Role of the hindbrain in dorsoventral but not anteroposterior axial specification of the inner ear

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Summary

An early and crucial event in vertebrate inner ear development is the acquisition of axial identities that in turn dictate the positions of all subsequent inner ear components. Here, we focus on the role of the hindbrain in establishment of inner ear axes and show that axial specification occurs well after otic placode formation in chicken. Anteroposterior (AP) rotation of the hindbrain prior to specification of this axis does not affect the normal AP orientation and morphogenesis of the inner ear. By contrast, reversing the dorsoventral (DV) axis of the hindbrain results in changing the DV axial identity of the inner ear. Expression patterns of several ventrally expressed otic genes such as *NeuroD*, *Lunatic fringe* (*Lfng*) and *Six1* are shifted dorsally, whereas the expression pattern of a normally dorsal-specific gene, *Gbx2*, is abolished. Removing the source of Sonic Hedgehog (SHH) by ablating the floor plate and/or notochord, or inhibiting SHH function using an antibody that blocks SHH bioactivity results in loss of ventral inner ear structures. Our results indicate that SHH, together with other signals from the hindbrain, are important for patterning the ventral axis of the inner ear. Taken together, our studies suggest that tissue(s) other than the hindbrain confer AP axial information whereas signals from the hindbrain are necessary and sufficient for the DV axial patterning of the inner ear.

Key words: Inner ear, Axis, Axial specification, Induction, Hindbrain, Rhombomere, Sonic hedgehog, SHH, Chicken

Introduction

Establishment of the body axes is an early event during vertebrate development, which provides positional information for development of later structures (Dale et al., 2002). Although asymmetric gene expression patterns are evident before the onset of gastrulation, the body axes are not morphologically obvious until formation of the primitive streak during gastrulation, closely followed by formation of the neural tube during neurulation in an anterior-to-posterior progression. Along the anteroposterior axis, members of the Hox gene family play important roles in conferring positional identity of the neural tube (Lumsden and Krumlauf, 1996), whereas BMPs/WNTs and SHH are thought to establish the dorsal and ventral axes, respectively (Harland et al., 2002). A properly patterned neural tube then relays positional information to adjacent tissues and organs. For example, cranial neural crest cells deriving from different segments of the hindbrain contribute to morphologically distinct structures and participate in the patterning of paraxial structures (Santagati and Rijli, 2003). In addition, BMPs/WNTs and SHH emanating from the neural tube are also responsible for somite differentiation (Tajbakhsh and Sporle, 1998).

The vertebrate inner ears are paraxial structures that originate as thickenings of the ectoderm, known as otic placodes, that form adjacent to rhombomeres 5 (R5) and 6 of the hindbrain. Normal development of the inner ear is dependent on the positional information provided by surrounding tissues, including the neural tube (Kiernan et al., 2002). Indeed, secreted signaling molecules, especially FGF family members, expressed in the hindbrain and paraxial mesoderm are implicated in otic placode induction (Ladher et al., 2000; Maroon et al., 2002; Phillips et al., 2001; Wright and Mansour, 2003a). The otic placode, once committed, begins to invaginate to form the otocyst, which undergoes elaborate morphogenesis to develop into a structurally complex inner ear (Kiernan et al., 2002; Torres and Giraldez, 1998).

An important step during early inner ear development is acquisition of axial identities from the surrounding tissues, which in turn influence the positional information and development of all inner ear components (Fekete and Wu, 2002). It is not clear when otic tissue acquires its axial identity, and the timing of this specification appears to vary across different species. In salamanders, the AP axis of the inner ear appears to be specified during or shortly after otic placode induction (Harrison, 1936). In chicken, the AP axis appears to be specified later in development (Wu et al., 1998). Nevertheless, results from both species indicate that the AP axis is specified before the DV axis, suggesting that axial specification occurs in multiple stages (Harrison, 1936; Wu et al., 1998). The mechanisms involved in acquiring axial identity, however, remain elusive in both species.

The role of hindbrain in inner ear development has been well

established (Kiernan et al., 2002). Hindbrain mutants with defects in the region of R5 are often associated with inner ear malformations. Therefore, it is possible that the hindbrain confers AP axial identity to the inner ear. Nevertheless, it is not clear from the typical hindbrain mutants such as *Hoxa1*$^{-/-}$ and kreisler whether their inner ear defects reflect a failure in AP patterning (Gavalas et al., 1998; Ruben, 1973). Furthermore, as the border between R5 and R6 corresponds to the AP midline of the otocyst, it has been postulated that unique signals from each rhombomere may provide AP axial information required for inner ear patterning (Brigande et al., 2000).

Although the role of the hindbrain in AP patterning of the inner ear is not clear, studies from inner ear analysis of *Shh* knockout mice have implicated the hindbrain in DV patterning of the inner ear (Riccomagno et al., 2002). The absence of ventral inner ear structures in *Shh*$^{-/-}$ mutants prompted the proposal that SHH secreted from the ventral midline (floor plate and notochord) is required to pattern the ventral axis of the inner ear (Riccomagno et al., 2002), similar to its role in the specification of ventral somites (Lassar and Munsterberg, 1996). However, a more recent study shows that immunoreactivity of SHH, as well as low levels of its mRNA (using RT-PCR) are detected in the mouse otocyst, unlike the situation in the somites (Liu et al., 2002). Thus, an issue remains of which source of SHH, the ventral midline or the otic tissue itself, is more important for patterning the ventral axis of the inner ear. Furthermore, as formation of multiple organs are disrupted in *Shh*$^{-/-}$ mutants, including the notochord (Chiang et al., 1996), it is possible that the inner ear phenotype observed in *Shh*$^{-/-}$ mutant is compounded by the loss of other structures that are also required for ear development.

In this study, we have tested the roles of hindbrain in axial specification of the inner ear in ovo. We demonstrate that the hindbrain is nonessential for AP but crucial for DV patterning of the inner ear. Furthermore, we show that SHH secreted from either the floor plate or notochord is required for ventral patterning of the inner ear.

Materials and methods

Microsurgical manipulations

Fertilized eggs (CBT farms, MD) were incubated at 37°C as specified. The number of specimens for each experiment is a representative of three or more independent experiments.

Hindbrain rotations

Embryos at embryonic day 1.5 (E1.5) equivalent to 10-13 somite stages (ss) or Hamburger Hamilton stage 10-11 (HH 10-11) were used (Hamburger and Hamilton, 1951). A segment of the neural tube including the notochord between R4 and R7 was surgically separated from its surrounding tissues using a microsurgical blade. The separated tissue was rotated either vertically (DV rotation) or horizontally (AP rotation) in ovo. The operated embryos were further incubated and subsequently harvested at E2.5-E3 for gene expression analysis using whole-mount in situ hybridization, or harvested at E7 for anatomical analysis using the paint fill technique (Bissonnette and Fekete, 1996).

Ablation of the midline structures

To ablate the ventral midline structures from 10-13 ss embryos, the neural tube and the notochord between R4-R7 were removed by making horizontal slits at R3-R4 and R7-R8 junctions, as well as longitudinal slits along both sides of the neural tube between R4 to R7. The dissected tissues were transferred to a Petri dish containing PBS, and the ventral region of the neural tube, including the notochord, was severed from the neural tube. The remaining dorsal part of the neural tube was returned to the embryo. To ablate the notochord alone, the isolated neural tube and notochord were transferred to a Petri dish containing 25% (v/v) dispase (Roche) in PBS, and the tissue was triturated through a narrow pipette tip until the notochord was separated from the neural tube. Only the neural tube was returned to the original embryo. To ablate the neural tube alone, a micro-surgical blade was carefully inserted between a partially freed neural tube segment and the underlying notochord in ovo, and the neural tube was then dislodged using a back and forth motion of the surgical blade.

Otic tissue transplantation

To reverse the AP axis of the otic tissue, a right otic cup of the host embryo was replaced with a left otic cup from an age-matched donor embryo at 11-16 ss (Wu et al., 1998). To guide the orientation and tracking of the transplanted tissue in host embryos, 0.05% CM-DiI (Molecular Probes) in 300 mM sucrose solution was injected into the anterior region of the left otic cup before transplantation. The transplanted otic tissues were monitored using a fluorescent microscope 24 hours after surgery, and embryos with appropriately transplanted otic tissues were further incubated and harvested as indicated.

Hybridoma cell implantation

Hybridoma cells secreting anti-SHH antibody (5E1) and anti-GAG antibody (3C2; Developmental Studies Hybridoma Bank, University of Iowa, IA) were labeled with 0.05% CM-DiI for 10 minutes at 37°C, washed three times with PBS, and resuspended in 300 mM sucrose solution containing 0.1% Fast Green (Sigma-Aldrich). The labeled cells were implanted either underneath the neural tube of E1.5 embryos or into the mesenchyme beneath the otocyst of E2.5 embryos using a pulled glass micropipette. The implanted cells were monitored using a fluorescent microscope.

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed as described (Wu and Oh, 1996). Riboprobes for *Lfng* (Laufer et al., 1997), *NeuroD* (Roztocil et al., 1997), *EphrinA4* (*EphA4*) (Patel et al., 1996), *Hoxb1* (Guthrie et al., 1992), *Shh* (Echelard et al., 1993), *Six1* (Heanue et al., 1999) and *Gbx2* (Niss and Leutz, 1998) were prepared as previously described. A 562 bp of 3′-untranslated region (nucleotides 1183-1745) of chicken *Otx2* (GenBank: AJ489221) was used to generate an antisense probe for *Otx2*.

Results

AP orientation of the hindbrain does not influence AP axial specification of the inner ear

Previous studies show that the AP axis for sensory tissues of the chicken inner ear is fixed by the otocyst stage (26 ss, HH 16, E2.5) (Wu et al., 1998). To better pinpoint the timing of AP axial specification, we reversed the AP axis of the otic tissue of chicken embryos between 11-16 ss (HH 10-12, E1.5-2), focusing mostly on 14-16 ss. The right otic cup of a host embryo was replaced with the left otic cup of an age-matched donor (Fig. 1A). Before transplantation, the anterior region of the donor otic cup was spotted with a DiI crystal, which serves as a marker for the orientation of the transplanted otic tissue (Fig. 1B,C). Operated embryos were further incubated and harvested at E2.5. We used the *Lfng* expression domain, which is restricted to the anteroventral otic region at E2.5, as a marker

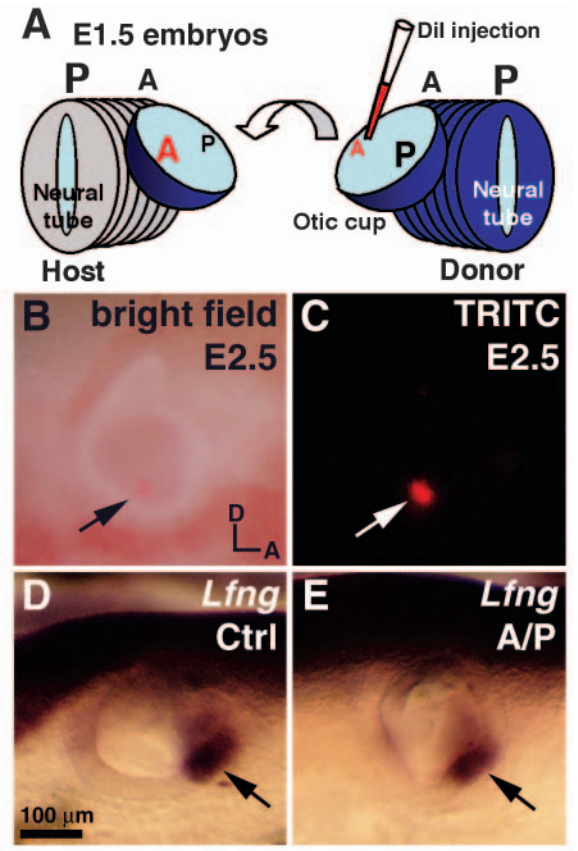

Fig. 1. Respecification of the AP axis of the inner ear at E1.5. (A) The surgery for AP reversal. DiI crystals were spotted in the anterior region of a right otic cup before replacing a left donor otic cup. (B) Bright-field and (C) fluorescent micrographs showing DiI labeling in a transplanted otocyst 24 hours after surgery. Arrows indicate DiI labeling in the transplanted otocyst. (D,E) Similar *Lfng* expression patterns (arrows) in controls (D) and AP rotated otocysts (E). A, anterior; D, dorsal. Scale bar: 100 μm.

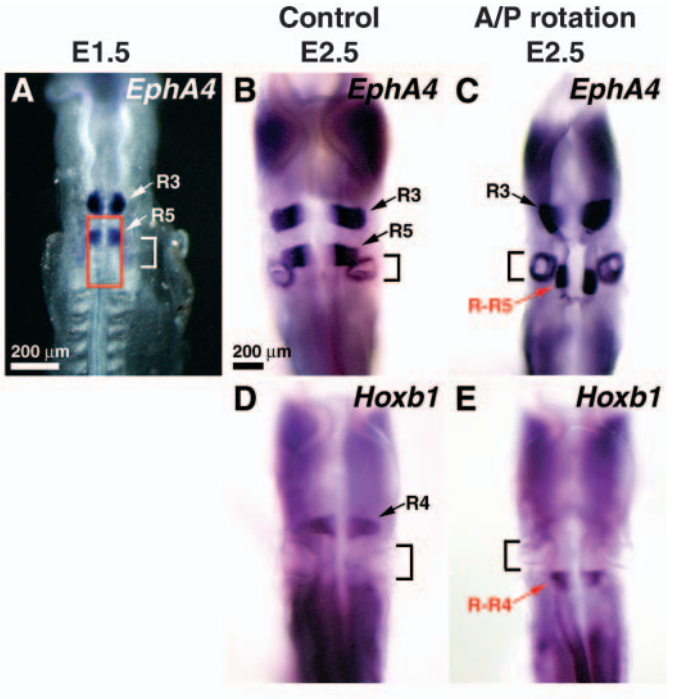

Fig. 2. Maintenance of rhombomere identities after AP axial rotation of R4 to R7. The AP axis of the hindbrain, R4-R7, is reversed at E1.5. (A) An E1.5 embryo showing *EphA4* expression in R3 and R5. The red rectangle indicates the region of hindbrain that is rotated. (B) *EphA4*-positive R5 is located next to the anterior half of the otocyst in controls. *EphA4* is also expressed in R3 and otocysts. (C) *EphA4* expression in R5 is relocated (R-R5) to the posterior half of the otocyst after AP rotation. (D,E) *Hoxb1*-positive R4 is located rostral to the otocyst in controls (D), but is relocated (R-R4) caudal to the otocyst after AP rotation (E). White and black brackets indicate otic locations. Scale bars: 200 μm in A; in B, 200 μm for B-E.

to assess AP orientation (Fig. 1D). In the transplanted ears, the *Lfng* expression pattern is similar to that in control embryos (Fig. 1E; n=10), indicating that the AP axis of the chicken inner ear is not fixed by the 16 ss.

To determine if the unique arrangement of the rhombomere segments in the hindbrain plays a role in conferring AP axial identity to the inner ear, we reversed the AP polarity of the hindbrain close to the otic tissue between R4 and R7 of 10-13 ss (HH 10-11, E1.5) chicken embryos (Fig. 2A). One day after surgery, the AP identity of the rotated hindbrain was verified using two rhombomere markers: *EphA4* for R3 and R5 (Cramer et al., 2000) and *Hoxb1* for R4 (Bell et al., 1999). From such an operation, the *EphA4*-positive R5, which normally adjoins the anterior half of the otocyst (Fig. 2B), is now associated with the posterior half of the otocyst (Fig. 2C, R-R5; n=9). R4, which is normally located rostral to the otocyst (Fig. 2D), is now located caudal to the otocyst (Fig. 2E, R-R4; n=8). This result indicates that R4 and R5 maintain their identities in the translocated positions. Consistent with this observation, it has been shown that transplantations of rhombomeres within the hindbrain area do not change their identities (Grapin-Botton et al., 1995; Guthrie et al., 1992; Kuratani and Eichele, 1993), whereas identities of the rhombomeres are altered when transplanted to a more caudal position, possibly owing to posteriorizing signals from somites (Itasaki et al., 1996). After R4-R7 rhombomere rotations, the AP orientation of the inner ear is normal based on the expression patterns of *Lfng* (Fig. 3A-D, arrows; n=12) and *NeuroD* (Fig. 3E-H, arrows; n=6) located in the anteroventral region of the otocyst. Moreover, the gross morphology of the inner ears in the operated embryos is also normal at E7 (Fig. 3I,J; n=5). These results indicate that changing the AP axis of the rhombomeres adjacent to the inner ears does not affect the AP axial orientation of the inner ear.

Signals from the ventral midline structures are critical for ventral inner ear patterning

Analysis of *Shh* knockout mice indicates that *Shh* is required for ventral patterning of the inner ear. However, in addition to *Shh* expression in the midline, SHH immunoreactivity has been reported within the otic epithelium in mice (Liu et al., 2002). As it cannot be easily determined which of these sources of SHH is more important for inner ear development in a knockout mouse model, we conducted ablation experiments in chicken. Chickens appear to be similar to mice in that *Shh* mRNA is not detectable in the otocyst using in situ hybridization technique, although signal is clearly visible in the

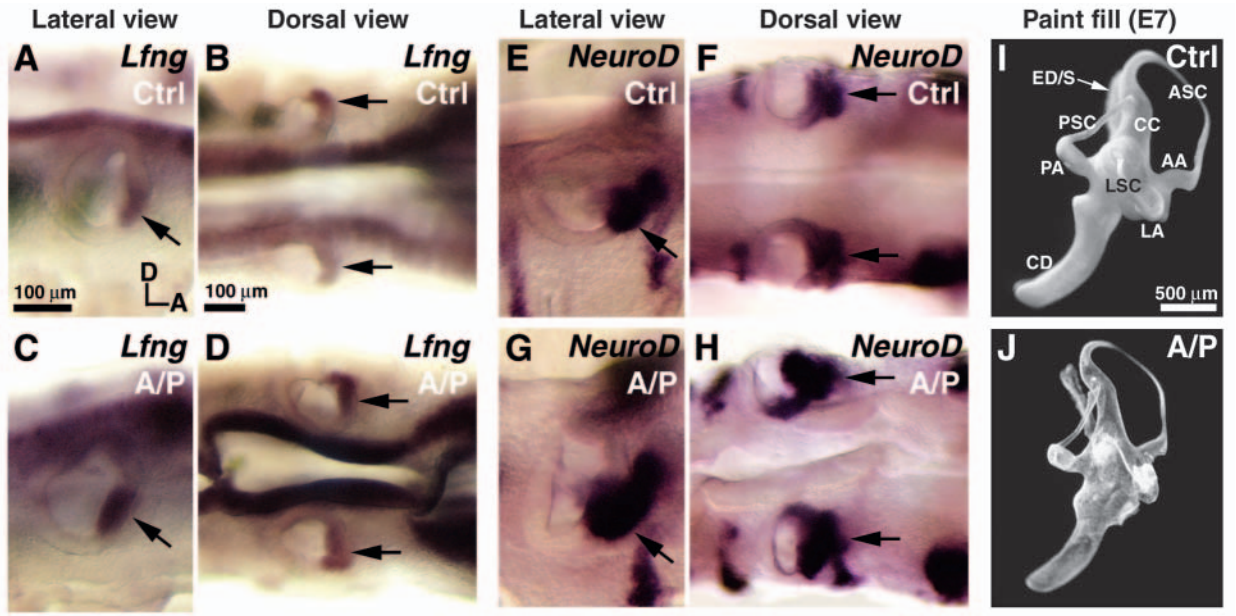

Fig. 3. The AP axis of the inner ear is not affected by AP axial rotation of the hindbrain. (A-D) *Lfng* expression patterns in control (A,B) and R4-R7 rotated (C,D) embryos at E2.5. Arrows indicate the normal *Lfng* expression patterns. (E-H) *NeuroD* expression patterns in control (E,F) and R4-R7 rotated (G,H) embryos. Arrows indicate the normal *NeuroD* expression in the otic epithelium as well as delaminated neuroblasts. (I,J) Anatomy of the inner ear after rhombomere rotation (J) is similar to controls (I) at E7. 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 A, 100 μm for A,C,E,G; in B, 100 μm for B,D,F,H; in I, 500 μm for I,J.

floor plate and the notochord (Fig. 4A,B) (Ozaki et al., 2004). However, both the SHH receptor, *Patched1,* and a gene activated by SHH, *Gli1*, are expressed in the chicken otic epithelium (data not shown) as they are in mice (Ozaki et al., 2004; Riccomagno et al., 2002), suggesting that otic epithelial cells in chicken respond directly to SHH as well. We ablated a region of the midline structures between R4-R7 of the hindbrain in 10-13 ss (HH 10-11, E1.5) chicken embryos in ovo. To monitor the success of operation, we assayed for the absence of *Shh* expression in the midline of the ablated region 24 hr after surgery at E2.5 (Fig. 4D). Transverse sections through the ablated region confirm the absence of the notochord and a smaller neural tube lacking the floor plate (Fig. 4E). At E7, operated embryos lack ventral inner ear structures, including the saccule and the basilar papilla (cochlea), compared with controls, whereas the dorsal vestibular structures are largely unaffected (Fig. 4F; n=16). These results demonstrate that signals emanating from the notochord and floor plate in the ventral midline are important for the ventral patterning of the inner ear.

To further determine whether signals from the floor plate or the notochord, or both, are required for ventral patterning of the inner ear, we removed either the notochord or the neural tube independently (Fig. 4G-L). Interestingly, the anatomy of the inner ear is normal with the removal of the notochord alone (Fig. 4I; n=8), indicating that SHH as well as other potential signals emanating from the notochord are not necessary for inner ear development. Next, we ablated the entire neural tube between R4-R7 (Fig. 4J,K). As shown in Fig. 4L, inner ears in embryos with neural tube ablation either lack or have malformed dorsal vestibular structures (n=15). However, relatively normal basilar papillae are present. Taken together, these results indicate that signals from either the notochord or the floor plate are sufficient for patterning ventral inner ear structures. The absence of vestibular components in inner ears after neural tube removal (Fig. 4L) also indicates that signals presumably emanating from the dorsal neural tube are crucial for the dorsal patterning of the inner ear. In cases where both the neural tube and the notochord were removed from R4-R7, the inner ears appear rudimentary with no obvious development of dorsal or ventral compartments (Fig. 4M-O, n=10), confirming the importance of the neural tube in inner ear patterning.

SHH from the ventral midline structures is required for inner ear patterning

To confirm that SHH is indeed the key molecule from the midline structures required for ventral inner ear patterning, we specifically inhibited its function in vivo using a function-blocking antibody. Hybridoma cells secreting anti-SHH antibody (5E1) (Ericson et al., 1996; Wechsler-Reya and Scott, 1999) or control anti-viral gag antibody (3C2) (Stoker and Bissell, 1987) were injected underneath the hindbrain region between R4-R7 at E1.5 (10-13 ss, HH 10-11; Fig. 5A). To facilitate visualization of hybridoma cells, they were pre-labeled with DiI before implantation and assessed 1 day after surgery (Fig. 5B,C). By E7, the inner ears from 5E1-implanted embryos show a complete absence of ventral structures including the basilar papilla and saccule (Fig. 5D; n=10), whereas embryos implanted with control hybridoma cells have no obvious malformations (data not shown). This inner ear phenotype elicited by 5E1 cells closely resembles embryos from which ventral midline structures are ablated (compare Fig. 4F with 5D). These results demonstrate that SHH

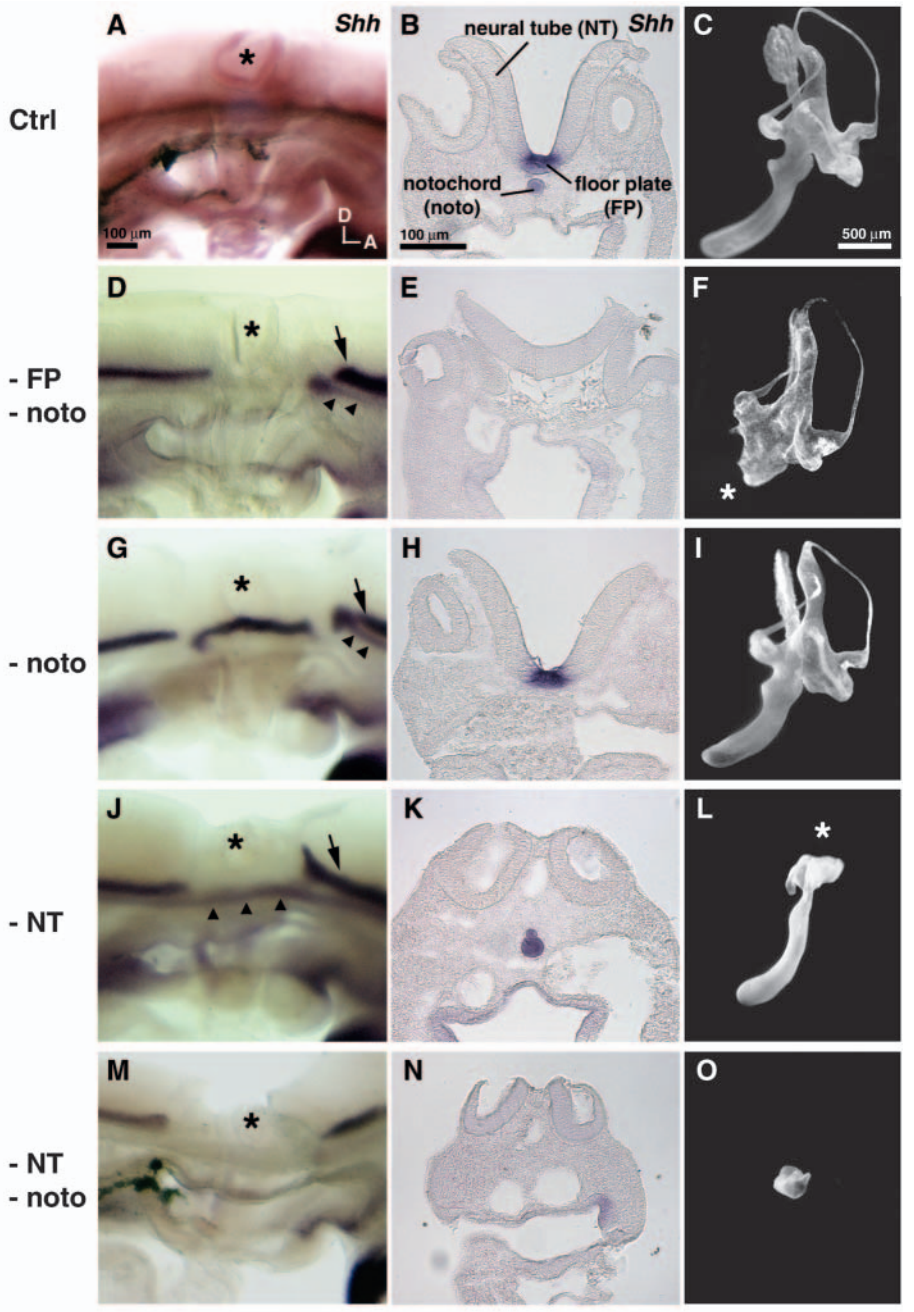

Fig. 4. Effects of ablating ventral midline structures on inner ear development. Chicken embryos are operated in ovo at E1.5, and further incubated and harvested at E2.5 for the assessment of *Shh* expression using whole-mount in situ hybridization (A,D,G,J,M) or at E7 for anatomical analysis of the inner ear using the paint-fill technique (C,F,I,L,O). Representative histological sections from ablated regions are also shown (B,E,H,K,N). Whole-mount (A) and section (B) showing *Shh* expression in the floor plate (FP) and notochord (noto) of control embryos. (C) Normal inner ear of controls at E7. (D-F) Both the notochord and ventral neural tube, including the floor plate are ablated in the region between R4-R7. (D,E) Floor plate (arrow) and notochord (arrowheads) associated *Shh* expression are missing in the ablated region. (F) The lack of ventral inner ear structures in an ablated embryo (asterisk). (G-I) Only the notochord underneath R4-R7 is ablated. (G,H) Neural tube-associated (arrow) but not notochord-associated (arrowheads) *Shh* expression is present in the ablated region. (I) The inner ear morphology of a notochord-ablated embryo is normal. (J-L) A region of the neural tube between R4-R7 is ablated. (J,K) Neural tube-associated *Shh* expression is missing in the ablated region (arrow), whereas the notochord-associated *Shh* expression is not disrupted (arrowheads). (L) Dorsal inner ear structures (asterisk) are missing after neural tube removal. (M-O) Both the notochord and neural tube are ablated. (M,N) *Shh* expression is absent from the ablated region. (O) A rudimentary inner ear results when both the notochord and neural tube are removed. Black asterisks indicate the positions of otocysts. Scale bars: in A, 100 µm for A,D,G,J,M; in B, 100 µm for B,E,H,K,N; in C, 500 µm for C,F,I,L,O.

expressed by the notochord or floor plate is indeed important for ventral patterning of the inner ear.

Previous studies have shown that blocking SHH function in the neural tube causes defects in DV organization of the neural tube (Briscoe et al., 2001). Therefore, the inner ear phenotype obtained from the 5E1-implanted embryos could be due to a lack of SHH function itself or secondary effects due to a disorganized neural tube. We further addressed this issue by restricting 5E1 cell implantation to the mesenchyme just beneath the otocysts of older embryos at E2.5 in order to minimize possible secondary effects of 5E1 cells on the neural tube (Fig. 5E,F). An additional advantage is that this allows us to address the temporal requirement of SHH in a way that was not previously possible in the mouse knockout model. The phenotype obtained from these older embryos is identical to that observed with earlier hybridoma implantations (Fig. 5G, n=9; compare with Fig. 5D). This result suggests that the loss of ventral inner ear structures by 5E1 implantation (Fig. 5D,G) is most probably due to the loss of direct SHH signaling in otic tissues, rather than due secondarily to the disorganization of the neural tube. Interestingly, 5E1 cells injected into the lumen of the otocysts of E2.5 embryos do not cause any obvious phenotype (data not shown, n=6), supporting the idea that SHH signaling external to the ear is required for inner ear patterning. Furthermore, our late implantation results suggest that there may be a continual requirement of SHH during inner ear patterning.

Signals from the hindbrain play a major role in the DV specification of the inner ear

Next, we tested the importance of hindbrain signaling in establishing the DV axis of the inner ear. As SHH expressed from the ventral midline is critical for the ventral inner ear patterning (Figs 4, 5), we investigated whether the ventral

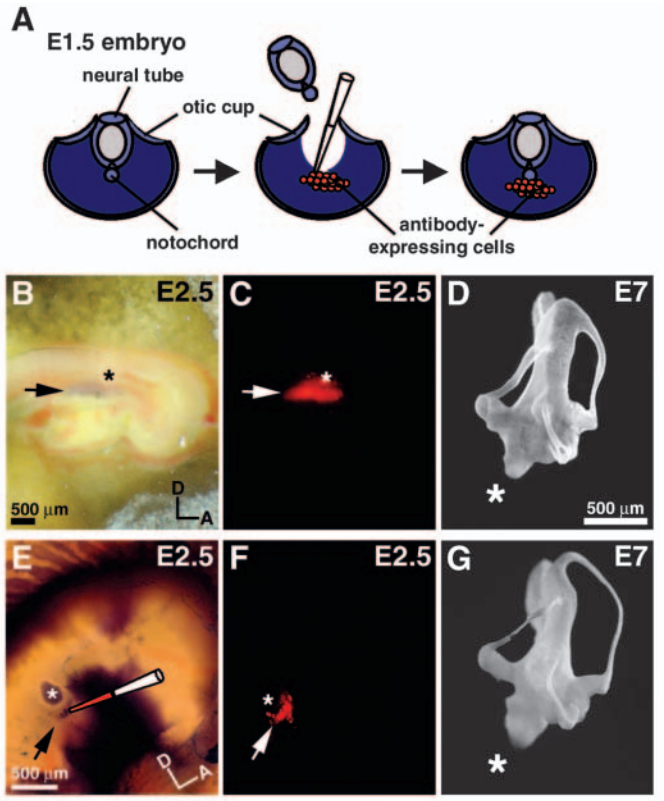

Fig. 5. Effects of blocking SHH function on inner ear development. (A-D) Hybridoma cells expressing anti-SHH antibody (5E1) are pre-labeled with DiI, and implanted underneath the region of the hindbrain adjacent to the otocysts at E1.5. (A) A schematic diagram summarizing the procedure. (B) Bright-field and (C) fluorescent micrographs showing the location of implanted hybridoma cells (arrows) 24 hours after implantation. Asterisks in B,C,E,F indicate otocyst positions. (D) Ventral inner ear structures are missing in 5E1-implanted embryos (asterisk). (E-G) 5E1 cells are injected into the mesenchyme underneath the otocyst at E2.5 as indicated in E. (E) Bright-field and (F) fluorescent micrographs taken immediately after injection of 5E1 cells (arrows). (G) An inner ear injected with 5E1 at E2.5 lacks ventral structures (asterisk). Scale bars: in B, 500 μm for B,C; in E, 500 μm for E, F; in D, 500 μm for D,G.

neural tube and the notochord are sufficient to confer ventral fates in dorsal otic tissues. This was accomplished by surgically rotating the neural tube between R4-R7 (including the notochord) along its horizontal axis between 10-13 ss (Fig. 6A), an age well before DV axis is specified in the inner ear (Wu et al., 1998). Presumably, such an operation would allow dorsal otic tissues to receive ventral signals from the rotated hindbrain in the presence of other potential dorsalizing signals from tissues such as the ectoderm and mesenchyme. The success of neural tube rotation was verified by examining the changes in gene expression in the neural tube 24 to 36 hours after surgery (E2.5-E3). In embryos in which the neural tube is rotated dorsoventrally, *Shh* expression is observed dorsally, closest to the dorsal (Fig. 6C,E) rather than the ventral region of the otocyst (Fig. 6B,D). Likewise, genes that are normally expressed in the dorsal neural tube such as *Bmp5* and *Msx1* are observed ventrally (data not shown). However, the original DV identity of the rotated neural tube is no longer maintained 48 hours after surgery, as evidenced by the loss of *Shh* expression in the dorsal region of the neural tube (data not shown). Therefore, the DV axial specification of the inner ears in the neural tube-rotated embryos was assessed prior to E3.5.

We determined the ventral fates of the inner ear in neural tube-rotated embryos using the expression patterns of *NeuroD* (Fekete and Wu, 2002), *Lfng* (Cole et al., 2000), *Six1* (Ozaki et al., 2004) and *Otx2* (Hidalgo-Sanchez et al., 2000). Normally, *NeuroD*, *Lfng* and *Six1* are expressed in anteroventral region of the otocyst (Fig. 6F,H,J). In otocysts of operated embryos, *NeuroD*, *Lfng* and *Six1* are expressed in the dorsoanterior region of the otocyst and are downregulated in the ventral region where these genes are normally expressed (Fig. 6G,I,K; *Lfng*, n=7; *NeuroD*, n=6; *Six1*, n=10). Similarly, the *Otx2* expression domain is expanded to the dorsal part of the otocyst, although the normal expression domain in the ventral region of otocyst persists (Fig. 6L,M; n=13). These results suggest that the dorsal otic tissue is ventralized when placed in proximity to the ventral neural tube. In addition, we show that rotating the neural tube alone without the notochord is also sufficient to induce these ventral fates in dorsal otic tissues (see Fig. S1 in the supplementary material).

We further assessed the dorsal fates of these inner ears using the expression pattern of *Gbx2*, which is normally associated with the dorsomedial region of the otocyst (Fig. 7A,D) (Hidalgo-Sanchez et al., 2000). Consistent with the expression patterns of ventrally expressed genes, *Gbx2* expression in the dorsal otic tissues is abolished in the operated embryos, indicating the loss of dorsal fates (Fig. 7B,E; n=14). However, *Gbx2* expression is not induced in the ventral otocyst of embryos with dorsoventrally rotated neural tube. Presumably, this is due to a considerable increase in distance between the original dorsal neural tube tissue and the otic epithelium after rotation (Fig. 7D,E; vertical bar). Interestingly, in some specimens, ectopic otocysts are present ventrally due to the inadvertent translocation of a part of the otic epithelium during neural tube rotation and *Gbx2* expression is induced in these ectopic ventrally positioned otocysts (Fig. 7C,F; arrows; n=9). This suggests that intrinsic signals in the dorsal neural tube are sufficient to confer dorsal otic fates, despite potential ventral signals from other surrounding tissues. Taken together, these experiments demonstrate the importance of the hindbrain in establishing the DV axis of the inner ear.

Discussion

Induction versus axial specification of the inner ear

Tissue transplantation experiments previously showed that the chicken otic placode is committed to its fate around 10 ss (HH 10) (Groves and Bronner-Fraser, 2000). By 15 ss (HH 12) or earlier, various genes such as *Bmp4*, *Ser1* and *Lfng* are asymmetrically expressed in the invaginating otic placode (Cole et al., 2000; Myat et al., 1996; Wu and Oh, 1996), suggesting that the AP axis of the inner ear might be committed by this stage. However, previous otocyst rotation experiments indicate a more complicated scenario (Wu et al., 1998). While the AP axis for the sensory components is committed by the otocyst stage (26 ss, HH 16), non-sensory components do not acquire AP identity until later (Wu et al., 1998). To refine the timing of axial specification, we have reversed the AP axis of the inner ear at an age before otocyst formation and found that the AP axis for the sensory components is not fixed at the 16

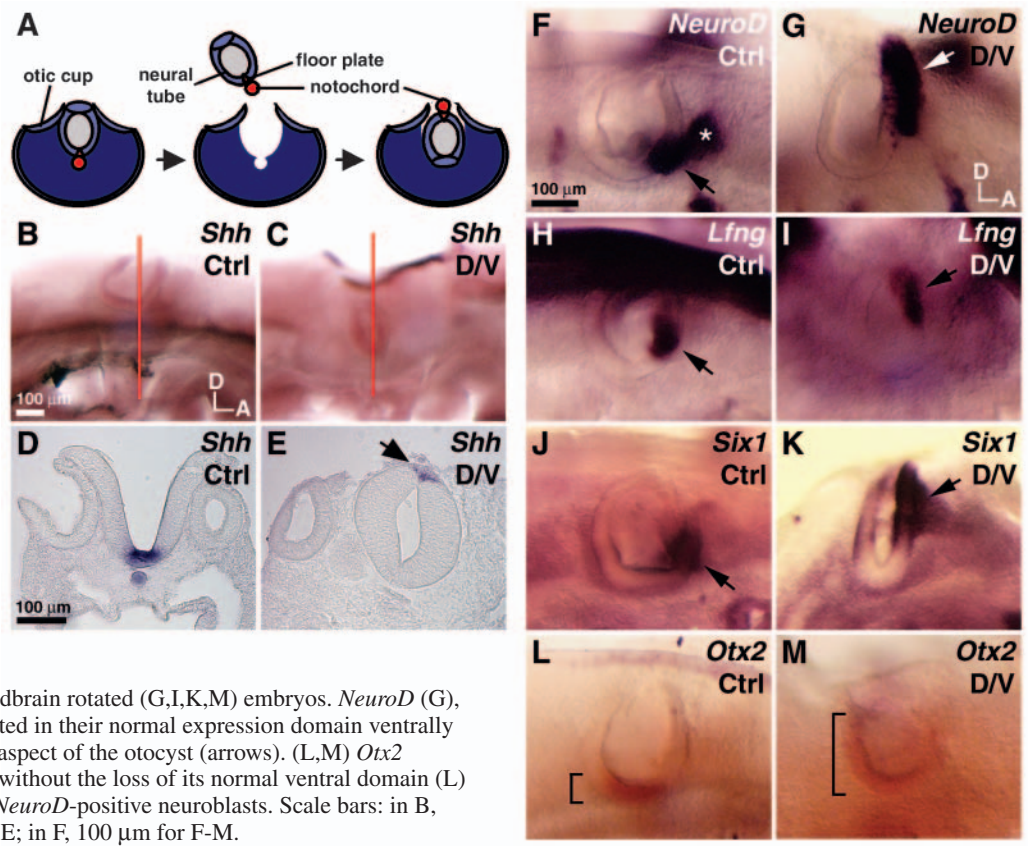

Fig. 6. Effects of DV axial rotation of the hindbrain/notochord on expression patterns of ventral otic genes. (A) DV rotation of the hindbrain and notochord between R4 and R7 at E1.5. (B,C) *Shh* expression pattern in whole-mount of control (B) and DV rotated hindbrain (C) embryos. (D,E) *Shh*-positive floor plate and notochord, which are normally located ventrally in control (D), are located dorsally in hindbrain rotated embryos (E, arrow). Red lines in B and C indicate the level of sections shown in D and E, respectively. (F-M) Expression patterns of *NeuroD*, *Lfng*, *Six1* and *Otx2* in control (F,H,J,L) and hindbrain rotated (G,I,K,M) embryos. *NeuroD* (G), *Lfng* (I) and *Six1* (K) are downregulated in their normal expression domain ventrally and upregulated in the dorsoanterior aspect of the otocyst (arrows). (L,M) *Otx2* expression is expanded dorsally (M) without the loss of its normal ventral domain (L) (brackets). An asterisk indicates the *NeuroD*-positive neuroblasts. Scale bars: in B, 100 µm for B,C; in D, 100 µm for D,E; in F, 100 µm for F-M.

ss (Fig. 1). Therefore, this axis must be specified between 17-26 ss (HH 12-16, E2-E2.5). Taken together, these experiments show that otic placode induction precedes axial specification in chicken. This temporal difference suggests that these two processes are independently regulated by distinct signaling mechanisms in the chicken. By contrast, in the salamander, ear induction and AP axial specification are temporally much more closely linked (Harrison, 1936). Whether the developmental time course of mammalian inner ear resembles that of the chicken or salamander is not yet known.

Roles of the hindbrain in AP axial specification of the inner ear

In all vertebrates, the inner ear develops in approximately the same position along the body axis, adjacent to R4-R6. Cooperative signals emanating from adjacent tissues including the hindbrain, mesoderm and endoderm are likely to contribute to the location of the ear (Baker and Bronner-Fraser, 2001). In particular, signals from the hindbrain have been implicated not only in induction but also in the later morphogenesis of the inner ear (Kiernan et al., 2002; Torres and Giraldez, 1998; Whitfield et al., 2002). For example, hindbrain mutants such as *Hoxa1*$^{-/-}$ and kreisler that are missing R5, display severe inner ear malformations (Gavalas et al., 1998; Ruben, 1973). However, despite the malformations, the AP axial identity of the inner ears appears to be unaffected (Gavalas et al., 1998) (D. Choo, personal communication). Our results indicate that the hindbrain has no major function in conferring AP axial identity to the otic tissue. Reversing the AP orientation of the hindbrain of R4 to R7 prior to axial specification of the ear had no adverse effect on inner ear development even when larger regions of the hindbrain were rotated (data not shown). Taken together, these observations demonstrate that despite the important role of rhombomeres in inner ear development, they are nonessential for conferring AP axial information to the inner ear. Thus far, tissue(s) responsible for conveying AP axial identity to the inner ear remains unknown.

SHH signaling from the ventral midline is required for inner ear patterning

Analyses of *Shh*-knockout mice demonstrate that SHH is required for ventral patterning of the inner ear (Riccomagno et al., 2002). However, the source of SHH, whether from the ventral midline or the otic tissue itself, was not clear (Liu et al., 2002). Using microsurgical ablation and localized inhibition with function-blocking antibodies in chicken, we demonstrate that SHH from the ventral midline is the primary signal for inner ear patterning in chicken, with either the floor plate or the notochord being sufficient to mediate this function. These conclusions are similar to those drawn in zebrafish (Hammond et al., 2003). Consistent with this idea, inner ear morphology is normal in *Gli2*-null mice that lack the floor plate (Ding et al., 1998; Matise et al., 1998) (D. Epstein and D.K.W., unpublished). The sufficiency of SHH from floor plate or notochord in conferring ventral inner ear fates is similar to their role in induction of ventral properties in somites (Brand-Saberi et al., 1993; Pourquie et al., 1993). Although we demonstrate that SHH from the midline is the primary source for establishing ventral inner ear patterning, these results do not preclude the possibility that SHH expressed within the otic tissues (Liu et al., 2002) and cochlear-vestibular ganglion may play a later role in inner ear development.

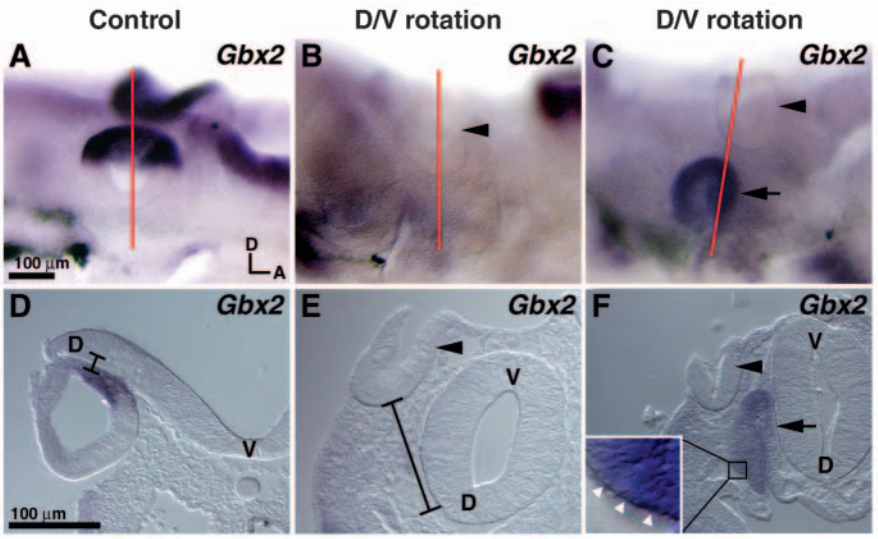

Fig. 7. Effect of DV axial rotation of the hindbrain on the expression of a dorsal otic gene, *Gbx2*. (A-C) Whole mounts and (D-F) sections of embryos showing expression patterns of *Gbx2*. Red lines in A-C indicate the level of sections shown in D-F. (A,D) *Gbx2* expression in dorsomedial region of control otocyst. (B,C,E,F) Absence of *Gbx2* expression in otocysts after DV rotation of the hindbrain (arrowheads). Arrows in C and F indicate *Gbx2* expression in the ectopic otocyst that was translocated ventrally during neural tube rotation. D and V in D-F indicate the original dorsal and ventral axes of the neural tube, respectively. Rules in D and E indicate the relative changes in the distance between the dorsal neural tube and otocyst after neural tube rotation. Arrowheads in the insert in F indicate the basement membrane of the ectopic otic epithelial tissue. Scale bars: in A, 100 μm for A-C; in D, 100 μm for D-F.

In zebrafish, *Hedgehog* signaling is required for AP patterning of the inner ear (Hammond et al., 2003), yet AP patterning in mouse $Shh^{-/-}$ inner ears appears normal (Riccomagno et al., 2002). Similarly, we do not observe any AP patterning defects in chicken inner ears in which the SHH signaling is disrupted. This suggests that the mechanisms for axial patterning in zebrafish are different from those of mice and chicken. Furthermore, in chicken embryos where the SHH signaling is disrupted, the dorsal region of the inner ear is normal. These results are also different from those observed in $Shh^{-/-}$ mice, which include dorsal inner ear defects (Riccomagno et al., 2002). As all axes of the $Shh^{-/-}$ mutants are correctly established initially, except for the ventral axis, we attribute these dorsal phenotypes observed in *Shh* mutants as secondary defects, possibly owing to disorganization of the neural tube (Riccomagno et al., 2002).

Possible mechanism of SHH function on inner ear patterning

How does SHH expressed in the ventral midline exert its effect on inner ear tissues? One possibility is that it travels from the midline and acts on otic tissues directly. Alternatively, it may mediate its effect indirectly by activating genes in the mesoderm, which in turn induce ventral inner ear development. There is evidence to support both scenarios. Both *Brn4/Pou3f4* and *Tbx1* expression levels in the mesoderm surrounding the inner ear have been shown to be regulated by SHH (Riccomagno et al., 2002). Although the specific role of mesodermal *Tbx1* in inner ear development is unclear, the lack of *Brn4/Pou3f4* in the mesoderm results in cochlear defects in mice (Phippard et al., 1999; Raft et al., 2004; Vitelli et al., 2003). However, SHH has been shown to be capable of traveling considerable distances to exert its function in other systems (Goetz et al., 2002). Several lines of evidence favor the idea of direct action of SHH on otic tissue. First, genes known to directly respond to SHH signaling, such as *Patched* and *Gli1*, are expressed in the otic epithelium of both mice and chicken (Ozaki et al., 2004; Riccomagno et al., 2002) (J.B., unpublished). Second, the expression of these SHH-responsive genes is upregulated when *Shh* is ectopically expressed in otic tissue in mice (Riccomagno et al., 2002) or when SHH-soaked beads are implanted to the developing otocysts in chicken (J.B., unpublished). Third, locally blocking SHH signaling beneath the otocyst at a later stage is sufficient to block ventral inner ear patterning (Fig. 5), further supporting the idea that SHH acts directly on the otic epithelium. These late implantation results also suggest that SHH action on otic tissue is required continuously. This is consistent with previous studies showing that specification of the DV axis, which is dependent on SHH, occurs well after otocyst formation (Wu et al., 1998).

SHH traveling from a source to a target area sets up a concentration gradient that is highest at the source and gradually decreases with the distance away (Goetz et al., 2002). This concentration gradient of SHH is thought to be an important mechanism in other systems for conveying unique positional information to cells and tissues located various distances from a source (Goetz et al., 2002). For example, it has been shown in the neural tube that different types of neurons are induced in response to a graded SHH concentration (Ericson et al., 1997; Ericson et al., 1996). Similarly, a SHH concentration gradient has been shown to be important for correct digit patterning during limb development (Yang et al., 1997). SHH may play a similar role in patterning the fine structure of the cochlea. The vertebrate cochlea is a tonotopically organized structure, such that each region of the cochlea is sensitive to a specific range of sound frequencies, with the base of the cochlea most sensitive to high frequency sounds and the apex to low frequency sounds (Davis, 2003). This tonotopic organization is reflected by a gradient of morphological and physiological differences along the cochlea (Davis, 2003). Since SHH function is crucial for the formation of the cochlea, an intriguing possibility is that a concentration gradient of SHH protein could be established during initiation of the cochlear outgrowth and may underlie the structural bases for the tonotopic organization that develops later.

Signals from the hindbrain play a major role in the DV axial specification of the inner ear

The present results show that rotating the DV axis of the neural tube, including the notochord is sufficient to convert presumably dorsal otic tissue to ventral fates, resulting in downregulation of dorsally expressed genes such as *Gbx2* and

upregulation of ventral specific genes such as *Lfng*, *NeuroD*, *Six1* and *Otx2* in dorsal otic tissues (Figs 6, 7). Similar results were obtained when the neural tube was rotated without the notochord (see Fig. S1 in the supplementary material), suggesting that the ventral neural tube alone is sufficient to confer the ventral inner ear fate, overriding any potential dorsalizing signals from surrounding ectodermal or mesodermal tissues. Concomitantly, most of the ventrally expressed genes, except *Otx2* are downregulated in the ventral otic tissue after DV rotation of the hindbrain (Fig. 6). It is not yet clear if ventral *Otx2* expression is sustained in the absence of SHH signaling, although in mice ventral *Otx2* expression is dependent on *Shh* (Riccomagno et al., 2002).

We did not observe upregulation of *Gbx2* in the ventral otocyst after DV axial rotation of the neural tube. We speculate that this is not because the dorsal neural tube is incapable of providing signals to confer dorsal fate to ventral otic tissue, but rather reflects the considerable increase in distance between the two tissues after rotation. This speculation is supported by the observation that *Gbx2* is upregulated in ectopically located otic tissues adjacent to the rotated dorsal neural tube. Taken together, these results suggest that even though the ectopic otic tissues are in a ventral environment, signals from the dorsal neural tube are still sufficient to upregulate a dorsally expressed gene in a ventrally located position. What might be the dorsalizing signals? In the neural tube (Mehler et al., 1997; Muroyama et al., 2002), secreted signaling molecules from the dorsal neural tube such as BMPs and WNTs are sufficient to confer dorsal fates in the neural tube as well as their adjacent somites (Fan et al., 1997; Marcelle et al., 1997). Interestingly, preliminary data suggest that WNT signaling from the dorsal neural tube might play an essential role in specification of the dorsal fate of the inner ear in mice (D. Epstein, personal communication).

Recently, *Six1* has been implicated in the ventral patterning of the inner ear in mice. Lack of *Six1* causes apoptosis of ventral otic tissues and a ventral expansion of dorsal-specific genes (Ozaki et al., 2004; Zheng et al., 2003). However, the two signaling pathways that regulate ventral inner ear patterning, *Shh* and *Six1,* appear independent. *Six1* expression in the otic tissue is not affected in *Shh*-null mutants, and the expression of SHH target genes in the otic epithelium are normal in *Six1*-null mutants (Ozaki et al., 2004). *Six1* expression in the otic epithelium is regulated by *Eya1* (Ozaki et al., 2004), although little is known about how *Eya1* expression is regulated in the inner ear. Interestingly, our data show that *Six1* expression in the otocyst is shifted after DV neural tube rotation, suggesting that the *Six1* expression in the otic tissue is also controlled by signal(s) from the neural tube.

In summary, our data show that there is a temporal difference between placode induction and axial specification in the chicken inner ear, suggesting that there may be distinct regulatory mechanisms for these two processes. Although multiple tissues appear to be capable of inducing placode formation, the specification of DV axis is mainly conferred by the hindbrain. Even though signals from specific segments of the hindbrain (R4-R6) participate in placode induction (Wright and Mansour, 2003b) and development of the inner ear at later stages (Kiernan et al., 2002), our data show that they do not play a role in conferring AP axial identity to the inner ear.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/9/2115/DC1

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