Placode Development of Xenopus laevis**

To confirm previous reports of BMP-4 expression in the otic placode, whole-mount in situ hybridization was performed on embryos ranging in age from late neurula to 3-day tadpoles (stages 20-37/38). The earliest expression of BMP-4 was detected in the posterior region of the otic placode of stage 25 embryos (Fig. 6A). This expression in the posterior region became stronger when the otocyst first formed (stages 27/28). Ventral expression was also apparent at this stage. BMP-4 was first observed in the anterior region of the otocyst at stage 28. The broad ventral–posterior expression observed at earlier stages was replaced by labeling in anterior and smaller posterior foci. Cross-sections through the otic placode and otocyst revealed that BMP-4 was expressed mainly in the lateral region of the placode and otocysts. Anterior and posterior expression was maintained throughout the remainder of otocyst stages (Figs. 6C and 6D). These expression patterns were consistent with those previously reported in Xenopus (Hemmati-Brivanlou and Thomsen, 1995), chick, and mouse (Morsli et al., 1998; Wu and Oh, 1996).

**BMP-4 Expression in Xenopus Sensory Organs**

While BMP-4 expression has been described for the early otocyst stage of Xenopus, its expression in the sensory organs has not been reported. We performed in situ hybridization on paraffin sections to examine whether inner ear sensory organs express BMP-4. At stage 45, when sensory organs have formed in the inner ear, all sensory organs (Fig. 7) expressed BMP-4 mRNA. Lateral and posterior cristae displayed the strongest BMP-4 mRNA expression (Figs. 7A, 7C, and 7E). Weak expression was seen in the anterior crista (Figs. 7A and 7B), macula utriculi, and macula sacculus (Figs. 7A and 7D). Interestingly, the combination of just lateral and posterior cristae labeling was never seen in the fate map (Table 1).

Sensory organs consist of mechanoreceptive hair cells and underlying cells called supporting cells. Based on the staining, it appears that both hair and supporting cells are labeled at least in the lateral and posterior cristae. In the remaining sensory organs, the staining was too weak to be certain if hair cells were labeled. The dorsal (2G) region of the otic placode and otocyst contributed to cells of the sensory organs, even though this region (Figs. 2G–2I) does not express BMP-4 mRNA (Figs. 6C and 6D). Anterior regions of otic placode, labeled at stages 24–27 just prior to BMP-4 expression (see Fig. 6), still contributed to sensory organs.

**DISCUSSION**

**Origin of Sensory Organs**

In this study, we present a fate map of the inner ear at placode and otocyst stages. Our results demonstrate that cellular contributions to the five sensory organs scored can arise from any quadrant of the placode or otocyst. This implies that there is less antero-posterior restriction of the prospective sensory organs at these stages than suggested by gene expression patterns. In contrast, a recent fate map of the chick otocyst revealed antero-posterior lineage restrictions, particularly in the dorsal region of the otocyst (Brigande et al., 2000). The chick study did not look at sensory organ development, so the contrasting results may be due to differences between either sensory organs and nonsensory organ tissues or species. More consistent with the chicken fate map results is our observation that the range of derivatives formed varied across quadrants. Still, the degree of cell mixing occurring during inner ear development is surprising and needs to be taken into account in any model of sensory organ formation. Inner ear fate maps will provide much needed references for interpreting gene expression patterns and designing experimental manipulations to study the development of the inner ear (Fekete, 1996; Woo et al., 1995).

Our results show that any sensory organ can arise from any quadrant at placode and otocyst stages, which suggest that sensory organs are not restricted to one region at these stages. One possible explanation is that the inner ear and its sensory organs are completely undetermined at these stages. Further work will be needed to determine the extent to which the sensory organs are determined during development.
stages, but this explanation is unlikely based on other studies. While the timing of sensory organ specification is unknown, the determination of the main axes of the inner ear has been the subject of much research (Harrison, 1936; Wu et al., 1998; Yntema, 1955). Embryological manipulations involving rotations of the otic placode in avian and salamander embryos demonstrate that the antero-posterior axis is determined before the dorsal-ventral axis (Harrison, 1936; Wu et al., 1998). The antero-posterior mixing suggested by our fate map, even at otocyst stages, needs to be reconciled with this antero-posterior axis determination. Our interpretation is that cells destined to give rise to anterior or posterior sensory organs are mixed along a determined antero-posterior axis. These cells then sort out into their respective sensory organs as development proceeds. Sensory organs in mouse have been shown to be specified by late otocyst stages and that, at this stage, anterior sensory organs are restricted to the anterior half of the otocyst, while posterior ones are restricted to the posterior half (Li et al., 1978). However, the mouse study was done at days 11 and 12, which is much later than our fate map stages and just prior to when sensory organ differentiation occurs in mouse (Morsli et al., 1998) (Sher, 1972). Cells from dorsal and ventral quadrants, an axis probably not determined at the stages we did our fate map, can also contribute to the prospective sensory organs. We do not know if, within a quadrant, there is further subdivision; for example, are cells that will contribute to anterior or posterior sensory organs restricted to these portions of the dorsal or ventral quadrants. Results in chick embryos would suggest that, within our dorsal quadrant, there may be a boundary dividing the prospective endolympathic duct into anterior and posterior halves (Brigande et al., 2000). Finer resolution fate maps would be required to address such questions.

Individual Sensory Organs Arise from Different Regions

In general, we observed only a few labeled cells in an individual sensory organ but with more than one sensory organ labeled. Even large injections that labeled a region greater than half the placode never resulted in all cells of a sensory organ being labeled. These results suggest that individual sensory organs arise from cells located in different regions of the placode or otocyst. This is consistent with single cell-labeling results, which show that different regions can contribute to the same sensory organ (unpublished data). It is possible that labeled cells from one quadrant would have labeled a whole sensory organ but the dye was diluted by cell division. This does not appear likely because we saw label in the rapidly dividing nonsensory tissues in every embryo labeled (Fig. 3). Also, to explain a pattern within an individual sensory organ, where brightly labeled cells are adjacent to unlabeled cells, would require a large difference in the proliferation rate of the same cell type. However, we do feel that dilution may help explain the scattered nature of the overall labeling (in particular that of the nonsensory tissue) and that the number of cells labeled after a week of development is less than might be predicted based on the initial injection size (Fig. 3). Finally, while the size of the initial DiD label did vary among embryos, the number of sensory organs labeled did not generally reflect the size of the initial injection. For example, the small injection shown in Fig. 2 resulted in labeled cells in four sensory organs (only two are shown in the figure), similar to what was seen in one of the large injections. In many embryos with small injections, we observed label in multiple sensory organs (maximum of five: Table 1).

Differences between the Range of Structures Formed by Different Regions

Although each quadrant of the placode or otocyst could contribute to the sensory organs of the inner ear, the range of derivatives formed varied by region. These differences confirm the value of constructing a fate map at the stages chosen. Our fate map reveals that marking a group of cells in the posterior quadrant is most likely to result in nonsensory tissue labeling, while the single most common result from ventral quadrant injections is labeling of the anterior and lateral cristae. The dorsal quadrant at otocyst stages was most likely to give rise to all three cristae, suggesting that, by this stage, there is some determination of these most dorsal sensory organs. Anterior and ventral quadrants never gave rise to just the posterior crista, while the posterior quadrant was the most likely to give rise to just posterior cristae. These results are consistent with the antero-posterior axis determination discussed above as they reveal differences between anterior and posterior quadrants.

The regionalization of the placode and otocyst suggested by our data would indicate that at least some of the cells within a given quadrant have prospective fates that differ from cells of other quadrants. While our data cannot reveal these cells’ commitment state at fate-map stages, the cell mixing seen in the double-labeled embryos used for our time-lapse analysis suggests that individual variation is not sufficient to explain the differences between quadrants. Single cell labeling could distinguish whether the reason posterior cristae alone are not labeled in anterior and ventral quadrant is because cells destined to contribute to the posterior crista are always adjacent to other sensory organ cells or if these sensory organs share a common progenitor in these quadrants. Single cell lineage analysis in the chick basal papilla using retroviral labeling suggests that specific sensory organs (including hair and supporting cells) and nonsensory structures have distinct lineages as early as the otocyst stage infected (Fekete et al., 1998). Preliminary results in Xenopus using single cell-labeling experiments tend to support such lineage segregation even at placode and early otocyst stages (unpublished observations). Such restriction is not inconsistent with the extensive mixing suggested by the fate map. The fate map labeled
populations of cells and could not distinguish the lineage of single cells, which could be more restricted in their sensory organ contribution.

There were no significant differences in the fate-map results between otic placode and otocyst stages. This was particularly evident when the data were grouped as in Fig. 4. When the data were looked at separately (Table 1), subtle differences between stages could be seen for all but the posterior quadrant. We expected to see a greater percentage of embryos giving rise to nonsensory structures by otocyst stage because, as the otocyst nears the time of sensory organ differentiation, the differences between the number of cells contributing to sensory versus the larger area of nonsensory structures should be accentuated. Also, otocyst injections were more likely to be labeling lateral rather than medial cells since the otocyst were injected from the lateral side. When anterior regions of slightly older embryos (stages 33–38) were labeled, in five out of six embryos, only nonsensory structures were labeled (data not shown). Thus, by stage 33, a considerably higher percentage of cells will form nonsensory structures than at earlier stages. Further analysis is needed to determine the exact timing of when cells in a given quadrant lose their broad contributions to the sensory organs.

**Direct Observation of Cell Mixing**

Our fate-map data suggested a developmental pattern that involves a great deal of cell mixing. Because fate-map experiments were not scored until approximately 1 week after injection, the exact timing of cell mixing could not be resolved. However, our time-lapse results showed that cell mixing occurs during placode and otocyst stages. Colocalization of different cell populations was evident within 4 h of injection (Fig. 5). The cell behavior evident in the time-lapse data is dynamic with cells moving together and apart repeatedly during the course of 10 h. Cell mixing might still be occurring at later stages than those observed, but later time-lapse observations would be needed to confirm such mixing.

We could be certain that the cell mixing observed was not just cells in the lateral versus the medial walls of the otocyst because multiple focal planes were collected at each time point. Mixing cells were always seen in the same focal plane, and other labeled cells in the mixing region were never more than one focal plane away. Cells moved into and out of the plane of focus, suggesting that mixing may also be occurring along the medial-lateral axis. Labeled cells were observed many focal planes away in some of the time lapses, but these cells were moving away from the region of mixing. Those cells that moved away ventrally probably populate the auditory ganglion, which is ventral and lateral to the otocyst, while those moving away dorsally may populate the elongating endolymphatic duct, which eventually extends over the hindbrain towards the embryo’s midline (Nieuwoop and Faber, 1967).

The question arises as to how cells are mixing: is it by active cell migration, cell intercalation during growth, or a combination of both? Our methodology does not allow us to resolve unambiguously between these possibilities. Cell protrusions are seen during time-lapse analyses, but higher resolution is needed to determine if these are migratory cells or just extensions between neighboring cells. The only migration apparent during inner ear development is the delaminating cells that will form the auditory ganglion neurons and this egression is mostly over by otocyst stages (Adam et al., 1998; Haddon and Lewis, 1996). Cell intercalation and dilution of the lineage marker, during the extensive growth characteristic of ear development, could account for the widespread distribution of labeled cells observed.

**Molecular Candidates for Sensory Organ Specification**

Molecular patterning of the developing inner ear is hypothesized to rely on sequential and combinatorial gene expression patterns for differentiation (Fekete, 1996; Torres and Giraldez, 1998; Wu and Oh, 1996). Mutant analyses seem to confirm such a model as the elimination of one gene never deletes the entire inner ear but can delete specific substructures (Fekete, 1999). A few genes have been identified as prospective sensory organ markers at early otocyst stages: the low-affinity nerve growth factor receptor (p75NGFR), brain-derived neurotrophic factor (BDNF), C-Serrate-1, and BMP-4 (Fekete, 1996). All these genes are expressed in two distinct regions (anterior and posterior), which are thought to represent the separation of prospective anterior from posterior cristae. This separation at early otocyst stages appears unlikely in frogs, even though they have a similar expression pattern for at least two of these genes (Serrate, unpublished observations, and BMP-4). Anterior sensory organs can arise from posterior regions and posterior ones can arise from anterior at these stages. This suggests that at least some cells destined to contribute to anterior sensory organs are still located in the posterior quadrant and vice versa. We would argue that this early molecular separation does not yet represent positional restriction of all the BMP-4-expressing cells. However, since BMP-4 expression remains in anterior and posterior regions at stages later than our initial fate map injections and these regions are where anterior and posterior cristae will form, it is likely that some or all of the BMP-4-expressing cells will contribute to cristae.

Of all the different genes that may have a role in sensory organ formation, BMP-4 is considered the best candidate because it is the only gene specifically expressed in all the sensory organs of the chicken inner ear prior to their morphological differentiation (Wu and Oh, 1996). Our in situ hybridization results confirmed previous reports of BMP-4 expression in anterior and posterior regions of the otic placode in the frog (Hemmati-Brivanlou and Thomsen, 1995). In addition, we observed BMP-4 expression in all sensory organs of the Xenopus inner ear. This is an impor-
tant prerequisite for interpreting any potential role for BMP-4 in sensory organ formation in this species. It is unlikely that all the BMP-4-expressing cells observed at otocyst stages are the same cells expressing BMP-4 in differentiated sensory organs because of the cell mixing observed during otocyst stages and the consistency of the BMP-4 expression pattern during these stages. It is more likely that cells are turning BMP-4 expression on and off during inner ear development.

The role of BMP-4 in sensory organ formation remains to be determined. Two studies in avian embryos using Noggin, a BMP-4 antagonist, indicate that BMP-4 is critical in the development of non-sensory structures of the inner ear, particularly the semicircular canals (Chang et al., 1999; Gerlach et al., 2000). They also observed defects in shape and reduction in size of sensory organs in these experiments. However, in both studies, sensory organs were missing only when the structures that house them were deleted. These studies may suggest that BMP-4 does not play a role in sensory organ formation, but they were done at otocyst stages and could not rule out an earlier role for BMP-4.

Other BMPs are expressed in the developing ear (Oh et al., 1996) and these may have a role in sensory organ formation. This does not appear likely since others, like BMP-7 and -2, are not expressed in all sensory organs. In Xenopus, BMP-7 has a domain of expression that is larger and broader than BMP-4 at placode and otocyst stages (unpublished data), as is seen in chick. Later expression in Xenopus has not been examined; in chicken embryos, BMP-7 is not expressed in hair cells in the few sensory organs in which it is expressed (Oh et al., 1996).

Our fate-map and time-lapse results suggest that the developing inner ear is a dynamic structure in terms of cell behaviors and sensory organ formation. These data provide an important context for future studies of ear development. The vestibular system and potential molecules involved in its formation appear to be highly conserved across vertebrates. Amphibians historically have been popular for studies of inner ear development, mainly because of the ease with which embryonic manipulations can be done (Yntema, 1955). The frog, Xenopus laevis, offers further advantages. Many molecules involved in Xenopus development have been isolated (Harland and Gerhart, 1997), and developing embryos provide an excellent system for gene function assays (Vize et al., 1991). Future studies will take advantage of this ease in doing gene function assays to test the role of different molecules in sensory organ formation. Extensive pattern formation and morphogenesis are required to develop a functional inner ear, yet it is only with recent advances in cellular and molecular biology that these processes have begun to be understood.

ACKNOWLEDGMENTS

We thank Stephen Brown, Ping Chen, Andrew Groves, Neil Segl, and Erik Waldman for discussions and comments on the manuscript. We thank Donna Fekete for sharing her manuscript before publication. This work was supported by grants from the National Institutes of Health (National Research Service Award to S.H.K. and RO1 to A.C.).

REFERENCES


Dorsal-ventral patterning and differentiation of Noggin-induced
Knowlton, V. Y. (1967). Correlation of the development of mem-
branous and bony labyrinths, acoustics ganglia, nerves, and brain
amphibians: Anatomy and physiology. In “Comparative Hearing:
Fish and Amphibians” (R. R. Fay and A. N. Popper, Eds.), Vol. 11,
mapping of the eleventh and twelfth day mouse otocyst: An in
vitro study of the sites of origin of the embryonic inner ear
Development of the mouse inner ear and origin of its sensory
Nieuwkoop, P. D., and Faber, J. (1967). Normal Table of Xenopus
laevis (Daudin). North-Holland, Amsterdam.
of bone morphogenetic proteins in the developing vestibular and
Saunders, Philadelphia.
Sher, A. E. (1972). The embryonic and postnatal development of the
and efficient procedure for non-isotopic in situ hybridization to
sectioned material. Trends Genet. 10, 75–76.
Vize, P. D., Melton, D. A., Hemmatibrivanlou, A., and Harland,
Woo, K., Shih, J., and Fraser, S. E. (1995). Fate maps of the zebrafish
sensory organs versus nonsensory structures of the chicken
Saunders, Philadelphia.

Received for publication October 3, 2000
Revised January 16, 2001
Accepted January 16, 2001
Published online April 6, 2001