Transient retinoic acid signaling confers anterior-posterior polarity to the inner ear

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Vertebrate hearing and balance are based in complex asymmetries of inner ear structure. Here, we identify retinoic acid (RA) as an extrinsic signal that acts directly on the ear rudiment to affect its compartmentalization along the anterior-posterior axis. A rostrocaudal wave of RA activity, generated by tissues surrounding the nascent ear, induces distinct responses from anterior and posterior halves of the inner ear rudiment. Prolonged response to RA by posterior otic tissue correlates with *Tbx1* transcription and formation of mostly nonsensory inner ear structures. By contrast, anterior otic tissue displays only a brief response to RA and forms neuronal elements and most sensory structures of the inner ear.

axial specification | developmental compartments | morphogen

Normal hearing and balance require that discrete patches of mechanosensory hair cells, each with a distinct function, be precisely positioned within the asymmetric membranous labyrinth of the inner ear (Fig. 1*A*). Five vestibular sensory patches are present in all vertebrate inner ears: the three cristae (anterior, lateral, and posterior) that detect angular head movements and two maculae (utricle and saccule) that detect linear acceleration. The specialized organ for detecting sound in chickens and mammals is the basilar papilla and organ of Corti, respectively.

The entire membranous labyrinth and its innervating neurons are derived from an ectodermal thickening adjacent to the hindbrain known as the otic placode. As the placode deepens to form a cup and then pinches off to form the otocyst, some cells of the otic epithelium delaminate to form neuroblasts of the cochleovestibular ganglion (CVG). Inner ear sensory organs, and the neurons that innervate them, are thought to arise from a neural-sensory competent domain (NSD), most of which is located in the anterior region of the otic cup (1). By contrast, posterior otic epithelium forms nonsensory tissues and only one sensory organ, the posterior crista. This basic organization of functional elements in the ear is thought to be governed by signals emanating from adjacent tissues (2, 3); however, molecular mechanisms that establish the initial anterior-posterior (A-P) asymmetry of the ear primordium are poorly defined. Here, we show that a rostrocaudal wave of retinoic acid activity provides signals to the ear rudiment and establishes structural asymmetries required for normal hearing and balance.

Results

Ectoderm Adjacent to the Otic Cup Confers A-P Polarity to the Otocyst. A clear manifestation of A-P asymmetry in developing amniote ears is the anterior expression of transcripts associated with cochleovestibular ganglion neurogenesis. We performed tissue transplantations in ovo to identify source(s) of signals that specify the otic A-P axis in the chicken. Transplantations were carried out at the otic cup stage (11–15 somite stages), before the otic A-P axis is specified (4). As expected, reversing the A-P orientation of the otic cup alone resulted in a high occurrence of otocysts with the axial plan of the host (Fig. 1 *C*, *D*, and *G* and Fig. S1*A*). However, a small percentage of transplants had either a posterior duplication of the NSD (double anterior) (Fig. S1 *F* and *I*) or a single posterior NSD, suggestive of an A-P inversion (Fig. 1*G*).

We hypothesized that A-P polarity inversion was due to an unintended transfer of the donor's A-P inductive signal into the host along with responding otic tissue. Because changing the A-P axis of the hindbrain has no apparent effect on A-P patterning of the inner ear (4), we modified our transplantation protocol to include ectoderm and underlying mesoderm adjacent to the otic cup (Fig. S1*C*). This modification increased the occurrence of A-P inversion (Fig. 1*G* and Fig. S1 *E* and *H*). Similarly, an increased occurrence of A-P inversion was obtained when ectoderm but not mesoderm was included in the otic cup transplant (Fig. 1 *B* and *E*–*G*), indicating that an activity within the periotic ectoderm influences A-P patterning of the inner ear.

To identify this activity, we sought candidate genes that are asymmetrically expressed in periotic ectoderm. Retinoic acid (RA) signaling has been implicated in patterning of the hindbrain and other embryonic structures (5–7). The location of the otic placode within a gap between domains of *retinaldehyde dehydrogenase2* (*Raldh2*), the earliest and most widely expressed gene encoding a RA-synthesizing enzyme, and *Cyp26* genes (*Cyp26A1, Cyp26B1*, and *Cyp26C1*), which encode P450-associated RA-catabolizing enzymes (8, 9), suggested a possible involvement of RA signaling in specifying the otic A-P axis (Fig. 2 *A*, *B*, *D*, and *E*). Our expression analyses confirmed previous reports of *Raldh2* and *Cyp26s* in tissues adjacent to the otic epithelium and showed *Cyp26C1* to be expressed in rostral but not caudal periotic ectoderm (Fig. 2*C*) (8, 9).

Responsiveness of the Otic Epithelium to RA Changes over Time. To determine whether otic tissue responds to RA, we used the transgenic mouse strain RARE-*lacZ*—in which *lacZ* is driven by a RA responsive element (10)—to assay for reporter expression within the otic epithelium. At embryonic day (E) 8.25, the anterior border of β-gal activity lies at the border of rhombomeres 4 and 5, rostral to the location of the otic placode (Fig. 3 *A* and *B*; ref. 11). One-half day later, β-gal staining is detectable only in the posterior half of the otic cup (Fig. 3 *C*–*F*), and by E9.5, β-gal is absent from the otocyst (Fig. 3 *G* and *H*). This gradual withdrawal of RA responsiveness, first from anterior otic tissue and then from posterior otic tissue, is a likely consequence of caudally shifting boundaries of *Raldh2* and *Cyp26* expression surrounding the ear (12).

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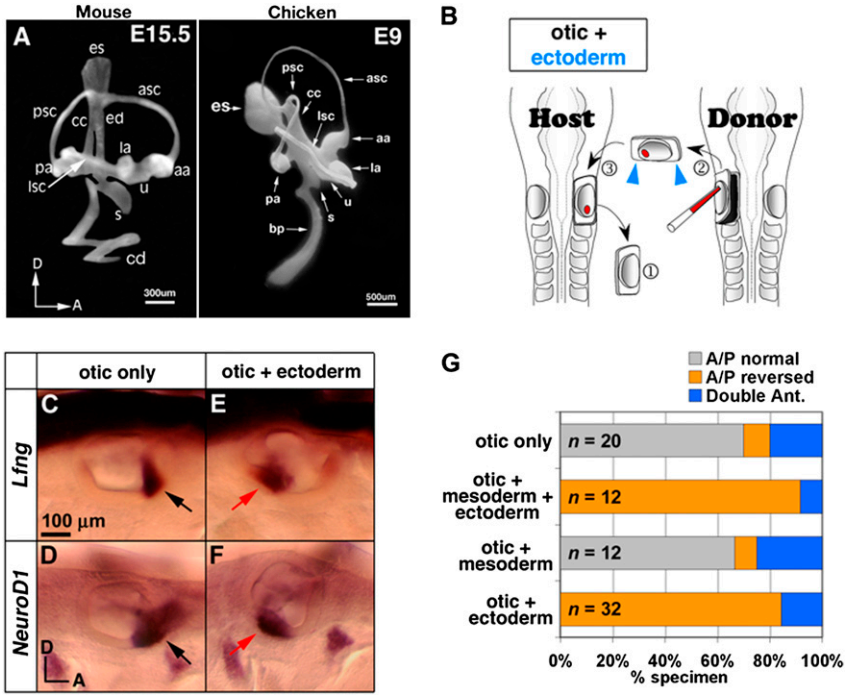

Fig. 1. (*A*) Paint-filled inner ears from mouse and chicken embryos. (*B*) The replacement of a host's right-sided otic cup with a donor's left-sided otic cup, including adjacent ectoderm (blue arrowheads). (*C* and *D*) Transplanting an otic cup alone results in normal patterning of anterior inner ear neurosensory markers, *Lfng* and *NeuroD1* (arrows). (*E* and *F*) Transplanting an otic cup plus adjacent ectoderm causes an inversion of *Lfng* and *NeuroD1* patterning (red arrows). (*G*) Percentages of samples from various surgical conditions with a normal or inverted A-P axis, or duplicated anterior domains. aa, anterior ampulla; asc, anterior semicircular canal; bp, basilar papilla; cd, cochlear duct; ed, endolymphatic duct; es, endolymphatic sac; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle.

RA Confers Posterior Identity to the Inner Ear. Administering RA to timed-pregnant RARE-*lacZ* mice at E7.75 (before otic placode formation) induced widespread *lacZ* activity throughout the embryo, including the entire otocyst, within 1 d (10). Similar administrations of RA to wild-type mice at E7.75, E8.25, and E8.5 down-regulated anterior expression of *Lunatic fringe* (*Lfng*) and *NeuroD1* in the otocyst (Fig. 4 *A*, *B*, *D*, *E*, *G*, and *H*) and caused ectopic anterior expression of *Tbx1*, which is normally expressed selectively in the posterior otic region (Fig. 4 *C*, *F*, and *I*). RA administered at the later gestational times caused less severe dysmorphology (Fig. 4 *G–I*) and fewer ears with altered gene expression patterns (Table S1). These results support the idea of a temporal window during which the ear rudiment is most sensitive to RA.

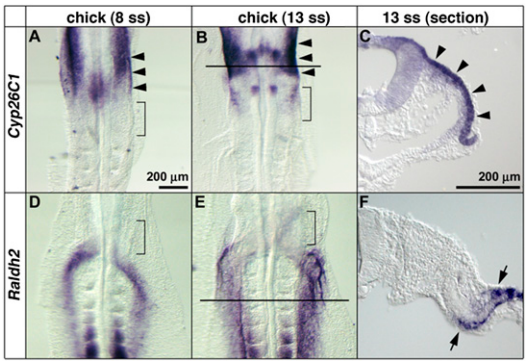

Fig. 2. Expression patterns of *Raldh2* and *Cyp26C1* in chicken embryos. *Cyp26C1* is expressed in the ectoderm anterior to the otic placode/cup region (*A–C*, arrowheads), whereas *Raldh2* is expressed in the mesodermal tissues caudal to the otic tissues such as somites and the lateral mesoderm (*D–F*, arrows). Brackets indicate the location of the otic placode/cup. Weak *Cyp26C1* expression in the otic region in *B* is associated with the mesoderm beneath the otic cup.

We next assayed for posteriorizing activity of RA on the ear rudiment of chicken embryos by implanting RA-soaked beads into the mesenchyme anterior to the otic cup at E1.5. An anterior source of exogenous RA reduced or abolished *Lfng* and *NeuroD1* expression in the otocyst (Fig. 4 *J*, *K*, *M*, and *N*) and induced ectopic expression of posterior otic genes *Tbx1* (Fig. 4 *L* and *O*) and *SOHo1* ($n = 11$) in the anterior otocyst. Implanting an RA-soaked bead posterior to the otic cup yielded similar results, indicating that otic patterning is highly sensitive to differences in effective RA concentration at a distance from the RA source. Interestingly, posterior implantation of RA-soaked beads 12–15 h later in development (E2) did not cause an expansion of the *Tbx1* expression domain, once again indicating that the competence of otic tissue to respond to RA is developmentally regulated. These results in both mouse and chicken strongly suggest that the changing patterns of RA responsiveness we characterized in the RARE-*lacZ* mouse (Fig. 3) reflect functionally relevant events in ear development.

RA bead implantations also caused posteriorization of the hindbrain, indicated by anterior expansion of genes normally expressed in the posterior hindbrain and down-regulation of genes associated with the anterior hindbrain (Fig. S2 *A–F*). To test whether the RA-induced inner ear phenotypes are indirect phenomena resulting from RA-induced changes in the hindbrain (Fig. S2 *A–F*; ref. 13), we surgically altered rhombomere patterns to mimic the molecular changes brought about by RA bead implantation. This alteration was accomplished by replacing the segment of hindbrain adjacent to the ear (r4–r6) with a block of caudal neural tissues containing r7 and spinal cord (Fig. S3). Such

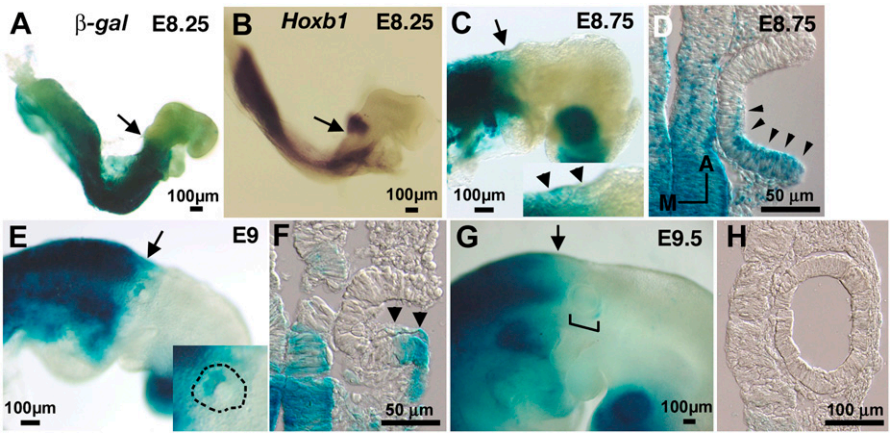

Fig. 3. β-Gal histochemical staining in RARE-*lacZ* embryos. (*A* and *C–H*) β-Gal histochemical staining at E8.25 (*A*), E8.75 (*C* and *D*), E9 (*E* and *F*), and E9.5 (*G* and *H*). Arrow in *A* indicates the anterior border of β-gal staining at E8.25, which is comparable to the r4/r5 boundary indicated in *B* (arrow) as assessed by *Hoxb1* expression. Arrows in *C*, *E*, and *G* indicate the anterior-most extent of β-gal staining, which lies at the r5/r6 boundary at E8.75 (*C Inset* and *D*) and posterior to r6 by E9.5 (*G*); arrowheads in *C*, *D*, and *F* indicate the anterior-most extent of otic epithelial β-gal staining. Otocyst: *E Inset*, dashed line, and *G*, bracket.

hindbrain operations failed to generate gene expression changes in ears similar to those with RA implantations (Fig. S3), suggesting that the effects of localized exogenous RA on inner ear development are independent of hindbrain-inner ear signaling.

If exogenous RA posteriorizes otic tissue, then blocking endogenous RA should have the opposite effect: namely, to anteriorize posterior otic tissue. Endogenous RA signaling can be blocked by using citral, an inhibitor of Raldh activity (14). Implanting a citral-soaked bead posterior to the otic cup down-regulated expression of posterior otic genes *Tbx1* and *SOHo1* (Fig. 5 *A–E*, brackets). In contrast, expression domains of the anterior genes *Lfng* and *NeuroD1* were duplicated or expanded into the posterior otic region (Fig. 5 *F–K*, red arrows). Interestingly, citral-bead implantations did not cause observable gene expression changes in the hindbrain (Fig. S2 *G–J*) (15), again suggesting that the effects of this perturbation on the inner ear are not mediated by the hindbrain.

The longer-term effects of early exogenous RA on inner ear development were determined at E7, when all gross structures are normally distinguishable (ref. 16 and Fig. 6*A*). Embryos in which an RA bead was implanted anterior to the otic cup had inner ears resembling a mirror image duplication of two posterior halves, each half consisting of a posterior-like canal and ampulla (Fig. 6 *B* and *C*). Anterior structures such as the anterior ampulla/canal, utricle, and saccule were missing (Fig. 6*A*, red labels). The lateral ampulla and canal, both of which are considered anterior structures (17), were also absent. The cochlear duct, composed of both A-P and medial-lateral components (1), was misshaped (Fig. 6 *B* and *C*). Consistent with these results, similarly treated embryos left to develop until E9 and immunostained for hair cells had inner ears with only two sensory patches resembling posterior cristae (Fig. 6 *E* and *F*).

RA Induction of Otic *Tbx1* Transcription Occurs Rapidly and Independently of Protein Synthesis. The T-box transcription factor gene *Tbx1* is implicated in the establishment of posterior otic identity (18, 19), making it a likely mediator of RA's effect on otic tissue. We therefore tested whether RA directly activates *Tbx1* transcription in otic epithelial cells, which would require that the response to exogenous RA be rapid and independent of protein synthesis. Indeed, *Tbx1* was up-regulated within 3 h of RA bead implantation (Fig. 7 *B* and *C*). Pretreating chicken embryos to inhibit protein synthesis (20) did not block this rapid RA-induced up-regulation of *Tbx1* in otic tissue (Fig. 7 *E* and *F*). In contrast, RA-induced down-regulation of mesodermal *Tbx1* in these same embryos, which has been shown to require protein synthesis (21), was blocked (Fig. 6 *C* and *F*, bracket), verifying that protein synthesis was inhibited in our experimental system. These results suggest that exposure to RA posteriorizes the rudimentary ear at least, in part, through direct transcriptional activation of *Tbx1*.

Low Concentrations of RA Are Required for Proper Anterior Gene Expression. Our finding that some critical concentration of RA is necessary and sufficient for posteriorizing the otic epithelium is consistent with gene expression analyses showing a close proximity of the otic posterior pole to mesodermal *Raldh2* expression (Fig. 2). However, developmental analyses of RARE-*lacZ* staining revealed an early, albeit brief, responsiveness of the anterior otic placode to endogenous RA (Fig. 3*A*). Furthermore, the inversion of A-P polarity achieved by rotating otic cup plus surrounding periotic ectoderm (Fig. 1 *B* and *G*) could be due to a posterior translocation of the rostral *Cyp26C1*-positive ectoderm, which might reduce the effective local RA concentration to a level suitable for stabilizing the anterior neural fate. We therefore asked whether a low concentration of RA (relative to that present posteriorly) promotes anterior otic identity. Presentation of low RA concentrations to the anterior otic epithelium during normal development could be due to distance from the mesodermal Raldh2 source, proximity to the catabolizing activity of rostral *Cyp26* gene products, or both. The activity of a catabolic "sink" may be of particular importance in controlling RA activity for ear development, given that RA synthesis unrelated to known sites of *Raldh1-3* expression has been reported in the neural tube anterior to the otic placode (22). We therefore sought to reduce the effective concentration of endogenous RA near the anterior otic cup by rostral implantation of a citral bead. This implantation resulted in a near complete loss of the anterior neurosensory marker *Lfng* and down-regulation of *NeuroD1* (Fig. 5 *L* and *M*), indicating that some effective concentration of RA activates or potentiates gene expression associated with anterior otic identity.

Discussion

Retinoic Acid Specifies the A-P Axis of the Inner Ear. In invertebrates, compartments and boundaries are thought to drive pattern formation. Cells within the embryo and primordial larval structures such as the wing imaginal disk of *Drosophila* are organized into compartments based on positional information in the form of morphogen gradients. Each compartment is then stabilized by the

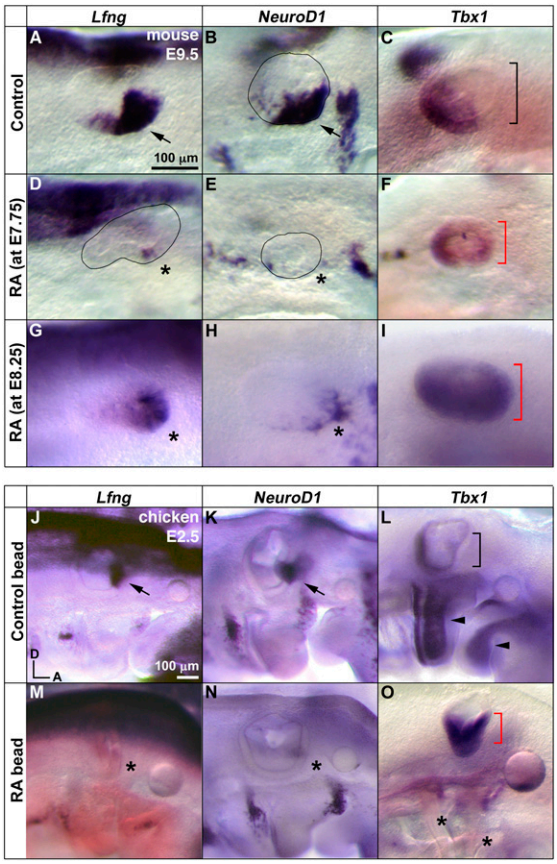

Fig. 4. RA signaling confers posterior identity to the inner ear. (*A–F*) Administering RA to pregnant mice at E7.75 affects the size of the otocyst, down-regulates anterior neurosensory markers *Lfng* (*D*, asterisk; *n* = 5) and *NeuroD1* (*E*, asterisk; *n* = 6), and up-regulates *Tbx1* anteriorly (*F*, bracket; *n* = 3), compared with controls at E9.5 (*A–C*). Similar gene expression changes are observed less frequently and with less severe dysmorphology when RA is administered at E8.25 (*G–I*). (*J–O*) Implantation of an RA bead in mesoderm anterior to the otic cup in chicken causes down-regulation of *Lfng* (*M*, asterisk; *n* = 16) and *NeuroD1* (*N*, asterisk; *n* = 15), and up-regulation of *Tbx1* anteriorly (*O*, bracket; *n* = 6) in comparison with controls wherein beads are soaked with DMSO alone (*J–L*). *Tbx1* expression in the branchial mesoderm is down-regulated in response to exogenous RA (*L* and *O*, asterisks).

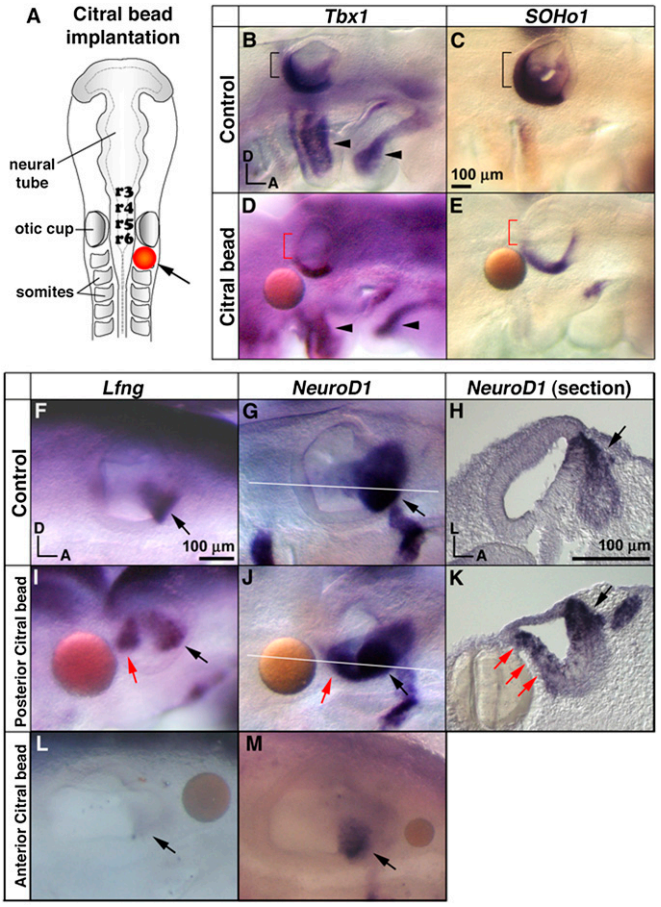

Fig. 5. Effects of implanting a citral bead posterior (*A–K*) or anterior (*L* and *M*) to the otic cup. (*A*) Posterior citral-bead (red) implantation diagram. (*B–E*) Posterior citral-bead implantation causes down-regulation of *Tbx1* (*D*, bracket; *n* = 5/10) and *SOHo1* (*E*, bracket; *n* = 6), compared with controls (*B* and *C*). No detectable change is seen in *Tbx1* expression in the branchial mesoderm (arrowheads). (*F–K*) Posterior citral-bead implantation causes ectopic expression of *Lfng* (*I*; *n* = 5/6) and *NeuroD1* (*J* and *K*; *n* = 11/18) in the posterior otocyst (red arrows). (*L* and *M*) Anterior citral-bead implantation causes down-regulation of *Lfng* (*L*, arrow; *n* = 16/18) and *NeuroD1* (*M*, arrow; *n* = 14/17).

activities of its intrinsic selector genes, which instruct cells as to their fate and how to interact with cells in adjacent compartments (23). A similar hypothesis of compartments and boundaries has been proposed for pattern formation of the inner ear (24).

In the early 1900s, mirror image "twinned" ears of either double anterior or double posterior identity were described as resulting from surgical rotations of the presumptive ear ectoderm in salamanders (25). These mirror image duplications suggest that the inner ear rudiment is at first equipotential along the A-P axis and later compartmentalized about its A-P midline. In recent years, similar mirror image duplications of inner ears in zebrafish and frogs have also been reported from perturbing hedgehog (hh) signaling (26, 27). Paradoxically, hedgehog signaling does not appear to be a primary determinant for A-P patterning of the inner ear in amniotes, even though it is essential for the dorsal-ventral (D-V) patterning (4, 28, 29). Although it remains unclear how a continuous source of hedgehog emanating from the ventral midline can impart A-P characteristics to inner ears of anamniotes, more recent data indicate that hh is also required for D-V patterning of the zebrafish inner ear, which underscores the similarities of inner ear patterning among vertebrates (30).

Our use of a localized source of exogenous RA to elicit a double posterior ear strongly supports the notion that RA is a key morphogen for patterning A-P compartments of the inner ear in amniotes. Perturbing RA signaling during a critical period of A-P specification affected A-P identity of the inner ear.

Dynamic RA Signaling May Pattern Multiple Cranial Structures in Parallel. The "source and sink" configuration of RA synthesis (caudal mesoderm) and RA degradation (rostrally in the neural tube and ectoderm) is an excellent model for explaining how signals that establish anterior and posterior compartments of the inner ear are generated (Fig. 7*G*). A critical feature of this model is its dynamism, with both synthetic and catabolic activity shifting caudally along the early embryo (9, 12). We describe here two results to suggest that A-P otic compartmentalization is effected within a limited time window by this dynamic process. First, we have used the RARE-*lacZ* reporter mouse to show a developmentally regulated withdrawal of RA responsiveness from the anterior and later from posterior otic epithelium. Second, we find in both chicken and mouse that the potency of exogenous RA to alter otic gene expression diminishes with advancing gestational age. Similar RA signaling dynamics are proposed to

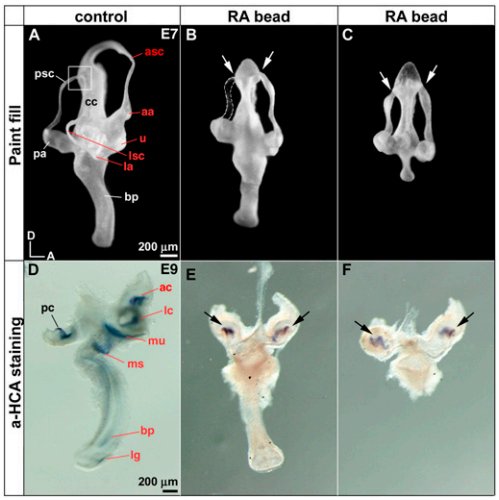

Fig. 6. Paint-filled chicken inner ears. Anterior structures (*A*, red labels) fail to develop in RA-treated ears, and only two posterior canal-like structures are evident (*B*, n = 5/9; *C*, n = 4/9). Box in *A* highlights the normal posterior canal's more ventral point of insertion into the common crus relative to the anterior canal's insertion and is comparable to regions highlighted by arrows in *B* and *C*. (*D*) Whole-mount anti-HCA staining of chicken inner ear at E9 showing locations within the membranous labyrinth of the auditory sensory organ, the basilar papilla (bp) and six vestibular sensory organs: anterior crista (ac), lagena (lg), lateral crista (lc), utriculi (mu), sacculi (ms), and posterior crista (pc). (*E* and *F*) RA-treated chicken ears lack all sensory organs except for two cristae, each associated with a posterior-like canal (n = 5).

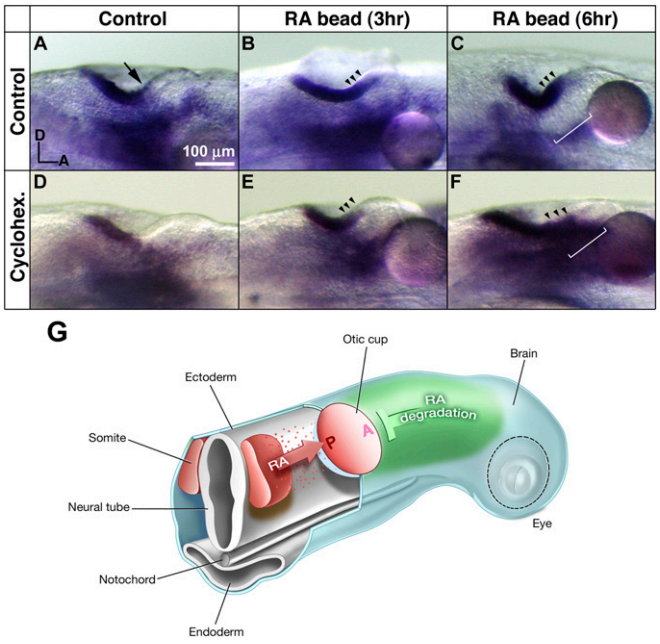

Fig. 7. RA induces otic *Tbx1* transcription in the presence of cycloheximide. (*A*–*C*) Ectopic *Tbx1* transcription in the anterior otic cup is observed within 3 h (*B*, arrowheads; n = 9/9) and 6 h (*C*, arrowheads; n = 12/12) of RA bead implantation. *Tbx1* expression is down-regulated in mesoderm at 6 h after RA bead implantation (*C*, bracket). (*D*–*F*) Ectopic *Tbx1* transcripts in the anterior otic cup within 3 h (*E*, arrowheads; n = 12/15) and 6 h (*F*, arrowheads; n = 17/19) of RA bead implantation in embryos pretreated with the protein synthesis blocker cycloheximide. Cycloheximide blocks the protein synthesis-dependent down-regulation of mesodermal *Tbx1* by RA (n = 17/19; compare brackets in *F* and *C*). (*G*) Summary diagram of the otic epithelium in relation to the source and sink configuration of RA synthesis and degradation enzymes, which is a key component in establishing the A-P axis of the inner ear.

specify the identity of anterior rhombomeres (12). More recent data in zebrafish suggest that the effects of RA signaling along the A-P axis of the body may not depend on simple diffusion of RA molecules from a posterior source alone, but rather on the complex regulation of genes involved in RA metabolism, in particular the *Cyp26s* (31). Regardless of the mechanisms involved in regulating RA signaling, our demonstration of the direct effects of RA on *Tbx1* expression in the otic epithelium and analyses of surgical hindbrain alterations (Fig. S3; ref. 4) strongly suggest that—at least in chicken—RA signaling patterns the hindbrain and inner ear independently of each other. It is possible that a wave of RA signaling coordinates the formation of multiple organs at these axial levels, and other structures such as the branchial arches and epibranchial placodes may rely on this dynamic regulation (32).

Perturbation of RA signaling at stages earlier than those used in the experiments reported here, as in vitamin-A–deficient quails and *Raldh2* knockout mice, affects the axial location of the otic placode (33, 34). These studies, taken together with results presented here, suggest that a dynamic source and sink configuration of RA regulation first specifies where the otic placode will be positioned along an animal's A-P axis and later specifies the A-P axis of the ear rudiment itself.

Molecular Mechanisms by Which RA Patterns the Otic A-P Axis. The stepwise and developmentally regulated withdrawal of RA responsiveness we have observed indicates that direct effects of RA on otic transcription are maintained for a longer period in the posterior than in the anterior region, a difference that could underlie the divergence of these otic regions into functionally distinct inner ear structures. Thus, a low RA concentration or short exposure should induce an anterior, neurosensory fate, whereas a high concentration or longer exposure of RA induces a posterior, largely nonsensory fate. Neurosensory genes directly regulated by RA in the anterior otic region remain to be determined. Recent data suggest that the anterior neurogenic fate in chicken depends on Fgf8 and Sox3 (35).

We have provided evidence for a direct effect of RA signaling on transcription of *Tbx1*, a gene that has been implicated in promoting posterior otic identity. In mice, lack of *Tbx1* causes a posterior expansion of the anterior neurosensory domain and duplication of the CVG, whereas transgenic overexpression of human *TBX1* results in a neurogenic domain and ganglion of reduced size (19). Results presented here indicate that at least some of RA's effects on A-P patterning are mediated by the activities of Tbx1.

In summary, RA, which is a well-known morphogen for somitogenesis, heart morphogenesis, and branchial arch patterning (7, 32, 36, 37), is also an essential determinant of A-P patterning for the amniote inner ear. The detailed mechanism by which RA confers A-P identity and promotes diverse otic fates at different concentrations/exposure durations demands further investigation and may prove to be of value to emerging techniques involving the use of pluripotent stem cells as a therapeutic approach to alleviate sensorineural hearing loss (38, 39).

Materials and Methods

Microsurgical Manipulations of Chicken Embryos. Fertilized eggs (CBT Farms) were incubated in a humidified chamber at 37 °C. Chicken embryos between E1.5 and E2 [equivalent to 11–22 somite stages (ss) or Hamburger Hamilton stage 11–14 (HH 11–14)] were used (40).

Otic tissue transplantation. Otic tissue transplantation procedures were performed as described with minor modifications (4). A right otic cup or otic cup with adjacent mesoderm and/or ectoderm (Fig. 1*B* and Fig. S1 *A*–*C*) from a host embryo was replaced with comparable left otic tissues of an age-matched donor. The otic cup was rotated such that the AP axis was reversed

relative to the host, but the other axes (D-V and medio-lateral) were unchanged. Before transplantation, 0.05% CM-DiI (Molecular Probes) in 300 mM sucrose solution was injected into the anterior region of the otic cup of the donor for orientation and tracking. Only embryos with appropriately transplanted tissues were used for further analyses.

Bead implantation and cycloheximide pretreatment. Bead implantation was carried out as described with minor modifications (41, 42). For delivery of RA (Sigma), AG1-X2 beads (Bio-Rad) were soaked in 0.5 mg/mL RA. For delivery of Citral (an inhibitor of retinaldehyde dehydrogenases; Sigma), SM2 beads (Bio-Rad) were soaked in 0.4 g/mL Citral solution diluted in DMSO. Anterior bead implantations were conducted by making a slit in the ectoderm rostral to the right otic cup, at the level of rhombomere 3/4 boundary, whereas posterior bead implantations were performed by making an incision in the ectoderm between the posterior otic cup and the first somite. A single bead soaked with specific reagents was pressed down into the slit by using the tip of a forcep, and implanted embryos were further incubated and harvested for whole mount in situ hybridization at E2.5–E3, paint fill analysis at E7, or anti-HCA (hair cell antigen) staining at E9 (17).

To inhibit protein synthesis, cycloheximide solution (2 mg/50 mL Tyrode's solution) was applied onto the chorioallantoic membrane of chicken embryos 2 h before bead implantations. Control embryos received 50 mL of Tyrode's solution alone.

RARE-*LacZ* Mice and RA Administration. RARE-*lacZ* mouse strain was generated by J. Rossant (10). RA solution emulsified in corn oil was administered to mice by gavage (50 mg/kg of body weight) between E7.75 and E8.5. Embryos were harvested at E9.5 and analyzed by whole-mount in situ hybridization or β-galactosidase histochemical staining. All animal procedures were approved and conducted according to the National Institutes of Health Animal Use and Care Committee guidelines.

Whole-Mount in Situ Hybridization and β-Galactosidase Staining. Whole-mount in situ hybridization and β-galactosidase histochemical staining were carried out as described (10, 43). Details of probes used are available upon request.

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