ABSTRACT

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Microsurgical manipulations were performed in ovo to identify the tissues that are required for conferring inner ear patterning. Our results show that the hindbrain, namely rhombomeres 5 and 6, are required for the formation and patterning of the cochlear duct (basilar papilla). Rhombomere 5 and its underlying notochord appear to be important for the growth of the cochlear duct, whereas rhomobomere 6 and its respective notochord are required for cochlear patterning. Rotating the segment of hindbrain from rhombomere 5 to rhombomere 6 along the anteroposterior axis affects cochlear duct formation but has no effect on the development of vestibular structures. The signaling molecules intrinsic to these tissues are distinct from Sonic Hedgehog, which has been shown to be required for cochlear duct outgrowth. In contrast, otic mesenchyme adjacent to the developing inner ear provides anteroposterior axial information to pattern the anterior and posterior canals and ampullae.

DIFFERENTIAL REQUIREMENTS OF THE HINDBRAIN AND MESENCHYME ON INNER EAR PATTERNING

By

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Table of Contents

List of Figures

Introduction

The inner ear emerges as a thickening of ectoderm, known as the otic placode, adjacent to the hindbrain. This otic epithelium undergoes a series of cell fate decisions giving rise to neural, sensory, and non-sensory cells. As development progresses, the otic placode begins to invaginate to form the otic cup and continues to deepen and close, forming the otic vesicle or otocyst (Appendix I). In chicken, the three axes; anteroposterior (AP), dorsoventral (DV), and mediolateral (ML), are thought to be specified during the otic cup to late otocyst stages, with the AP axis committed before the DV axis (Bok et al., 2007b; Wu et al., 1998).

Axial identity of the inner ear is specified by interactions of otic and surrounding tissues and is vital to establish the position and pattern in which the inner ear components subsequently develop (Fekete and Wu, 2002). Such tissues include the endoderm, hindbrain, notochord, neural crest, and mesenchyme. For instance, the endoderm is a source of Fgf8 that initiates an FGF signaling cascade required for induction of the otic placode (Ladher et al., 2005). The dorsal hindbrain is a source of Wnt signaling required for dorsal otic patterning and inner ear morphogenesis of both dorsal and ventral structures (Riccomagno et al., 2005). The ventral hindbrain and the notochord are a source of Sonic Hedgehog (Shh) signaling required for the formation of ventral inner ear structures (Bok et al., 2005; Riccomagno et al., 2002). Additionally, reciprocal epithelial-mesenchymal signaling, which is required for the morphogenesis of diverse organs, is required for the inner ear as well. *Brn4* and *Tbx1* expressed in the mesenchymal cells surrounding the otocyst have been shown to mediate proper cochlear outgrowth (Braunstein et al., 2008; Phippard et al., 1999; Xu

et al., 2007). While these tissues are known to contribute to inner ear formation, the molecular pathways involved are still under investigation.

The role of hindbrain signaling on inner ear patterning has been of particular interest because of the well-known hindbrain mutants that exhibit inner ear abnormalities, such as *kreisler* and *Hoxa1*$^{-/-}$ (Deol, 1964; Kiernan, 2002; Mark et al., 1993). Additionally, it has been shown that removal of a large segment of hindbrain resulted in a rudimentary inner ear (Bok et al., 2005). During development, the hindbrain is organized into transient, molecularly defined segments known as rhombomeres. These rhombomeres are thought to dictate the positions where specific neurons develop along the AP axis of the hindbrain and the axial levels where neural crest cells exit the hindbrain. Several rhombomeres are affected in the *kreisler* and *Hoxa1*$^{-/-}$ mutants generating interest in the specific contribution of individual rhombomeres to the morphogenesis of the inner ear. The precise alignment of the developing otocyst along rhombomeres 5 and 6 (r5 and r6) had prompted the proposal that r5 and r6 confer AP axial identity to the inner ear (Brigande et al., 2000). However, reversing the AP orientation of r4 to r7 does not appear to affect the delamination of the cochleovestibular ganglia or gene expression along the AP axis of the otocyst (Bok et al., 2005). Yet, proper alignment between the rhombomeres and the otocyst is critical for cochlear outgrowth in both chicken and mice (Bok et al., 2007a).

To determine the contribution of each tissue source that dictate inner ear patterning, we conducted three types of surgical experiments in chicken: 1) Removing r5 and r6 combined or individually to determine the distinct contribution of each

rhombomere for inner ear patterning, 2) Reversing the AP polarity of r5 to r6 (r5-r6) with and without the notochord to determine whether AP patterning of the inner ear is affected, and 3) Translocating anterior otic mesenchyme to the posterior, and vice versa to determine if the mesenchyme provides AP axial information to the inner ear. In these studies, we demonstrate that there is a differential requirement of r5 and r6 for cochlear duct growth and patterning. The correct AP orientation of r5-r6 and the notochord is essential for proper patterning of the cochlear duct. Additionally, we show that the mesenchymal tissues anterior and posterior to the developing otic cup provide AP axial information to the formation of vestibular structures.

Materials and Methods

Microsurgical manipulation

Hindbrain removals

Fertilized eggs (CBT farms, MD) were incubated at 37°C until 10 to 25 somite stages (ss) depending on the type of surgeries performed. Embryos at embryonic day 1.5 (E1.5) equivalent to 10-13ss or Hamburger Hamilton stage 10-11 (HH 10-11) were used (Hamburger and Hamilton, 1992). Individual rhombomere, r5 or r6, including the notochord beneath was surgically removed from its surrounding tissues by making longitudinal cuts along the neural tube, as well as horizontal cuts at the rhombomere junctions, using a microsurgical blade (Appendix II). Sham operations were performed such that after the rhombomere and notochord was separated from its environment, it was placed back into the hindbrain. The operated embryos were incubated further and harvested at E2.5-E3 and analyzed for gene expression using whole mount in situ hybridization, or harvested at E7.5 for inner ear analysis using the paint-fill technique (Bissonnette and Fekete, 1996).

Hindbrain and notochord rotations

A segment of neural tube and notochord between r5 and r6 was surgically separated from its surrounding tissue and rotated along its AP axis in ovo. Sham operations were performed such that the separated tissue segment was placed back into the hindbrain. Operated embryos were incubated further and subsequently harvested for whole mount in situ hybridization or paint-fill analysis.

Hindbrain rotation without the notochord

R5-r6 and the notochord were surgically separated from the surrounding tissues and transferred to a Petri dish containing 0.6 units/ml dispase (Roche) in PBS. The notochord was separated from the hindbrain in the presence of dispase. Then, dissected r5-r6 was returned to the operated embryo in either its normal or AP rotated orientation.

Hindbrain rotation with intact notochord

A dissected segment of r5-r6 after removing the notochord in dispase (described above) was AP rotated and transplanted into a host embryo with an intact notochord and r5-r6 removed. This operation was designed to ensure high quality of the transplanted r5-r6. Operated embryos were incubated further and harvested for paint-fill analyses.

Mesenchyme transplantation

A piece of mesenchyme posterior to the right otocyst of a host embryo was replaced with an equivalent piece of mesenchymal tissue anterior to the left otocyst of a donor embryo. Using a micropipette, 0.05% CM-DiI (Molecular Probes) in 300 mM sucrose solution was spotted on the medial side of the donor tissue and rotated along its AP axis before implantation such that the relationships of the donor tissue with the hindbrain and otic tissues remain the same in the host. The opposite surgery of replacing anterior mesenchyme with posterior mesenchymal tissue was carried out in a similar manner. Sham operations were performed such that anterior or posterior

mesenchymal tissue from a donor was replaced in the anterior or posterior position in a host, respectively. Operated embryos were incubated further and harvested for paint-fill analyses.

In situ hybridization and Paint-fill analyses

Whole-mount in situ hybridization was carried out as described (Wu and Oh, 1996). Riboprobes for chicken *EphrinA4 (EphA4)* (Patel et al., 1996), *Hoxb1* (Guthrie et al., 1992), *Hoxb3* (Sham et al., 1992), and *Hoxd4* (Searcy and Yutzey, 1998), were prepared as previously described. Chicken embryos were harvested and fixed overnight in Bodian's fixative. Specimens were then dehydrated in ethanol and cleared in methyl salicylate. Inner ears were visualized by injecting 0.1% white correction fluid in methyl salicylate into the membranous labyrinth as described previously (Bissonnette and Fekete, 1996).

Results

Rhombomeres 5 and 6 or their underlying notochord is important for cochlear duct formation

Previous results showed when a block of hindbrain spanning r4 to r7 and the notochord beneath were removed, a cyst-like inner ear developed (Bok et al., 2005). To narrow the scope of those results, we investigated the distinct contribution of hindbrain segments r5 and r6 located directly adjacent to the inner ear (Fig. 1). Complete r5-r6 removal including the underlying notochord resulted in some cyst-like inner ears (Fig. 1B; n=3/9), which resemble ears with r4-r7 removal (Bok et al., 2005). The remaining specimens were malformed with severe vestibular and cochlear defects (Fig. 1C; n=6/9). These data suggest that r5-r6 plays a major role in inner ear patterning.

When either the notochord (Fig. 1E-F) or r5-r6 was removed (Fig. 1H-I), the cochlear duct was relatively normal showing similar shape and curvature as controls (Fig. 1E,H; compare to Fig. 1D,G). In some specimens, cochlear ducts were wider in width (Fig. 1F,I). These results indicate that the presence of either r5-r6 or notochord is sufficient for cochlear patterning, similar to results obtained with r4-r7 removal. Shh is thought to be a key molecule that is redundantly provided by both the floor plate of the rhombomeres and the notochord required for cochlear patterning (Bok et al., 2005). Formation of vestibular structures was more affected by removing r5-r6 than removing the notochord (Fig. 1I, asterisks; n=4/7). These vestibular results are also in accordance with previous findings involving r4-r7 removals (Bok et al., 2005). Taken together, these results suggest that either r5-r6 or the notochord beneath is

important for proper cochlear patterning, whereas rhombomeres alone are required for vestibular formation.

Rhombomere 5 is important for cochlear duct growth, whereas rhombomere 6 is more critical for cochlear duct patterning

To examine the specific contribution of r5 and r6 to inner ear patterning, we removed each rhombomere individually, including the corresponding segment of notochord beneath (Fig. 2A). The success of rhombomere removal was assessed by whole mount in situ hybridization using rhombomere specific markers 36 to 48 hrs after operations. All eight r5-removed embryos exhibited a lack of r5- but an intact r3-associated *EphA4* domain (Fig. 2B-C). In addition, some operated embryos were probed simultaneously for *Hoxb1* and *Hoxb3* transcripts. Normally, *Hoxb3* is expressed in the neural tube starting at the border of r5/r6 and extends posteriorly (Fig. 2D, white bar), whereas *Hoxb1* is expressed in r4. Thus, probing for both *Hoxb1* and *Hoxb3* transcripts labels r4 and the region of hindbrain caudal to the r5/r6 junction, leaving a gap at r5 (Fig. 2D, double headed arrow). In the operated embryos, the unlabeled gap between r4 and r6 was substantially reduced indicating that the ablation was specific to r5 (Fig. 2E). A normal cochlear duct is curved; its proximal end is in a posterior and lateral position and it arcs in an anterior and medial direction with the distal end pointing towards the posterior (Fig. 2F). The majority of cochlear ducts from r5-removed embryos were similar to those in control and sham operated specimens (Compare Fig. 2F-G to 2H; n=12/21). In some r5-removed embryos, the cochlear duct was slightly shorter (Fig. 2I, arrow; n=9/21), yet the overall curvature persisted, suggesting that r5 is more important for the growth rather than the

patterning of the cochlear duct (Appendix III). Vestibular structures appeared to be unaffected by r5 removal.

We next examined the effects of r6 removal on inner ear development. Proper ablation of r6 was determined by comparing the expression patterns of *EphA4* and *Hoxd4* between operated and control embryos. Normal *Hoxd4* expression in the neural tube starts at the border of r6/r7 and extends posteriorly (Fig. 3A, white bar). The removal of r6 resulted in a reduction of the gap between the expression domains of r5 and r7 validating the lack of r6 (Fig. 3B,C, double headed arrow). Inner ears of sham-operated embryos are indistinguishable from controls (Fig. 3D; compare to Fig. 2F). However, inner ears with r6-removal resulted in moderate to severe cochlear phenotypes ranging from a shortened (Fig. 3E, arrow; n=12/26) to an absent cochlear duct (Fig. 3F, asterisks; n=12/26). In contrast to the shortened cochlear ducts with r5-removal, in which the typical cochlear curvature was maintained, all of the shortened cochlear ducts in r6-removed embryos were relatively straight showing no apparent curvature (Fig 3E). These results suggest that while r5 and r6 are both important for cochlear duct outgrowth, r6 also plays an important role in cochlear duct patterning (Appendix III). Vestibular structures are more affected in r6 than r5 removals with absence of resorption in the posterior canal as the most prevalent phenotype (Fig. 3F, double asterisks; n=9/12).

AP orientation of r5-r6 is crucial to the patterning of the cochlear duct but not the vestibular structures

Our data indicate that as far as cochlear patterning is concerned, r6 is more critical than r5. To determine whether the positional placement of r5 and r6 confers

any AP axial information to cochlear duct formation, we rotated r5-r6 including the underlying notochord along the AP axis. Operated embryos were hybridized with *EphA4* and the expression pattern was compared with controls. Figure 4C shows an embryo with an *EphA4*-positive r5 domain adjacent to the posterior rather than the normal anterior half of the otic region (bracket; n=5). The effects of this rotation resulted in three cochlear phenotypes: 1) wide and shortened (Fig. 4E, double arrow; n=11/30), 2) thin and pointed (Fig. 4F, arrow; n=11/30), and 3) absent or stunted (Fig. 4G, asterisks; n=7/30). The shortened cochlear ducts have no clear direction of duct curvature, suggesting the patterning of the duct is affected when the relative positions between r5-r6 and the otocyst is disrupted.

Correct AP orientation of both r5-r6 and the notochord is required for proper cochlear duct patterning

The rhombomere rotation experiments performed thus far have included both the hindbrain tissue and the underlying notochord. To distinguish whether r5-r6 or the notochord, or both are required for proper patterning of the cochlear duct, we removed the notochord and rotated the rhombomeres (Fig. 5B-D). The resulting cochlear phenotypes appear similar to those with both the rhombomeres and notochord rotated (compare Figs. 5B to 4E; compare Figs. 5C to 4F; compare Figs. 5D to 4G), suggesting that the correct orientation of r5 and r6 is indeed required for proper cochlear duct patterning.

Next, we wanted to examine any possible contribution of the notochord orientation to cochlear patterning. However, due to the technical difficulty of maintaining a lone piece of rotated notochord in position, we indirectly tested this

idea by rotating only the rhombomeres leaving the notochord intact. This was accomplished by transplanting an AP rotated r5-r6 from a donor embryo into a host with an intact notochord and r5-r6 removed (Fig. 5F-G). We reasoned if the notochord is contributing to the patterning of the cochlear duct, then some rescue of phenotypes caused by r5-r6 rotation should result. Indeed, the majority of inner ears had a cochlear duct with a relatively normal curvature (Fig. 5F; n=4/5) and only one embryo failed to form a cochlea (Fig. 5G). Thus, these results suggest that proper position of the notochord also contributes to normal cochlear duct patterning. The patterning of the vestibular structures does not appear to be affected after r5-r6 rotation. The incomplete canal resorption observed is probably due to the younger ages of the harvested embryos.

Mesenchymal tissue adjacent to the otocyst appears to be important for providing AP axial identities to vestibular structures

The relatively normal vestibular development in embryos with r5 or r6 removal or r5-r6 rotated suggests that the hindbrain does not play a major role in vestibular patterning. However, previous transplantation results suggest that vestibular patterning is dependent on signaling from surrounding tissues (Wu et al., 1998). Therefore, we focused our attention on the mesenchymal tissues adjacent to the otic region. Mesenchymal transplantation was performed by replacing a piece of mesenchyme posterior to the right otocyst of a host embryo with an equivalent size of mesenchymal tissue anterior to the left otocyst of a donor embryo (Fig. 6A). The donor mesenchymal tissue was rotated along its AP axis before implantation such that the relationships of the donor tissue with the hindbrain and otic tissues remain the

same in the host. If the mesenchymal tissue plays a role in patterning the AP polarity of vestibular structures then signaling from the transplanted anterior mesenchyme should cause the posterior canal and ampulla to take on anterior characteristics.

Normally, there are four main features that distinguish between the normal anterior and posterior semicircular canals and ampullae (Fig. 6B,F). First, the positions where the canals insert on the common crus differ such that the anterior canal inserts into the common crus dorsal to where the posterior canal inserts. Second, the anterior and posterior canals are oriented in an approximately 90 degree angle to one another; the larger anterior canal is positioned sagittally and the smaller posterior canal is positioned in a coronal plane (Fig. 6F, red line). Third, the anterior ampulla develops in a vertical c-shape and the posterior ampulla develops in a horizontal n-shape. Finally, the anterior and posterior semicircular canals are different in shape such that the anterior canal is larger and stands as the highest point of the inner ear whereas the posterior canal is smaller and is set lower aligning with the middle region of the common crus. These four criteria were used to quantify the vestibular phenotype and each embryo was scored on a zero to four-point scale. One point was gained for each criterion that adopted an anterior-like characteristic (see Appendix IV for additional examples of scored embryos). More than half of the twenty-two operated embryos had a score of two points or higher showing the adoption of anterior characteristics (Fig. 6C-E, G-I, J). An example of an operated embryo scoring 2 Points shows the anterior and posterior canals both inserting at the same dorsal position on the common crus (Fig. 6C, #1) and the posterior canal oriented in a sagittal plane creating a 180 degree angle (Fig. 6G, #2). Figure 6D,H

shows an example of a 3 Point scored embryo with the added characteristic of the posterior ampulla developing in a vertical c-shape. Finally, the only 4 Point scored specimen is shown in Figure 6E,I with the additional point of two anterior-like canals. The distribution of embryos displaying each anterior characteristic is represented in a Venn diagram (Fig. 6J). Together, these results suggest that transplanting anterior mesenchymal tissue to a posterior position causes the posterior canal and ampulla to adopt anterior characteristics.

Next, the converse experiment was performed by replacing a piece of mesenchymal tissue anterior to the right otocyst of a host embryo with a piece of AP-rotated mesenchymal tissue posterior to the left otocyst of a donor embryo (Fig. 7A). Among 28 specimens scored, three embryos illustrate the anterior canal mirroring posterior canal characteristics garnering each a 3 Point score. First, all three embryos show the anterior and posterior canals inserting at the same ventral position on the common crus (Fig. 7C-E, #1). Second, the anterior-positioned canal is oriented in the coronal rather than the normal sagittal plane, creating an acute canal angle (Fig. 7G-I, #2). Third, both canals are similar in pattern and resemble two posterior canals (Fig. 7C-E, #3). Although this double posterior canal phenotype is consistent with the hypothesis that posterior mesenchyme should cause the anterior canal and ampulla to take on posterior characteristics, a majority of inner ears were normal (Fig. 7B,F; n=25/28). No intermediate phenotypes were obtained. Interestingly, the transplantation of either anterior or posterior mesenchyme did not affect the overall development of the cochlear duct in growth and shape. Only a slightly less angle of cochlear duct curvature is noticed after transplantation of anterior mesenchymal

tissue. Taken together with the rhombomere removal and rotation experiments, these results suggest that the mesenchymal tissues adjacent to the otocyst are important for providing AP axial identities to the vestibular components, whereas the rhombomeres are more important for proper cochlear duct growth and patterning.

Discussion

Involvement of rhombomeres 5 and 6 in patterning the cochlear duct

Complete removal of r5-r6 and the notochord resulted in a rudimentary inner ear, demonstrating the importance of these tissues for inner ear morphogenesis. These findings are largely similar to inner ear phenotypes after r4-r7 removal. Moreover, only in the presence of the rhombomeres or the notochord did the cochlear duct form properly, further supporting the evidence that Shh is a key signaling molecule provided by the floor plate within the rhombomeres and the notochord beneath (Bok et al., 2005).

Interestingly, individual rhombomere removal and rhombomere rotation experiments indicate there is a differential requirement for r5 and r6 in cochlear duct patterning; while r5 appears to be important for the growth only, r6 is more critical for patterning the cochlear duct. It is possible that this differential function of the rhombomeres is involved in conferring the AP axial identity of the cochlear duct. Yet, the lack of forming a cochlear duct that is a mirror image of a normal duct after AP rotation of r5-r6 suggests that r5 and r6 cannot be the only tissues conferring AP identity to the cochlear duct. Existing data indicate that proper cochlear duct outgrowth is guided by multiple factors. For example, when chicken otocysts were rotated in ovo along their AP axis at a time after the AP axis was already fixed, some of the resulting inner ears have cochlear ducts that were reversed in their AP orientation while others were short and stunted (Wu et al., 1998). These results indicate that intrinsic otic signaling plays a role in mediating cochlear duct patterning.

In addition, lack of *Tbx1* and *Brn4* in the otic mesenchyme in mice has been shown to affect cochlear duct growth and coiling (Braunstein et al., 2008; Phippard et al., 1999; Xu et al., 2007). Our mesenchymal transplantation results also support a role of the mesenchyme in cochlear duct patterning (see below). Therefore, it is likely that intrinsic signaling from the otic epithelium as well as extrinsic signaling from r5-r6 and the surrounding mesenchyme could all contribute to proper cochlear duct patterning. Thus, absent or misshaped cochlear ducts of r5-r6 rotated specimens could be resulting from a disparity among all the signaling molecules that normally pattern the cochlea. The specific positional requirement of r5 and r6 for inner ear formation is consistent with previous results showing that a relative shift in the position of the rhombomeres in relation to the developing inner ear affected cochlear duct formation in both chicken and mice (Bok et al., 2007a).

Shh or other contributing molecules

What are the signaling molecules from the rhombomeres and notochord that confer cochlear duct growth and patterning information? The necessity of Shh signaling from the ventral midline (floor plate and notochord) was previously shown to be required for cochlear formation (Bok et al., 2005; Riccomagno et al., 2002). Nevertheless, our results show an absent or stunted cochlear duct after AP rotation of r5-r6. Since such operations should not affect Shh signaling, which is ubiquitously expressed in the ventral midline, these results suggest that other signals from r5-r6 and the notochord in addition to Shh are required for cochlear outgrowth. Alternatively, while there is no report of differential *Shh* expression along the AP axis

of the midline at the transcriptional or translational level, Shh signaling in the r5 and r6 region may not be the same. In zebrafish, hedgehog (Hh) signaling is required for correct AP patterning of the otic vesicle and affects the ear in a dose-dependent manner (Hammond et al., 2003). Ectopic activation of the Hh pathway in zebrafish ears via injection of Shh RNA resulted in a mirror image duplication of the posterior region and a loss of anterior otic structures (Hammond et al., 2003). Despite the presumed ubiquitous expression of Hh in the midline, the expression of *ptc1*, a Hh receptor activated by Hh, is only highly expressed in the posterior but not anterior otic region. It was postulated that Fgf3 from r4 and Fgf8 in the otic epithelium is antagonizing Hh activity in the anterior otic vesicle (Hammond et al., 2003). However, preliminary expression analyses of two genes that are regulated by Shh in the chicken otocysts, *Gli1* and *Patched1*, did not reveal any obvious differential patterns along the AP axis of the otocyst and neural tube. Therefore, since Shh signaling does not appear to be differential along the inner ear region, we propose that other signaling molecules specific to r5-r6 are required for cochlear duct patterning, additional to Shh.

While we suggest that signaling molecules other than Shh from r5 and r6 are important for cochlear duct patterning, the notochord appears to facilitate cochlear duct patterning as well. This view is based on the milder cochlear phenotypes obtained when r5-r6 rotation is conducted in the presence rather than the absence of a normal notochord. It is not clear whether the signaling molecules in the notochord are different from those in the rhombomeres.

Mesenchymal tissue confers AP identity to the vestibular structures

Formation of the semicircular canals is thought to be dependent on secreted signals emanating from the presumptive cristae such as Fgfs and Bmps (Chang et al., 2004). However, patterning of the semicircular canals is thought to be independent of the cristae (Wu et al., 1998). This conclusion is based on previous otocyst transplantation experiments. When the AP axis of the chicken inner ear was reversed at a time when the otocyst is almost formed, the resulting inner ear is one in which the AP axis of all the sensory structures, including the cristae, formed according to the pre-established AP axis of the donor whereas the canals are patterned correctly according to the AP axis of the host (Wu et al., 1998). These results indicate that at the age of transplantation, the AP axis of the sensory components was already specified, whereas the non-sensory structures was not and the non-sensory tissues were able to form according to the new axial information of the host. The ability of the canals to form according to a different axis from that of its respective crista indicates canal patterning is independent of signaling from its sensory crista. Instead, canal patterning is most likely specified later than the cristae and is dependent on signaling from other tissues such as the hindbrain and/or mesenchyme.

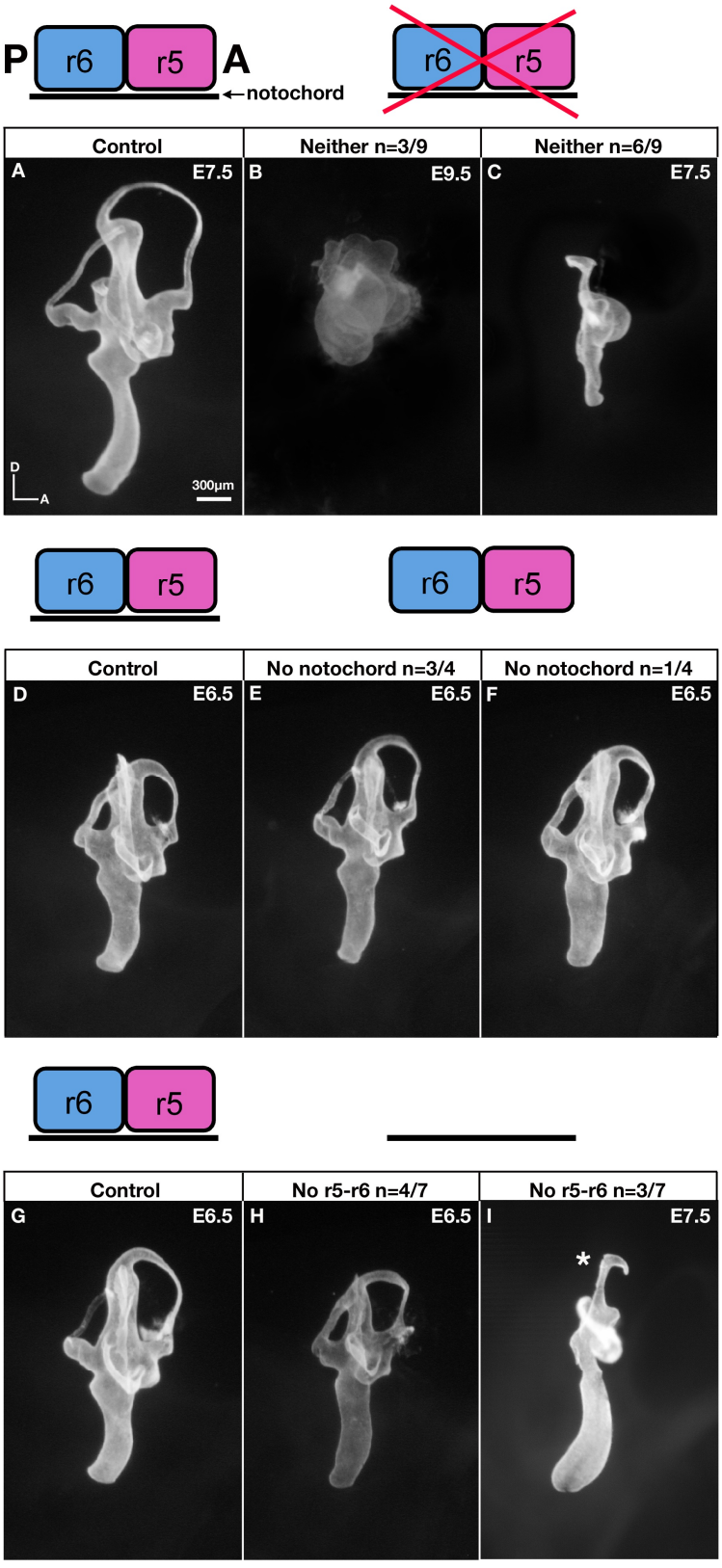
The normal vestibular structures in our rhombomere and notochord rotated specimens indicate that these tissues are not involved in conferring canal patterning or its AP identity. Instead, the mesenchyme adjacent to the otocyst appears to confer AP axial information to both the ampullae and their accompanying canals. The majority of the time, our anterior to posterior mesenchymal transplantations resulted in the

posterior canal and ampulla taking on anterior characteristics. We generated a dual anterior environment abutting the otocyst and signaling specific to the anterior mesenchyme seems to drive the patterning of anterior characteristics. Additionally, though much less frequently, we obtained double posterior characteristics when the opposite transplant was performed. The cause for this low frequency is unclear and does not seem to improve with heterochronic transplants.

Interestingly, these mesenchymal transplantations affected the AP identity of the ampullae and canals, but the cochlear duct patterning is relatively intact. Only a slight reduction in the angle of cochlear curvature is noticed after the anterior to posterior mesenchyme transplants suggesting that in addition to relaying AP axial information to the vestibular structures, signaling from the anterior mesenchyme also contributes to cochlear duct patterning. In summary, our results show that not only are multiple tissues required to coordinate proper inner ear formation and patterning, but that the tissue requirements are also different between the vestibular and cochlear duct structures. Future studies will focus on identifying the signaling molecules within the interacting tissues that are required for vestibular and cochlear duct patterning.

Figure 1. Rhombomeres 5 and 6 or their underlying notochord is important for proper cochlear patterning. Control inner ears at E7.5 (A) and E6.5 (D,G). Removing both r5-r6 and the notochord resulted in inner ears with a cyst-like structure (B) or malformed vestibular and cochlear components (C) ranging from E6.5-E9.5. (E-F) Removing the notochord results in a fairly normal inner ear with intact vestibular structures. The cochlear duct in (F) is slightly broader. (H-I) Inner ears with hindbrain removal result in cochlear patterning that is similar to controls such that the curvature of the cochlear duct remains relatively intact. In some specimens, the width of the cochlear duct is wider than controls (I). Vestibular structures can be quite affected after rhombomere removal (I, asterisk). Schematic diagram of the surgery is shown above each panel. Scale bar: 300 μm.

Figure 1


P
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Control
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E7.5
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Figure 2. Removal of r5 affects cochlear duct growth. (A) A diagram showing the surgery for r5 removal. R5 and the underlying notochord were removed from the hindbrain of chicken embryos at E1.5. (B) An E2.5 embryo showing *EphA4* expression in r3 and r5. *EphA4*-positive r5 is located adjacent to the anterior half of the otocyst. (C) *EphA4* expression associated with r5 is lost 36 hrs after surgery. (D) In controls, *Hoxb1* is expressed in r4 and the expression domain of *Hoxb3* starts at the border of r5/r6 (white bar) and extends posteriorly. A double-headed arrow points to the length of the r5 domain. (E) Loss of r5 tissue (double-headed arrow) 36 hrs after surgery. (F-I) Lateral views of control (F), sham-operated (G), and r5 removed (H,I) paint-filled right inner ears at E7.5. The patterning of most inner ears after r5 removal (H) is similar to controls (F) but some show a shorter and wider cochlear duct (I, arrow). Cochlear ducts maintain their curvature after r5 removal. The patterning and formation of the vestibular structures are normal. Brackets indicate the location of the otocysts. A, anterior; D, dorsal; AA, anterior ampulla; ASC, anterior semicircular canal; CC, common crus; CD, cochlear duct; ED/S, endolymphatic duct and sac; LA, lateral ampulla; LSC, lateral semicircular canal; PA, posterior ampulla; PSC, posterior semicircular canal. Scale bars: in B, 200 µm for B-E; in F, 300 µm for F-I.

Figure 2

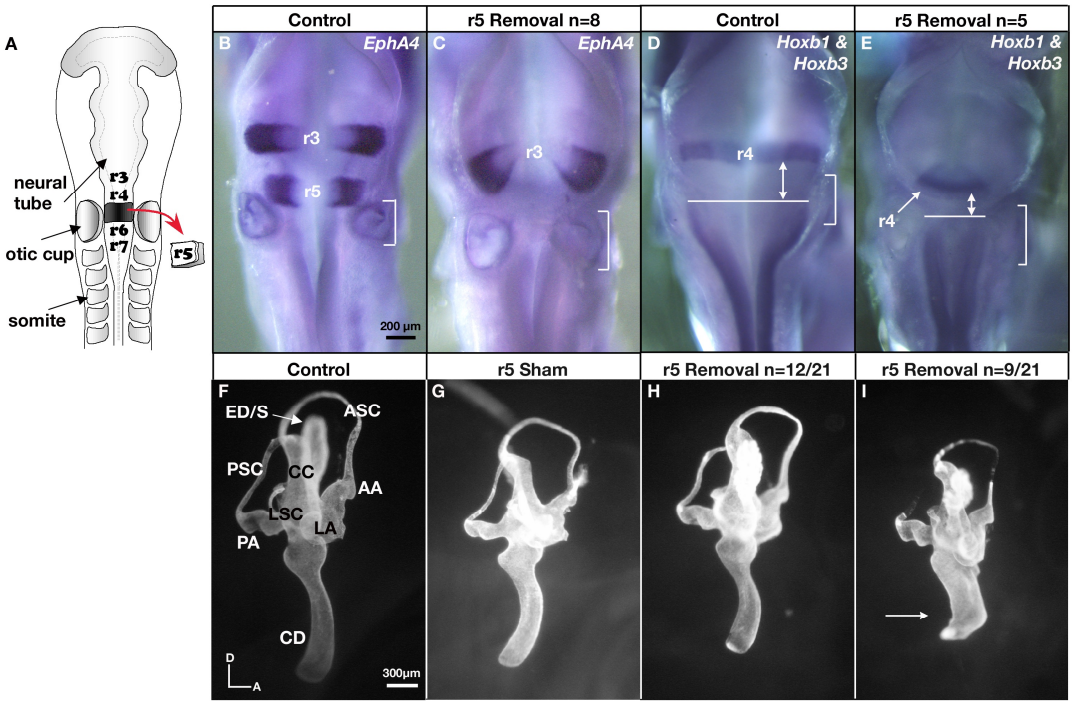

A
neural tube
otic cup
somite
r3
r4
r6
r7
r5
Control
r5 Removal n=8
Control
r5 Removal n=5
B EphA4
C EphA4
D Hoxb1 & Hoxb3
E Hoxb1 & Hoxb3
r3
r5
r3
r4
r4
200 µm
Control
r5 Sham
r5 Removal n=12/21
r5 Removal n=9/21
F
ED/S
ASC
PSC
CC
AA
LSC
LA
PA
CD
D
A
300µm
G
H
I

Figure 3. Removal of r6 affects cochlear duct growth and patterning. R6 and the underlying notochord are removed from the hindbrain at E1.5. (A) *EphA4* is expressed in r3 and r5 and *Hoxd4* expression begins at the r6/r7 border (white bar) in controls at E2.5. (B,C) Examples of embryos after r6 removal showing a reduced r6 domain (double-headed arrow). (D) Sham operated inner ear at E7.5. (E,F) Inner ears after r6 removal show a loss of duct curvature with a pointy and shorter cochlea (arrow), or an absent or stunted cochlear outgrowth (asterisk). Some inner ears show a lack of posterior canal resorption (double asterisks) after r6 removal. Two embryos are normal (2/26). Brackets indicate the location of the otocysts. Scale bars: in A, 200 µm for A-C; in D, 300 µm for D-F.

Figure 3

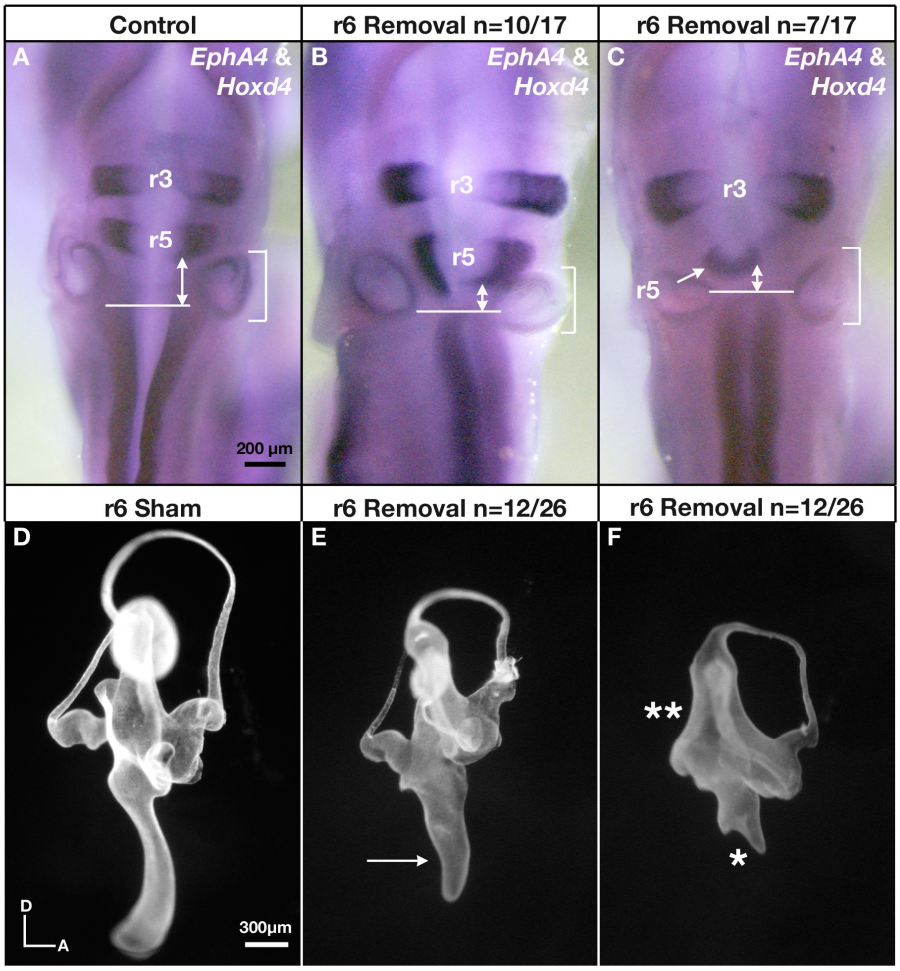

Control
r6 Removal n=10/17
r6 Removal n=7/17
A
EphA4 &
Hoxd4
r3
r5
200 µm
B
EphA4 &
Hoxd4
r3
r5
C
EphA4 &
Hoxd4
r3
r5
r6 Sham
r6 Removal n=12/26
r6 Removal n=12/26
D
D
A
300µm
E
F
**
*

Figure 4. Cochlear duct patterning is affected after AP axial rotation of r5 and r6 including the notochord. (A) The red rectangle in the diagram indicates the region of hindbrain and notochord that is rotated at E1.5. (B) An E2.5 embryo showing *EphA4* expression in r3 and r5. *EphA4*-positive r5 is located adjacent to the anterior half of the otocyst in controls. (C) *EphA4*-positive r5 is relocated adjacent to the posterior half of the otocyst after AP rotation. (D) Inner ears after a sham operation are similar to controls (Fig. 2F). (E,F,G) Inner ears at E7.5 after r5-r6 AP rotation lose the normal curvature of the cochlear duct, which normally starts from the posterior and curving slightly anterior as it extends ventrally. Three types of cochlear malformations are observed in operated ears: wide and shortened (E, double arrow), thin and pointed (F, arrow), or absent or stunted (G, asterisk). One embryo is normal (1/30). The patterning of the vestibular structures is normal. Brackets indicate the location of the otocysts. Scale bars: in B, 200 µm for B, C; in D, 300 µm for D-G.

Figure 4

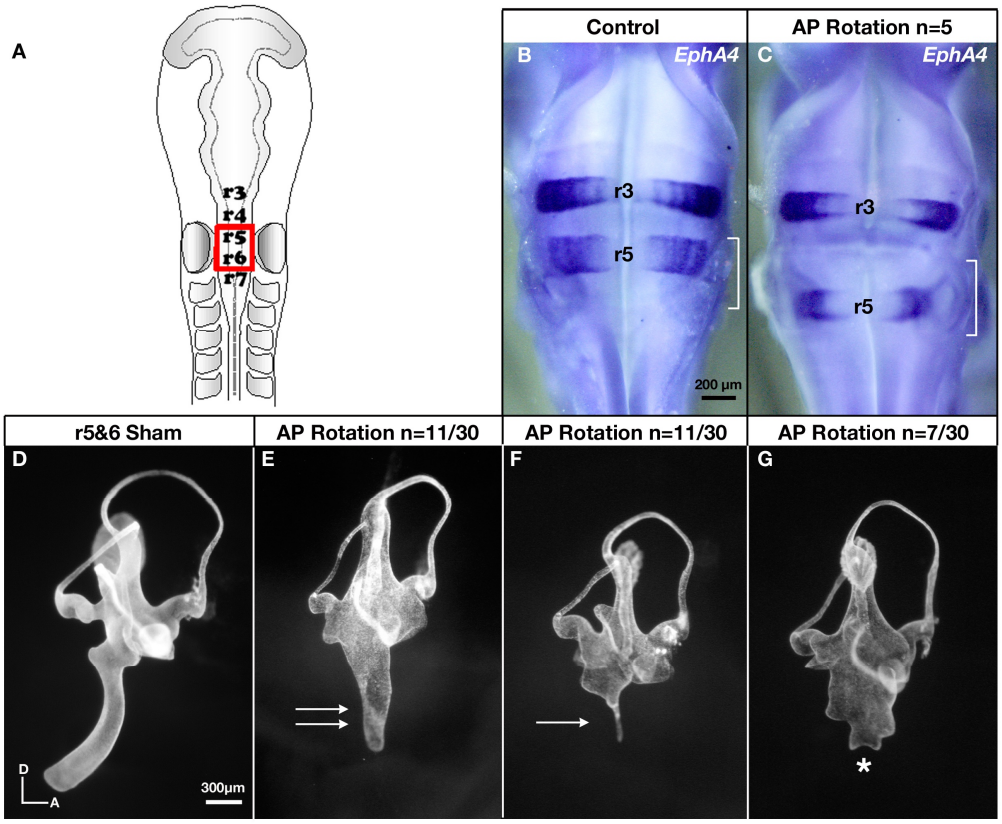

A
r3
r4
r5
r6
r7
Control
AP Rotation n=5
B
EphA4
C
EphA4
r3
r5
r3
r5
200 µm
r5&6 Sham
AP Rotation n=11/30
AP Rotation n=11/30
AP Rotation n=7/30
D
E
F
G
D
A
300µm
*

Figure 5. Recovery of cochlear duct patterning with the notochord in a normal AP orientation. (A,E) Normal inner ear of controls at E6.5. (B-D) Inner ears at E6.5 with r5-r6 AP reversal in the absence of notochord results in loss of cochlear duct curvature with three types of cochlear malformations: wide and shortened (B, double arrow), thin and pointed (C, arrow), or absent or stunted (D, asterisk). (F) Inner ears at E6.5 with AP reversal of r5-r6 result in a rescue of cochlear patterning when the underlying notochord remains intact. (G) The same operation as in F but the cochlear malformation is similar to C. Schematic diagrams of the surgeries are shown above the panels. Scale bar: 300 µm.

Figure 5

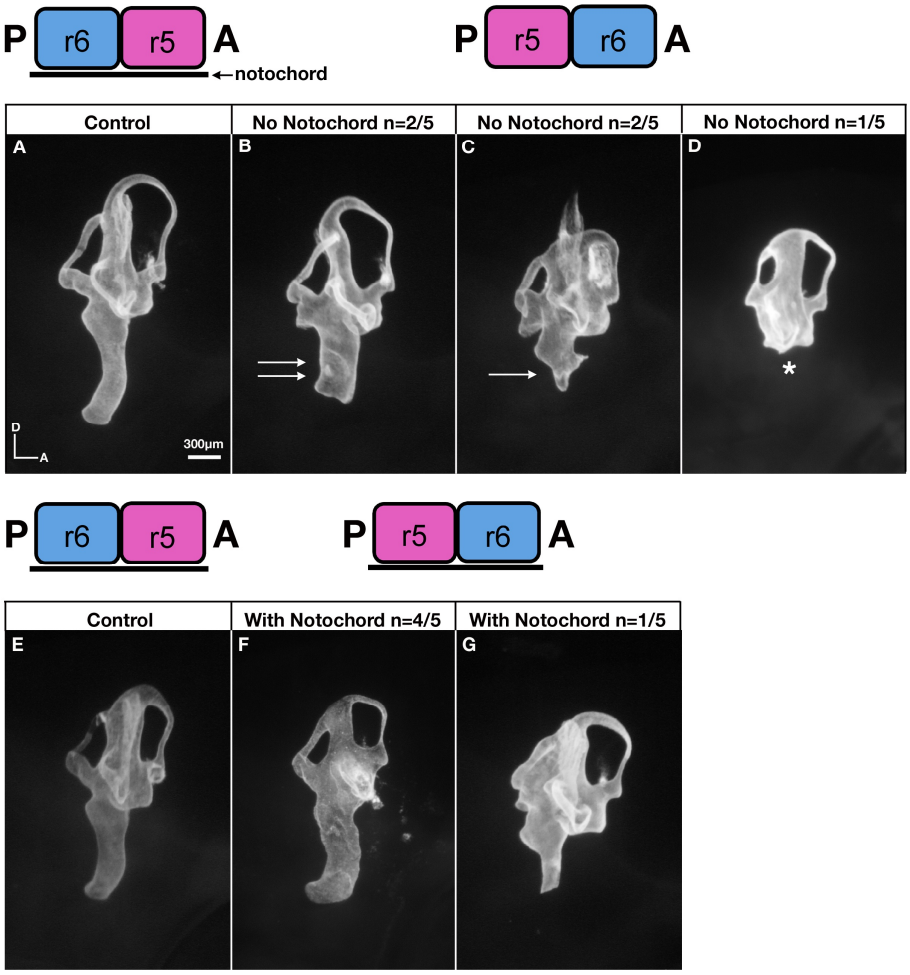

P
r6
r5
A
←notochord
P
r5
r6
A
Control
No Notochord n=2/5
No Notochord n=2/5
No Notochord n=1/5
A
B
C
D
D
A
300µm
*
P
r6
r5
A
P
r5
r6
A
Control
With Notochord n=4/5
With Notochord n=1/5
E
F
G

Figure 6. Transposing anterior otic mesenchyme to the posterior causes posterior canals to take on anterior canal characteristics. (A) A diagram showing the surgery of replacing mesenchyme posterior to the right otocyst of a host embryo with mesenchymal tissue anterior to the left otocyst of a donor embryo at E1.5. DiI crystals were spotted on the medial side of the donor tissue and rotated along its AP axis before implantation such that the normal relationships of the donor tissue with the hindbrain and otic tissues remain the same in the host. Lateral (B-E) and corresponding dorsal (F-I) views of control (B,F) and operated inner ears (C-E,G-I) at E7.5. (B,F) A normal inner ear showing the four features that distinguish between the anterior and posterior canals and ampullae: 1) Insertion point on the common crus - the insertion point of the ASC is more dorsal than the PSC (B), 2) Canal orientation - the ASC and the PSC form a 90 degree angle (F, red line), 3) Shape of the ampulla - the AA is c-shaped and the PA is n-shaped (B), and 4) Canal size - the ASC is larger than the PSC (B). (C-E,G-I) Operated specimens showing the posterior canal and/or ampulla adopting anterior canal characteristics scored 2 to 4 Points. (C,G) 2 Points: the ASC and the PSC both insert at the same dorsal position on the CC (C, #1) and the PSC is oriented in the sagittal plane creating a 180 degree angle (G, #2). (D,H) 3 Points: same 2 Points as in (C,G) and in addition, the PA is a vertical ‘c’ shape (D, #3). (E,I) 4 Points: same 3 Points as in (D,H), and the PSC is larger in size similar to the ASC (E, #4). The overall patterning of the cochlear duct appears relatively intact after mesenchyme transplantation. (J) A Venn diagram summarizing the distribution of vestibular phenotypes obtained, points scored are shown in parentheses. Three out of 22 embryos are normal and scored 0 pts. A, anterior; D, dorsal; L, lateral; AA,

anterior ampulla; ASC, anterior semicircular canal; CC, common crus; CD, cochlear duct; ED/S, endolymphatic duct and sac; LA, lateral ampulla; LSC, lateral semicircular canal; PA, posterior ampulla; PSC, posterior semicircular canal. Scale bar: 300 µm.

Figure 6

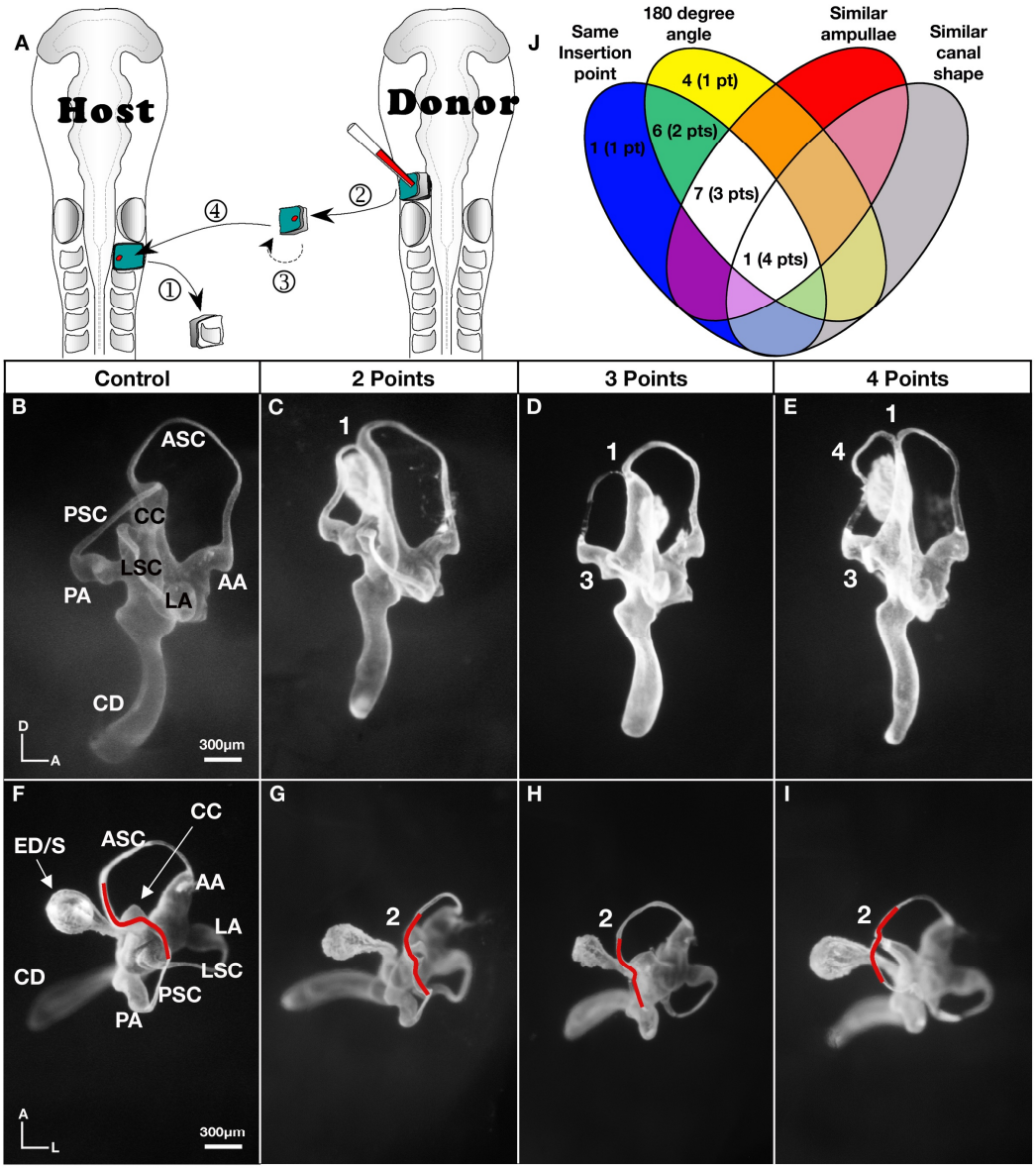
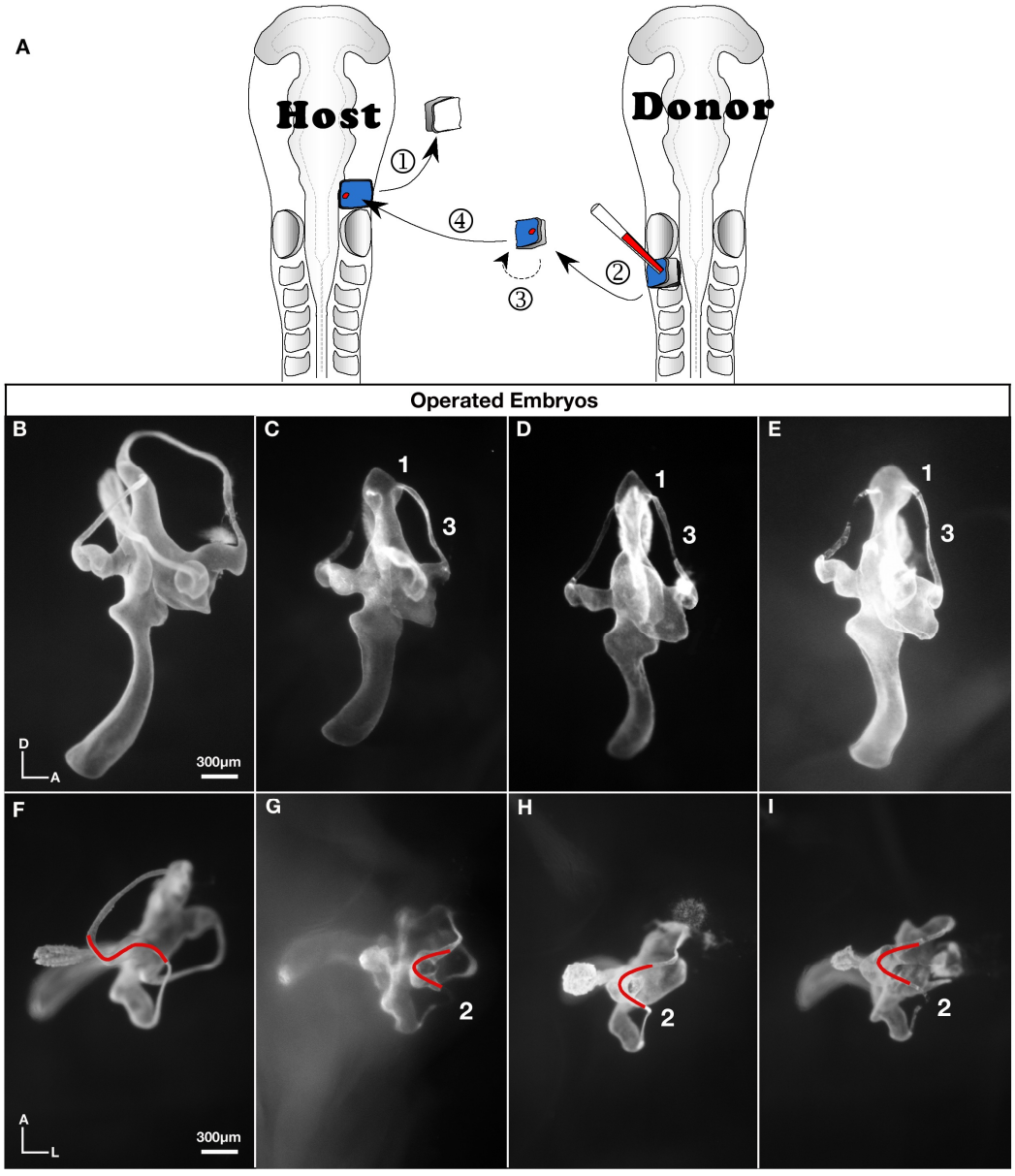

A
Host
Donor
①
②
③
④
J
Same Insertion point
180 degree angle
Similar ampullae
Similar canal shape
4 (1 pt)
6 (2 pts)
1 (1 pt)
7 (3 pts)
1 (4 pts)
Control
2 Points
3 Points
4 Points
B
ASC
PSC
CC
LSC
PA
LA
AA
CD
D
A
300μm
C
1
D
1
3
E
1
4
3
F
CC
ED/S
ASC
AA
LA
CD
LSC
PSC
PA
A
L
300μm
G
2
H
2
I
2

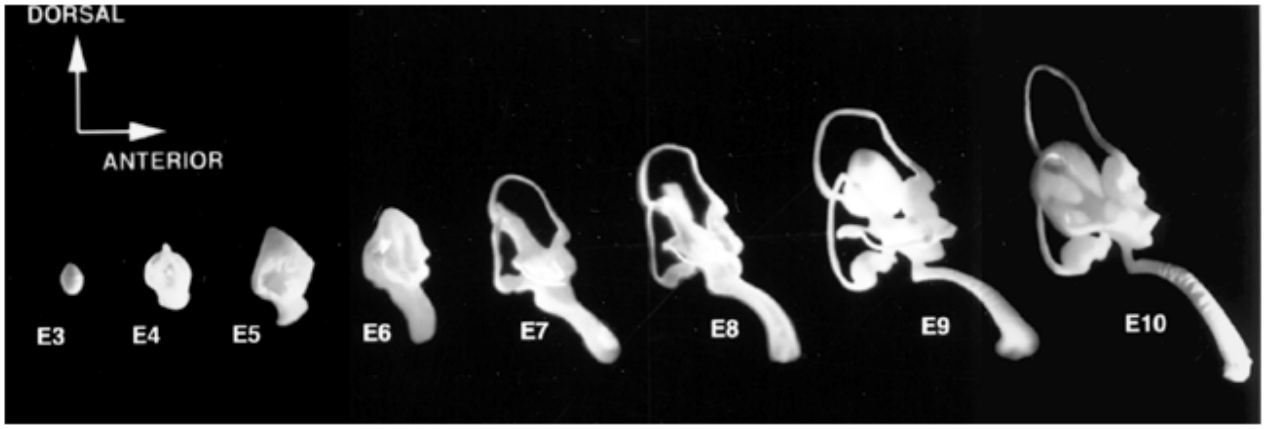
Figure 7. A small percentage of anterior canals take on posterior canal characteristics under the influence of posterior otic mesenchyme. (A) A diagram showing the surgery opposite of that shown in Fig. 6. Lateral (B-E) and corresponding dorsal (F-I) views of operated inner ears at E7.5. (B,F) Most inner ears after surgery are indistinguishable from controls, n=25/28. (C-E,G-I) Three inner ears out of 28 operated embryos show the anterior canal displaying posterior characteristics, each scoring 3 Points. Point 1: ASC and the PSC both insert from the same ventral position on the CC (C-E). Point 2: the ASC is oriented in the coronal plane creating an acute canal angle (G-I). Point 3: the ASC is smaller in shape (C-E). The patterning of the cochlear duct remains intact after mesenchyme transplantation. A, anterior; D, dorsal; L, lateral. Scale bar: 300 µm.

Figure 7



Appendix

I. Progression of chick inner ear development



The inner ear develops from a cyst into a complex structure with a dorsal vestibular region responsible for the detection of linear and angular acceleration and a ventral auditory region responsible for the detection of sound. The orientation and position at which the inner ear components will develop is specified early at the otic cup stage based on signaling from surrounding tissues.

II. Methods

One-inch segments of stainless steel wires were bent at a 90 degree angle at one end and hammered down to a thin and flattened shape. The bent and flattened end was sharpened at one edge using a multi-tool drill kit (Drumel) under a microscope. The microsurgical blade was then inserted into a needle holder and used for the surgical manipulations described in the Materials and Methods section.

III. Vocabulary

Proper Growth of the cochlear duct refers to its size, relating to the normal length and width of the duct.

Proper Patterning of the cochlear duct refers to its shape and curvature.

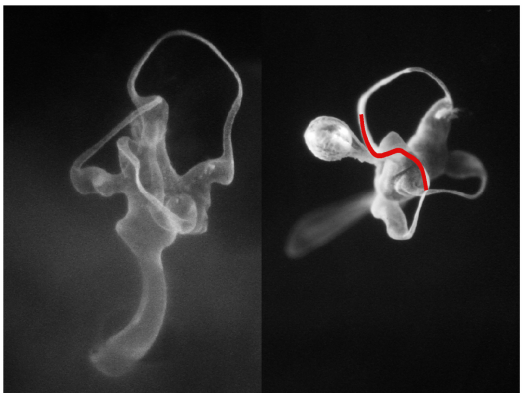
IV. Scoring system

There are four main characteristics that establish the anterior region of the vestibular domain. 1) Insertion point on the common crus - the insertion point of the ASC is more dorsal than the PSC; 2) Canal orientation - the ASC is situated in a sagittal plane and the PSC is situated in a coronal plane forming a 90 degree angle; 3) Shape

of the ampulla - the AA is c-shaped and the PA is n-shaped; and 4) Canal size - the ASC is larger and set higher on the common crus than the PSC. Anterior to posterior mesenchymal transplant embryos were scored on a zero to four-points scale, receiving one point for each adopted anterior vestibular-like characteristic.

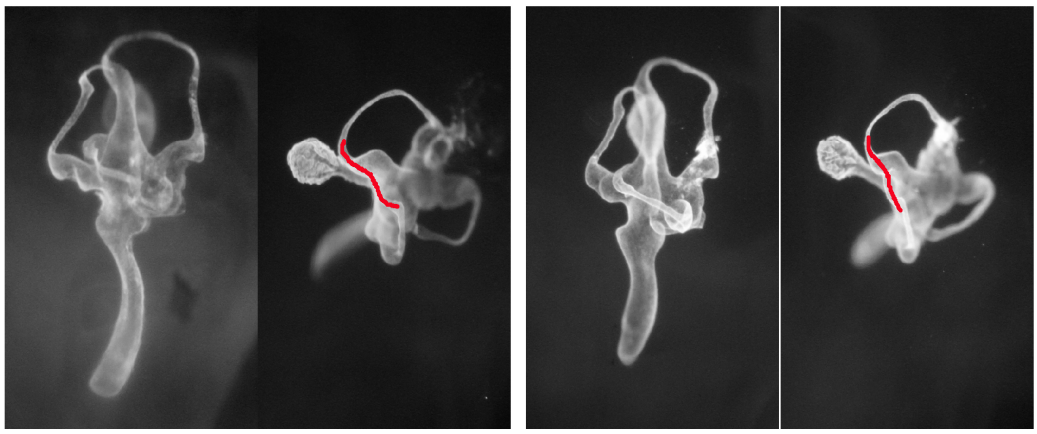
Examples of scored embryos

Control embryo



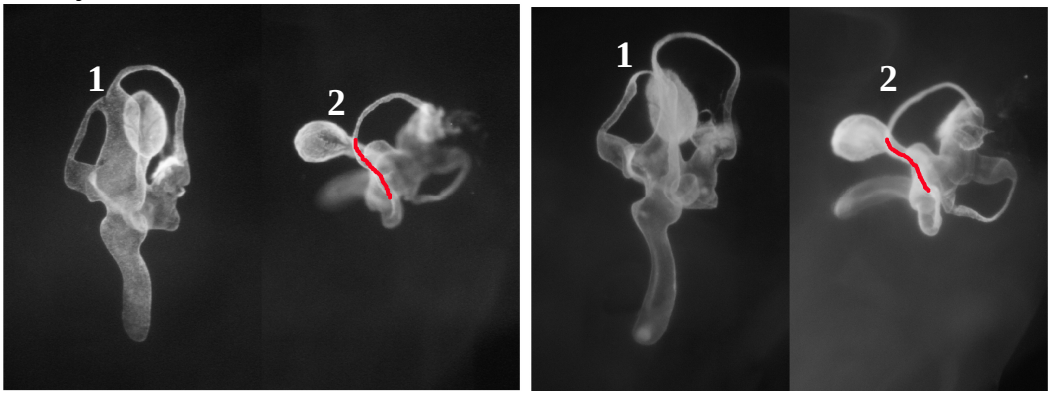
Control figure shows the anterior and posterior canals inserting on the common crus at different points and the differently shaped ampullae. The dorsal view shows the 90 degree canal angle (red line).

Embryos with a 1 Point score



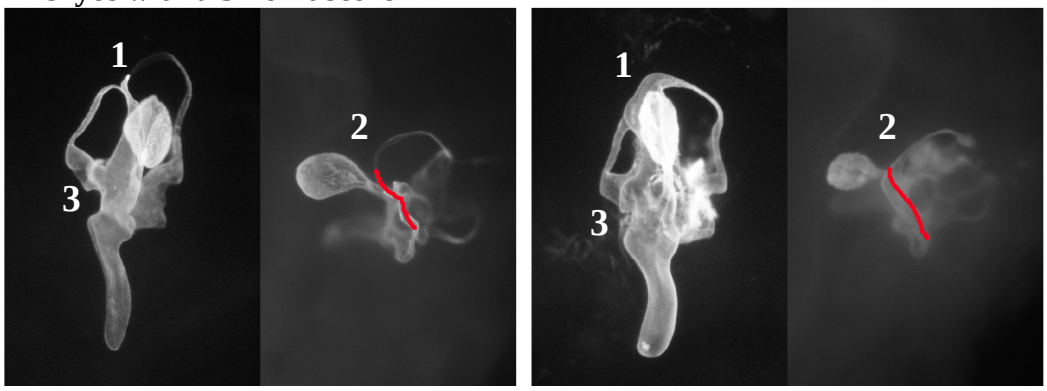
These two examples are embryos that are scored with 1 point. The 180 canal angle is evident from the dorsal view (red line). The insertion points of the anterior and posterior canals on the common crus and the shape of their respective ampullae are normal.

Embryos with a 2 Point score



These two examples show the canals inserting on the common crus at the same place (#1). The dorsal view shows the 180 degree canal angle (#2, red line).

Embryos with a 3 Point score



These two examples show the canals inserting on the common crus at the same place (#1) and the two ampullae are similar in shape (#3). The dorsal view shows the180 degree canal angle (#2, red line).

References

Bissonnette, J. P., Fekete, D. M., 1996. Standard atlas of the gross anatomy of the developing inner ear of the chicken. J Comp Neurol. 368, 620-30.

Bok, J., Bronner-Fraser, M., Wu, D. K., 2005. Role of the hindbrain in dorsoventral but not anteroposterior axial specification of the inner ear. Development. 132, 2115-24.

Bok, J., Brunet, L. J., Howard, O., Burton, Q., Wu, D. K., 2007a. Role of hindbrain in inner ear morphogenesis: analysis of Noggin knockout mice. Dev Biol. 311, 69-78.

Bok, J., Chang, W., Wu, D. K., 2007b. Patterning and morphogenesis of the vertebrate inner ear. Int J Dev Biol. 51, 521-33.

Braunstein, E. M., Crenshaw Iii, E. B., Morrow, B. E., Adams, J. C., 2008. Cooperative Function of Tbx1 and Brn4 in the Periotic Mesenchyme is Necessary for Cochlea Formation. J Assoc Res Otolaryngol. 9, 33-43.

Brigande, J. V., Kiernan, A. E., Gao, X., Iten, L. E., Fekete, D. M., 2000. Molecular genetics of pattern formation in the inner ear: do compartment boundaries play a role? Proc Natl Acad Sci U S A. 97, 11700-6.

Chang, W., Brigande, J. V., Fekete, D. M., Wu, D. K., 2004. The development of semicircular canals in the inner ear: role of FGFs in sensory cristae. Development. 131, 4201-11.

Deol, M. S., 1964. The Abnormalities of the Inner Ear in Kreisler Mice. J Embryol Exp Morphol. 12, 475-90.

Fekete, D. M., Wu, D. K., 2002. Revisiting cell fate specification in the inner ear. Curr Opin Neurobiol. 12, 35-42.

Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R., Lumsden, A., 1992. Neuroectodermal autonomy of Hox-2.9 expression revealed by rhombomere transpositions. Nature. 356, 157-9.

Hamburger, V., Hamilton, H. L., 1992. A series of normal stages in the development of the chick embryo. 1951. Dev Dyn. 195, 231-72.

Hammond, K. L., Loynes, H. E., Folarin, A. A., Smith, J., Whitfield, T. T., 2003. Hedgehog signalling is required for correct anteroposterior patterning of the zebrafish otic vesicle. Development. 130, 1403-17.

Kiernan, A. E., Steel, K. P. and Fekete, D. M., Development of the mouse inner ear. Mouse Development: Patterning, Morphogenesis, and Organogenesis. Academic Press, San Diego, 2002, pp. 539-566.

Ladher, R. K., Wright, T. J., Moon, A. M., Mansour, S. L., Schoenwolf, G. C., 2005. FGF8 initiates inner ear induction in chick and mouse. Genes Dev. 19, 603-13.

Mark, M., Lufkin, T., Vonesch, J. L., Ruberte, E., Olivo, J. C., Dolle, P., Gorry, P., Lumsden, A., Chambon, P., 1993. Two rhombomeres are altered in Hoxa-1 mutant mice. Development. 119, 319-38.

Patel, K., Nittenberg, R., D'Souza, D., Irving, C., Burt, D., Wilkinson, D. G., Tickle, C., 1996. Expression and regulation of Cek-8, a cell to cell signalling receptor in developing chick limb buds. Development. 122, 1147-55.

Phippard, D., Lu, L., Lee, D., Saunders, J. C., Crenshaw, E. B., 3rd, 1999. Targeted mutagenesis of the POU-domain gene Brn4/Pou3f4 causes developmental defects in the inner ear. J Neurosci. 19, 5980-9.

Riccomagno, M. M., Martinu, L., Mulheisen, M., Wu, D. K., Epstein, D. J., 2002. Specification of the mammalian cochlea is dependent on Sonic hedgehog. Genes Dev. 16, 2365-78.

Riccomagno, M. M., Takada, S., Epstein, D. J., 2005. Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of Shh. Genes Dev. 19, 1612-23.

Searcy, R. D., Yutzey, K. E., 1998. Analysis of Hox gene expression during early avian heart development. Dev Dyn. 213, 82-91.

Sham, M. H., Hunt, P., Nonchev, S., Papalopulu, N., Graham, A., Boncinelli, E., Krumlauf, R., 1992. Analysis of the murine Hox-2.7 gene: conserved alternative transcripts with differential distributions in the nervous system and the potential for shared regulatory regions. EMBO J. 11, 1825-36.

Wu, D. K., Nunes, F. D., Choo, D., 1998. Axial specification for sensory organs versus non-sensory structures of the chicken inner ear. Development. 125, 11-20.

Wu, D. K., Oh, S. H., 1996. Sensory organ generation in the chick inner ear. J Neurosci. 16, 6454-62.

Xu, H., Viola, A., Zhang, Z., Gerken, C. P., Lindsay-Illingworth, E. A., Baldini, A., 2007. Tbx1 regulates population, proliferation and cell fate determination of otic epithelial cells. Dev Biol. 302, 670-82.