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Spatiotemporal Expression of Zic Genes During Vertebrate Inner Ear Development

Andrew P. Chervenak,[1,2] Ibrahim S. Hakim,[1] and Kate F. Barald[1–3]*

Background: Inner ear development involves signaling from surrounding tissues, including the adjacent hindbrain, periotic mesenchyme, and notochord. These signals include SHH, FGFs, BMPs, and WNTs from the hindbrain and SHH from the notochord. *Zic* genes, which are expressed in the dorsal neural tube and act during neural development, have been implicated as effectors of these pathways. This report examines whether *Zic* genes' involvement in inner ear development is a tenable hypothesis based on their expression patterns. Results: In the developing inner ear of both the chick and mouse, all of the *Zic* genes were expressed in the dorsal neural tube and variably in the periotic mesenchyme, but expression of the *Zic* genes in the otic epithelium was not found. The onset of expression differed among the *Zic* genes; within any given region surrounding the otic epithelium, multiple *Zic* genes were expressed in the same place at the same time. Conclusions: *Zic* gene expression in the region of the developing inner ear is similar between mouse and chick. *Zic* expression domains overlap with sites of WNT and SHH signaling during otocyst patterning, suggesting a role for *Zic* genes in modulating signaling from these pathways. *Developmental Dynamics 242:897–908, 2013*. © **2013 Wiley Periodicals, Inc.**

Key words: *Zic* gene expression; inner ear; embryogenesis; mouse; chick

Key Findings:

- *Zic* genes are expressed in the dorsal neural tube and mesenchyme surrounding the developing inner ear in both mouse and chick.
- *Zic* genes are not expressed in the otic epithelium of either mouse or chick.
- Differential spatiotemporal expression of *Zic* genes (*Zic*1-5 in mouse, *Zic*1–4 in chick) is seen during inner ear development.

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INTRODUCTION

The *Zic* (zinc finger of the cerebellum) genes comprise a family of transcription factors found in both vertebrates and invertebrates (reviewed in Ali et al., 2012) that are related to the *Drosophila Zic* homologue, *odd-paired* (*opa*) (Nusslein-Volhard and Wieschaus, 1980; Benedyk et al., 1994). Among vertebrate species, humans and mice have five *Zic* genes (*Zic1–5*); other species have more (zebrafish, 8 *Zic* genes; frog, 6 *Zic* genes) or fewer (chicken, 4 *Zic* genes) *Zic* genes. Structurally, ZIC proteins are characterized by a zinc finger region that contains five tandem C_2H_2 zinc finger domains (Aruga, 2004; Merzdorf, 2007; Ali et al., 2012). The amino acid sequence of zinc fingers 2 though 5 is almost identical among *Zic1–5* for a given species, while the sequence outside of this region can be highly variable (Aruga, 2004; Grinberg and Millen, 2005).

The *Zic* genes are involved in a multitude of developmental processes. *Zic* gene function has predominantly been studied during neural

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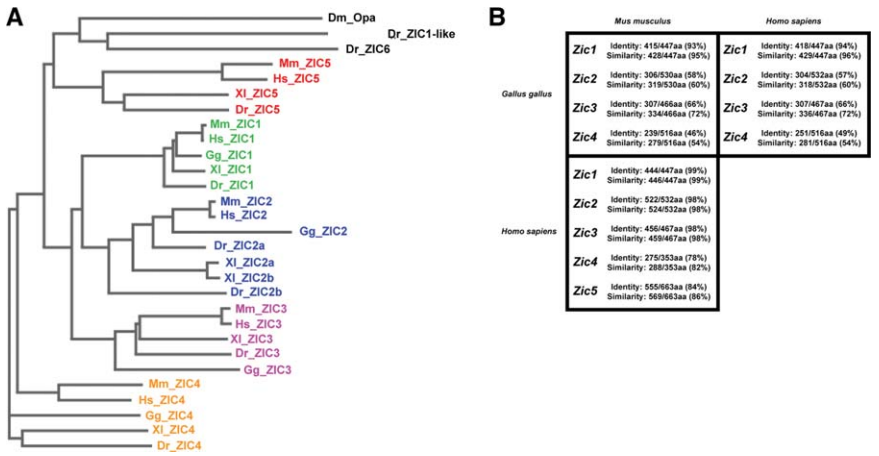

Fig. 1. Comparison of *Zic* genes across species. **A:** Phylogenetic tree showing the relationships among the *Zic* genes from human, mouse, chick, zebrafish, and frog, as well as the ancestral gene *odd-paired* (*opa*) from *Drosophila*. The complete amino acid sequences were used for the construction of the tree. **B:** Percent identity and similarity of the amino acid sequences among the mouse, chick, and human ZIC proteins. The chick ZIC2, ZIC3, and ZIC4 protein sequences have not been finalized, so comparisons to these protein sequences result in much lower percent identity and similarity. Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*.

development, where it has roles in controlling neural patterning, generation of neural crest, cerebellar development, and the formation of the neural tube (Aruga, 2004; Merzdorf, 2007). However, *Zic* genes also function in somite myogenesis (Pan et al., 2011), left-right asymmetry (Herman and El-Hodiri, 2002), and retinal development. Although there have been a number of studies focused on *Zic* gene function, the exact role these genes play in vivo has yet to be elucidated. In vitro, ZIC2 inhibits WNT/β-catenin signaling through a direct interaction with TCF4 (Pourebrahim et al., 2011) and ZIC proteins interact with GLI transcription factors to either suppress or enhance GLI-mediated transactivation (Koyabu et al., 2001; Mizugishi et al., 2001; Pan et al., 2011). In *Xenopus, Zic* genes both activate and inhibit WNT/β-catenin signaling (Merzdorf and Sive, 2006; Pourebrahim et al., 2011; Fujimi et al., 2012), and in zebrafish *Zic* genes regulate expression of *shh* and *nodal* and modulate Hedgehog-mediated gene expression (Maurus and Harris, 2009; Sanek et al., 2009).

Given the importance of *Zic* gene expression in the neural tube, as well as the ability of *Zic* genes to modulate WNT and SHH signaling, key pathways known to be involved in dorsoventral patterning of the inner ear (Riccomagno et al., 2002, 2005), surprisingly little attention has been paid to *Zic* gene expression and function in and around the developing inner ear. One early study identified *Zic2* as one of the first genes up-regulated in the regenerating sensory epithelium of the chicken inner ear after noise exposure (Gong et al., 1996). Conflicting data exist regarding the location of *Zic* gene expression during inner ear development (Warner et al., 2003; McMahon and Merzdorf, 2010). Although some *Zic* expression patterns have been described in the mouse (reviewed in Merzdorf, 2007; Ali et al., 2012), to date *Zic* expression in the developing mouse inner ear has not been examined. Given the discrepancies in the *Zic* gene expression patterns described in the earlier studies, we have undertaken a more extensive comparative spatiotemporal analysis of *Zic* mRNA expression in the otic regions of both the chick and mouse during the early stages of inner ear development.

RESULTS

Evolutionary Conservation of the *Zic* Genes Across Species

Phylogenetic analysis of ZIC proteins from mouse (ZIC1–5), human (ZIC1–5), chicken (ZIC1–4), zebrafish (Zic1/Opl, Zic1-like, Zic2a and b, and Zic3–6), frog (Zic1, Zic2a and 2b, and Zic3–5), and fly (Odd-paired; OPA) indicate significant conservation among species (Fig. 1A). ZIC proteins from across species clustered together into five groups, with each representing one of the 5 *Zic* genes. In addition to these 5 clusters, Zic6 and Zic1-like from zebrafish grouped together with the ancestral protein from the fly, odd-paired, into a 6th cluster. The ZIC1, ZIC2, and ZIC3 clusters group together, the ZIC5 and odd-paired clusters group together, and the ZIC4 proteins form two groups (mouse/human, and chick/frog/fish) that cluster together away from all of the other Zics. Within each of the five major clusters (ZIC1–5), the mouse and human genes are closely related to one another, and more distantly related to those from chicken, zebrafish, and frog.

Identity and similarity comparisons among the complete amino acid sequences of the mouse, human, and chicken ZIC proteins indicate that the mouse and human ZIC1, ZIC2, and ZIC3 proteins are almost identical, but the ZIC4 and ZIC5 proteins differ slightly (Fig. 1B). Chicken Zic1 is very similar to both mouse and human ZIC1 proteins, but chicken Zic2–Zic4 differ greatly from mouse and human ZIC2–4, with Zic4 being the most different. Zic1–3 are the most similar between chicken and mouse and human, while Zic4 is not as conserved. ZIC1–3 contain a Zic-opa conserved (ZOC) domain, resulting in a higher level of homology among these proteins, while ZIC4 and ZIC5 do not. The ZOC domain is required for activation of target gene transcription in vitro, as well as for binding I-mfa, a myogenic repressor protein (Mizugishi et al., 2004). Further, the intron-exon boundaries are conserved between ZIC1–3, but not between ZIC1–3 and either ZIC4 or ZIC5 (Grinberg and Millen, 2005). This suggests that the *Zic4* and *Zic5* genes are the most divergent and may have evolved novel functions. ZIC1 is almost identical in mouse, human, and chicken and, therefore, may have the same function in all three organisms. ZIC2 and ZIC3 are almost identical between mouse and human, but differ when compared to chick Zic2 and Zic3, suggesting that they may have different functions in birds and mammals.

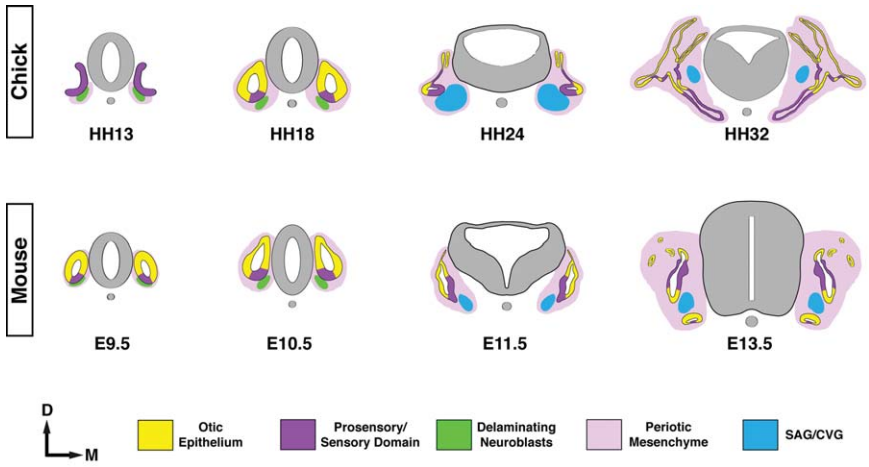

Fig. 2. Inner ear development in chicken and mouse. Schematic depicting inner ear development in chicken from stage 13 to stage 32 (top row) and in mouse from E9.5 to E13.5 (bottom row). Gray shaded regions correspond to the neural tube and the notochord. Images are based upon transverse sections through the middle of the otocyst, but are not to scale. SAG, statoacoustic ganglion; CVG, cochleovestibular ganglion; D, dorsal; M, medial.

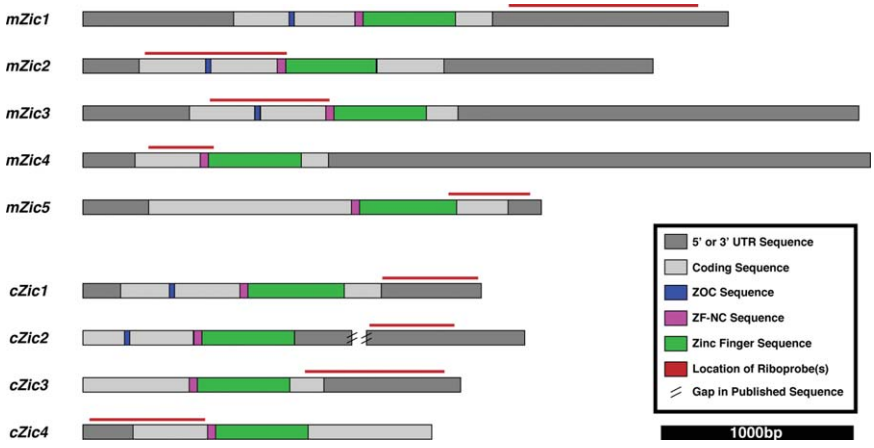

Fig. 3. Location of riboprobes within the mRNA sequence of the *Zic* genes. Diagrams of the mRNA sequence for each *Zic* gene illustrate the location of the coding sequence (light gray boxes), UTRs (dark gray boxes), and functional domains (blue, magenta, and green boxes) within each of the *Zic* genes. Probes for each of the *Zic* genes in mouse (*Zic1–5*) and chick (*Zic1–4*) were designed to target the less-conserved 5′ and 3′ UTR regions of each gene (red lines above the mRNA diagram show the location of the riboprobes). The published sequences for chick *Zic2–4* are still being annotated and updated, so the most 5′ and 3′ untranslated regions of the mRNA have not been defined. UTR, untranslated region; ZOC, Zic-opa conserved domain; ZF-NC, zinc-finger nucleocapsid domain.

Comparison of Otic Development in Chick and Mouse

The early stages of inner ear development are similar in both the chick and in the mouse (summarized in Fig. 2), and require signaling from different tissues to specify and pattern the otic placode (underlying mesoderm), otocyst (mesenchyme, neural tube, notochord), and inner ear (mesenchyme, neural tube, notochord). Initially, cells in the dorsal ectoderm adjacent to rhombomeres 5 and 6 of the hindbrain thicken, forming the otic placode [HH stage 10 in chick (Hamburger and Hamilton, 1992), embryonic day 8 to 8.5 (E8–E8.5) in mouse]. Subsequently, the placode invaginates to form the otic cup, and neuroblasts begin to delaminate from the ventral region of the otic cup and migrate away to form the cochleovestibular ganglion (CVG)/statoacoustic ganglion (SAG) (HH stage 13 in chick, E8.75–E9 in mouse). The otic cup closes completely and pinches off from the overlying ectoderm, forming the otic vesicle/otocyst (HH stage 17 in chick, E9.5 in mouse). Over the next several days, the otocyst undergoes complex morphological changes, transforming into a complex structure comprised of three semicircular canals, the utricle, the saccule, and the cochlea (mouse)/basilar papilla (chick).

Expression of the *Zic* Genes During Otic Development in Chick and Mouse

We used in situ hybridization to examine the expression of the *Zic* genes (*Zic1–4* in chick, *Zic1–5* in mouse; refer to Fig. 3 for the location of the riboprobes within each gene) in the hindbrain and in the adjacent region of the developing inner ear in the chick (HH stage 13 to HH stage 32) and in the mouse (E9.5 to E13.5). *Pax2* expression in this region, which has previously been reported for both chick (Hutson et al., 1999; Hidalgo-Sanchez et al., 2000; Sanchez-Calderon et al., 2002; Li et al., 2004; Sanchez-Calderon et al., 2005) and mouse (Nornes et al., 1990; Puschel et al., 1992; Rinkwitz-Brandt et al., 1995, 1996; Lawoko-Kerali et al., 2002; Burton et al., 2004), is included in the same series of in situ hybridizations as an internal control for otic epithelial gene expression.

Expression of *Zic* Genes in the Developing Chick Inner Ear

At HH stage 13, *Pax2* was expressed throughout the epithelium of the otic cup but not in the adjacent neuroepithelium of the developing neural tube (Fig. 4A). By HH stage 18, the otic cup has closed to form the otocyst (Fig. 2, top row) and *Pax2* expression was restricted to the medial and ventral walls of the otic epithelium (Fig. 4F). At this stage, *Pax2* expression was also detected in the ventral neural tube. Between HH stages 18 and 24, the spherical otocyst elongates to form the early inner ear with identifiable dorsal (endolymphatic duct and sac) and ventral (basilar papilla)

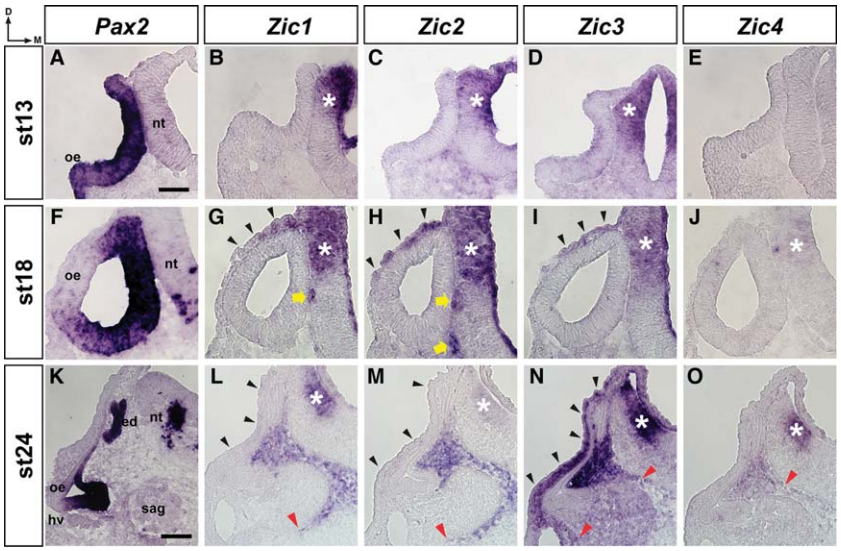

Fig. 4. *Zic* expression in the otic region of early stage chick embryos. In situ hybridization on 12-μm transverse sections through the otocyst of stage-13 (**A–E**), stage-18 (**F–J**), and stage-24 (**K–O**) chick embryos using probes for *Pax2* (A, F, K), *Zic1* (B, G, L), *Zic2* (C, H, M), *Zic3* (D, I, N), and *Zic4* (E, J, O). Note that *Pax2* is expressed in sensory regions of the otic epithelium, while *Zic* gene expression is restricted to the surrounding mesenchyme. oe, otic epithelium; ed, endolymphatic duct; nt, neural tube; sag, statoacoustic ganglion; hv, head vein; D, dorsal; M, medial. Asterisks identify *Zic* expression in the neural tube (B, C, D, G, H, I, J, L, M, N, O). Black arrowheads indicate *Zic*-expressing cells between the outer epithelium and the otic epithelium (G, H, I, L, M, N). Yellow arrows indicate *Zic*-expressing cells between neural tube and otic epithelium (G, H). Red arrowheads indicate the ventral-most extent of *Zic* expression in the mesenchyme (L, M, N, O). Scale bar in A = 50 μm (applies to A–J); scale bar in K = 100 μm (applies to K–O).

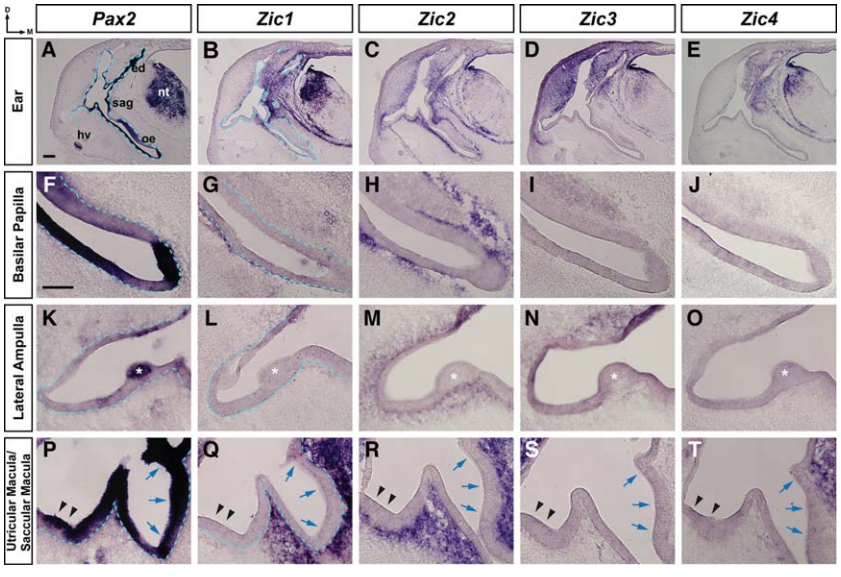

Fig. 5. *Zic* expression in the otic region of stage-32 chick embryos. In situ hybridization on 25-μm transverse sections through the otocyst of stage-32 chick embryos using probes for *Pax2* (**A, F, K, P**), *Zic1* (**B, G, L, Q**), *Zic2* (**C, H, M, R**), *Zic3* (**D, I, N, S**), and *Zic4* (**E, J, O, T**). Expression at low magnification in the ear (A–E), and at higher magnification in the basilar papilla (F–J), Lateral ampulla (K–O) and utricular/saccular maculae (P–T). Note that *Pax2* is expressed in sensory regions of the otic epithelium, while *Zic* gene expression is restricted to the surrounding mesenchyme. In L–N, the otic epithelium at the upper right corner is darkened as a result of tissue folding. oe, otic epithelium; ed, endolymphatic duct; nt, neural tube; sag, statoacoustic ganglion; hv, head vein; D, dorsal; M, medial. Asterisks identify the lateral ampulla (K–O). Black arrowheads indicate the utricular macula and blue arrows denote the saccular macula (P–T). Blue dashed lines define border between the otic epithelium and the mesenchyme (first two columns). Scale bar in A = 200 μm (applies to A–E); scale bar in F = 100 μm (applies to F–T).

structures (top row, Fig. 2). *Pax2* expression at HH stage 24 (Fig. 4K) was further restricted to the endolymphatic duct and prosensory regions of the otic epithelium, and was maintained in the ventral neural tube. In contrast to *Pax2*, expression of the *Zic* genes was not detected in the otic epithelium at HH stages 13, 18, or 24 (Fig. 4). Expression of *Zic1–3* was detected in the dorsal neural tube at HH stages 13, 18, and 24, but *Zic4* expression was not detected in the dorsal neural tube until HH stages 18 (weak) and 24 (asterisks in Fig. 4B, C, D, G, H–J, L, M–O). Following otocyst closure at HH stage 18, *Zic1–3* expression was observed laterally around the outside of the otocyst (black arrowheads, Fig. 4G–I), and *Zic1* and *Zic2* expression was detected medially between the otic epithelium and the neural tube (yellow arrows, Fig. 4G, H). By HH stage 24, expression of *Zic1–4* was observed medially between the developing inner ear and the neural tube, with the ventral extent of expression varying among the *Zic* genes (Fig. 4L–O; the red arrowheads indicate the ventral extent of *Zic*-expressing cells). Weak *Zic1* and *Zic2* expression, as well as strong *Zic3* expression, was observed lateral to the developing inner ear (black arrowheads, Fig. 4L–N).

Between HH stages 24 and 32, the developing inner ear has undergone further morphological changes, resulting in an inner ear with clearly defined structures with identifiable prosensory domains (Fig. 2, top row; Fig. 5). *Pax2* was expressed in the endolymphatic duct and the neuroepithelium of the neural tube (Fig. 5A; blue dashed line marks border between otic epithelium and the surrounding mesenchyme) and in the emerging sensory patches within the otic epithelium of the basilar papilla (Fig. 5F), the crista of the lateral semicircular canal (asterisk, Fig. 5K), the utricular macula (arrowheads, Fig. 5P), and the saccular macula (blue arrows, Fig. 5P). *Zic1–4* expression was not detected in the otic epithelium at HH stage 32 (Fig. 5). However, *Zic1–4* expression was detected in the dorsal neural tube and adjacent to the otic epithelium (Fig. 5B–E; a blue dashed line in Fig. 5B marks the border between the otic

epithelium and the surrounding mesenchyme). *Zic2* was the only *Zic* gene expressed surrounding the entire otic epithelium, including the developing basilar papilla (Fig. 5H), lateral semicircular canal (Fig. 5M), and the utricular and saccular maculae (Fig. 5R). Expression of the other *Zic* genes was restricted to the mesenchyme adjacent to specific regions of the otic epithelium. *Zic1* expression was limited to the dorsomedial region of the mesenchyme between the otic epithelium and neural tube, but was also detected adjacent to a portion of the ventral basilar papilla (Fig. 5G), next to the dorsolateral wall of the ear (Fig. 5L), and surrounding the saccular macula but not the utricular macula (Fig. 5Q). *Zic3* expression was restricted to cells surrounding the dorsal region of the developing inner ear, including cells adjacent to the dorsal wall of the lateral semicircular canal (Fig. 5N), but was not found in cells adjacent to the basilar papilla (Fig. 5I) or the utricular and saccular maculae (Fig. 5S). *Zic4* had the most restricted expression pattern, expressed in the mesenchyme in the dorsomedial region between the otic epithelium and neural tube, including in cells adjacent to the dorsomedial portion of the saccular macula (Fig. 5T), but not in cells adjacent to the basilar papilla (Fig. 5J) or in cells adjacent to the lateral semicircular canal (Fig. 5O).

Expression of *Zic* Genes in the Developing Mouse Inner Ear

At E9.5, *Pax2* was expressed in the ventromedial wall of the otocyst but not in the adjacent neuroepithelium of the developing neural tube of the mouse (Fig. 6A). By E10.5, the otocyst has begun to elongate (Fig. 2, bottom row) and *Pax2* expression remained in the lateral and ventromedial wall of the otocyst (Fig. 6G). Between E10.5 and E11.5, the otocyst undergoes further morphological changes, elongating along its dorso-ventral axis and becoming compacted along its medio-lateral axis, forming the endolymphatic duct at the dorsal end and the start of the cochlear duct at the ventral end (Fig. 2, bottom row). At E11.5, *Pax2* was expressed in the neural tube, in the endolymphatic duct, and in the developing sensory patches of the otic epithelium, but not in the mesenchyme surrounding the developing inner ear (Fig. 6M; a blue dashed line marks the border between the otic epithelium and the surrounding mesenchyme). In contrast to *Pax2*, expression of *Zic1–5* was not detected in the otic epithelium at E9.5, E10.5, or E11.5 (Fig. 6). *Zic1–5* expression was seen in the dorsal neural tube at E9.5, E10.5, and E11.5 (asterisks in Fig. 6B–F, H–L, N–R). *Zic1- and Zic2*-expressing cells were found in the mesenchyme between the ventral neural tube and the otic epithelium and adjacent to the dorsolateral side of the otocyst at E9.5 and E10.5, although the *Zic2*-expressing cells were not seen as far ventrally as were the *Zic1*-expressing cells (yellow arrow and black arrowheads, Fig. 6B, C, H, I). *Zic3*- and *Zic4*-expressing cells were also found adjacent to the dorsolateral side of the otocyst at E10.5 (black arrowheads, Fig. 6J, K). By E11.5, *Zic1* expression was seen in cells located medially between the otic epithelium and the neural tube (Fig. 6N; a blue dashed line marks the border between otic epithelium and the surrounding mesenchyme), while *Zic2*- and *Zic5*-expressing cells surrounded the entire otic epithelium, although Zic5 was expressed more strongly adjacent to the dorsal region of the otic epithelium (Fig. 6O, 6R). *Zic3* and *Zic4* expression was restricted to the mesenchyme surrounding the dorsal half of the otic epithelium, with *Zic3* expression extending further ventrally than *Zic4* on the lateral side of the otic epithelium and *Zic4* expression extending further ventrally than *Zic3* on the medial side of the otic epithelium (Fig. 6P, Q; red arrowheads denote the ventral extent of expression).

At E12.5 and E13.5, *Pax2* was expressed in the neural tube, endolymphatic duct, and the developing sensory patches of the otic epithelium (Fig. 7A, G; a blue dashed line marks the border between the otic epithelium and the surrounding mesenchyme), including the developing cochlear duct (Fig. 7A', G'), but was not found in the mesenchyme surrounding the developing inner ear. *Zic1–5* were expressed in the dorsal neural tube (data not shown), but in contrast to *Pax2*, they were not expressed in the otic epithelium (Fig. 7B–F, B'–F', H–L, H'–L'). *Zic1* was expressed in cells in the mesenchyme adjacent to all portions of the otic epithelium except for the ventral portions of the ear and the CVG (Fig. 7B, B', H, H'; a blue dashed line marks the border between the otic epithelium and the surrounding mesenchyme). *Zic2* and *Zic5* expression was seen in cells in the mesenchyme that surrounded the entire otic epithelium, including the cochlea, although the expression of *Zic5* was much weaker (Fig. 7C, C', F, F', I, I', L, L'). Expression of *Zic3* was restricted to cells surrounding the dorsal half of the developing inner ear and was strongest in cells adjacent to the dorsolateral region of the otic epithelium (Fig. 7D, D', J, J'). *Zic4* expression was the most restricted of the *Zic* genes at these stages, with *Zic4* -expressing cells only detected medially in the mesenchyme between the neural tube and otic epithelium (Fig.7E, E', K, K').

Identifying *Zic*-Expressing Cells

We examined the expression of *Axin2, Brn4, Tbx1*, and *Sox10* in sections at similar levels through the otocyst at E10.5 to attempt to characterize the *Zic*-expressing cells in the mesenchyme outside of the neuroepithelium and otic epithelium. *Axin2*, which identifies cells with active Wnt signaling (Jho et al., 2002), was expressed in the dorsal neural tube, the dorsomedial otic epithelium, and in cells adjacent to the neural tube along its entire dorso-ventral axis (Fig. 8A, D). A subset of *Axin2*-expressing cells adjacent to the dorsal neural tube also expressed *Zic1–4* (see Figs. 6H–K and 8A, D). In the mesenchyme between the ventral neural tube and the otic epithelium, *Axin2* expression overlapped with the expression of *Zic1* and *Zic2* (compare Figs. 6H, I and 8A, D). *Brn4*, a marker of condensing mesenchyme (Phippard et al., 1998; Riccomagno et al., 2002), was expressed throughout the neural tube and in cells adjacent to the ventrolateral and ventromedial otic epithelium, but was not expressed in a band

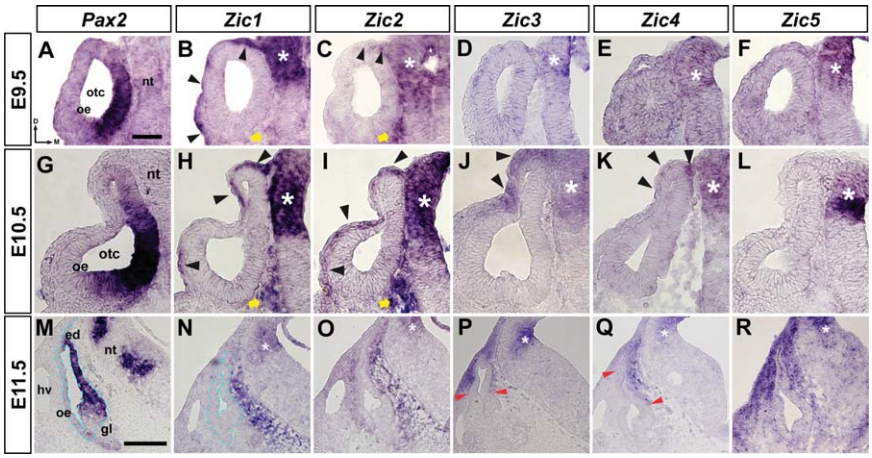

Fig. 6. *Zic* expression in the otic region of mouse embryos between E9.5 and E11.5. In situ hybridization on 12-μm transverse sections through the otocyst of E9.5 (**A–F**), E10.5 (**G–L**), and E11.5 (**M–R**) mouse embryos using probes for *Pax2* (A, G, M), *Zic1* (B, H, N), *Zic2* (C, I, O), *Zic3* (D, J, P), *Zic4* (E, K, Q), and *Zic5* (F, L, R). Note that *Pax2* is expressed in the otic epithelium, while *Zic* gene expression is restricted to the surrounding mesenchyme. otc, otocyst; oe, otic epithelium; nt, neural tube; ed, endolymphatic duct; gl, ganglion; hv, head vein; D, dorsal; M, medial. Asterisks identify *Zic* expression in the neural tube (B–F, H–L, N–R). Black arrowheads indicate *Zic*-expressing cells between the surface ectoderm and the otic epithelium (B, C, H, I, J, K). Yellow arrows indicate *Zic*-expressing cells between neural tube and otic epithelium (B, C, H, I). Red arrowheads indicate the ventral-most extent of *Zic* expression in the mesenchyme (P, Q). Blue dashed lines define border between the otic epithelium and the mesenchyme (M, N). Scale bar in A = 50 μm (applies to A–L); scale bar in M = 200 μm (applies to M–R).

of cells immediately adjacent to the neural tube (Fig. 8B, E). This expression overlapped with that of *Zic1* and *Zic2* in the mesenchyme between the ventral neural tube and the otic epithelium; however, the region of $Brn4^{+}$ cells only partially overlapped the region of Zic^{+} cells (compare Figs. 6H, I and 8B, E). Expression of *Tbx1*, a marker of the periotic mesenchyme (Riccomagno et al., 2002; Vitelli et al., 2003; Raft et al., 2004), was also detected in the lateral wall of the otocyst, in most of the cells below the ventral otic epithelium, and between the ventral neural tube and the otic epithelium (Fig. 8C, F). Most of the *Brn4*-expressing cells also expressed *Tbx1*, which was expected given that *Tbx1* marks the periotic mesenchyme, and *Brn4* is expressed in condensing cells of the periotic mesenchyme (compare Figs. 8B, E and C, F). The mesenchymal expression of *Zic1* and *Zic2* between the ventral neural tube and the otic epithelium overlapped with

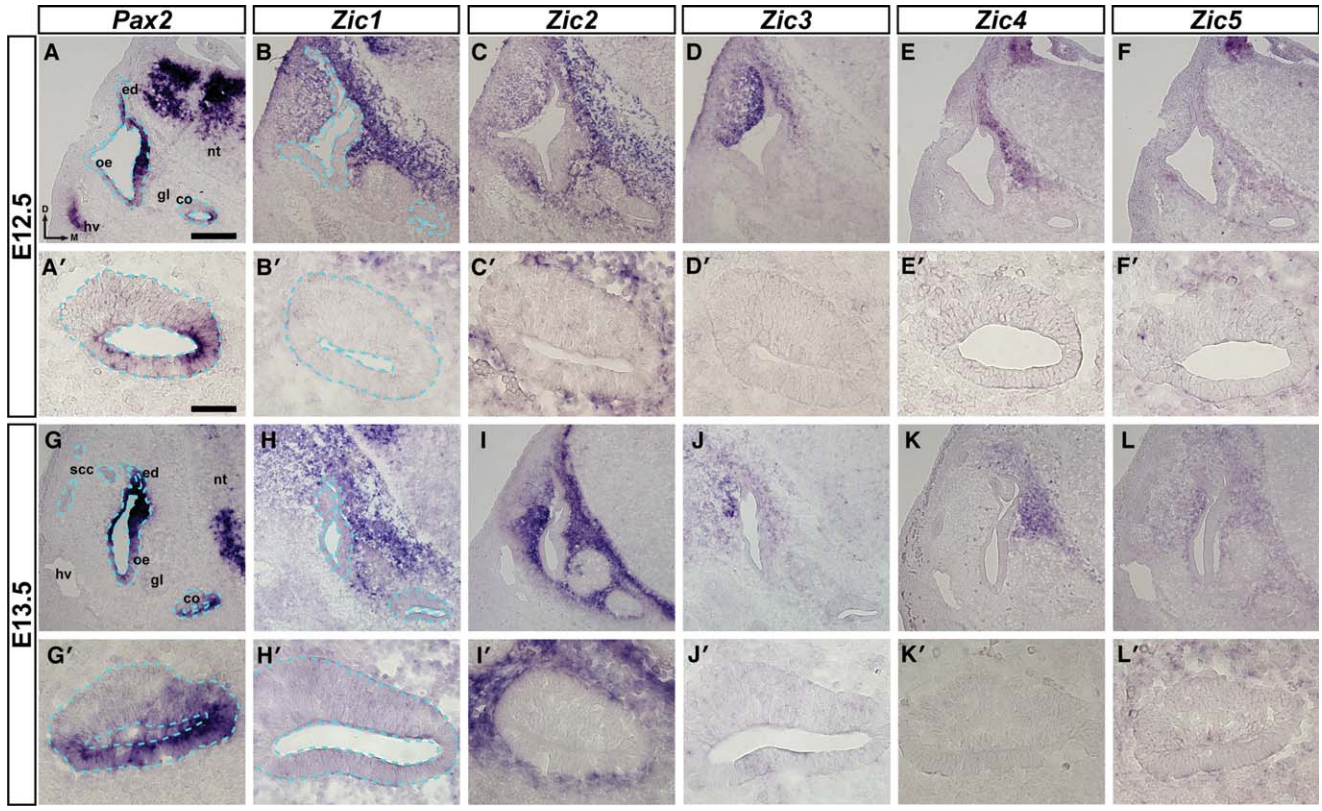

Fig. 7. *Zic* expression in the otic region of E12.5 and E13.5 mouse embryos. In situ hybridization on 12-μm transverse sections through the otocyst of E12.5 (**A–F, A'–F'**) and E13.5 (**G–L, G'–L'**) mouse embryos using probes for *Pax2* (A, A', G, G'), *Zic1* (B, B', H, H'), *Zic2* (C, C', I, I'), *Zic3* (D, D', J, J'), *Zic4* (E, E', K, K'), and *Zic5* (F, F', L, L'). Note that *Pax2* is expressed in the otic epithelium, while *Zic* gene expression is restricted to the surrounding mesenchyme. oe, otic epithelium; ed, endolymphatic duct; co, cochlea; scc, semicircular canals; nt, neural tube; gl, ganglion; hv, head vein; D, dorsal; M, medial. Blue dashed lines define border between the otic epithelium and the mesenchyme (A, A', B, B', G, G', H, H'). Scale bar in A = 200 μm (applies to A–L); scale bar in A' = 50 μm (applies to A'–L').

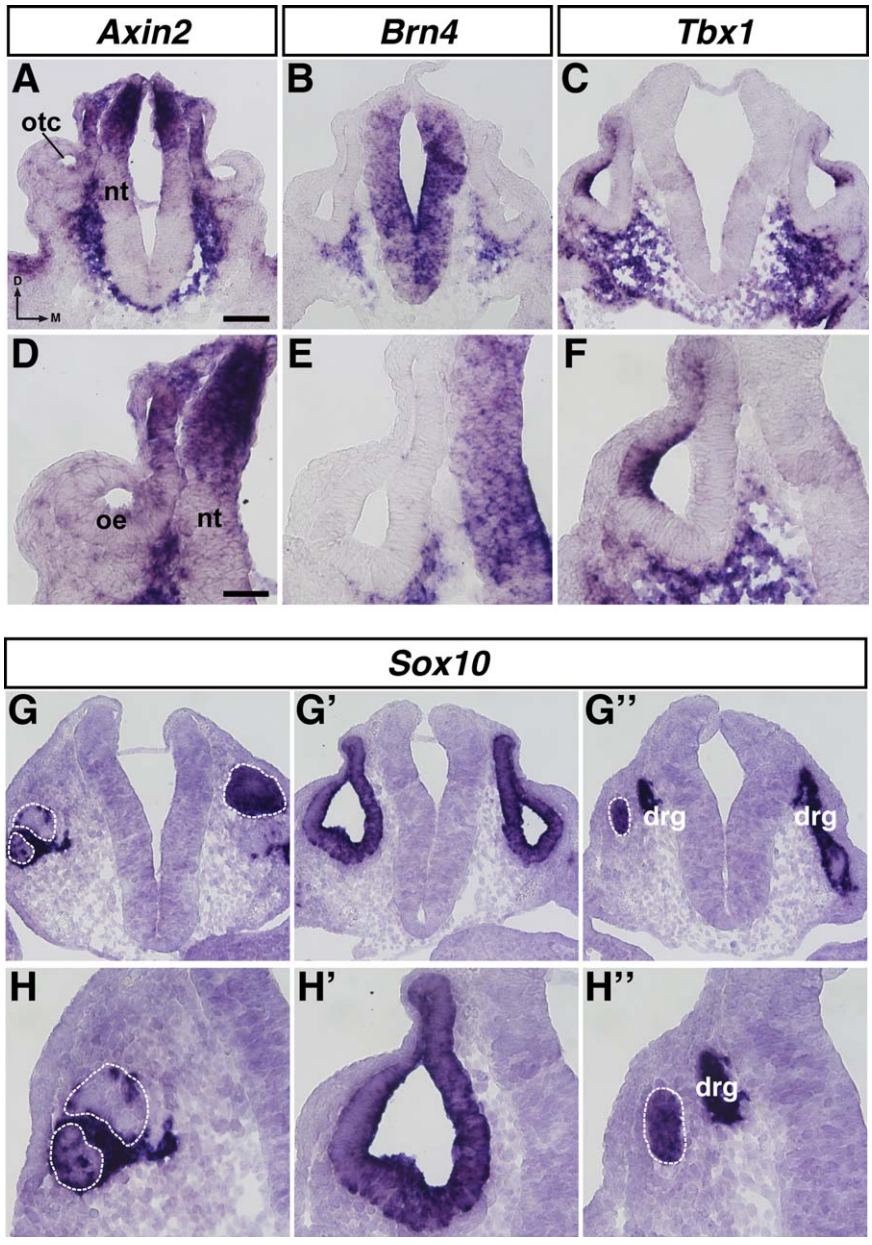

Fig. 8. Characterization of *Zic*-expressing cells in the periotic mesenchyme of E10.5 mouse embryos. In situ hybridization on 12-μm transverse sections through the otocyst of E10.5 mouse embryos using probes for *Axin2* (**A, D**), *Brn4* (**B, E**), *Tbx1* (**C, F**), and *Sox10* (**G–G", H–H"**). oe, otic epithelium; otc, otocyst; nt, neural tube; drg, dorsal root ganglion; D, dorsal; M, medial. White dashed line indicates anterior (G, H) or posterior (G", H") edge of otocyst. Scale bar in A = 100 μm (applies to A–C, G–G"); scale bar in D = 50 μm (applies to D–F, H–H").

Tbx1 expression (compare Figs. 6H, I and 8C, F).

Expression of *Sox10*, which identifies neural crest cells and is also expressed by cells in the otic epithelium (Watanabe et al., 2000; Breuskin et al., 2009; Bronner, 2012), was examined at three different axial levels through the otocyst: anterior to the otocyst (Fig. 8G, H), through the otocyst (Fig. 8G', H'), and posterior to the otocyst (Fig. 8G", H"). Anterior to the otocyst, *Sox10* expression was detected in the wall of the otocyst but not in the mesenchyme (Fig. 8G, H; white dashed lines outline the otocyst). Similar expression was observed through the otocyst (Fig. 8G', H'). Posterior to the otocyst, *Sox10* expression was again found in the wall of the otocyst (white dashed line) as well as in neurons of the neural crest–derived dorsal root ganglion adjacent to the neural tube (drg; Fig. 8G", H").

Taken together, these results indicate that the Zic^+ cells surrounding the otocyst are not neural crest cells because *Sox10* expression was not seen in the same regions in which Zic^+ cells were located (Fig. 8G–G", H–H"). The ventral-most Zic^+ cells are most likely periotic mesenchyme, a subset of which is condensing cartilage (that will later form the otic capsule), because they expressed either *Tbx1* or *Tbx1* and *Brn4* (Fig. 8B, C, E, F). The Zic^+ region in the ventral mesenchyme and adjacent to the dorsal neural tube overlapped with the $Axin2^+$ region (Fig. 8A, D), suggesting that WNT signaling was active in these cells.

DISCUSSION

Distinct Spatiotemporal Expression of *Zic* Genes During Inner Ear Development in the Chick and Mouse

This study presents a comprehensive analysis of *Zic* gene expression in the developing chick and mouse inner ear. *Zic* genes from both mouse (*Zic1–5)* and chick (*Zic1–4*) were found to be expressed in similar patterns during inner ear development, in the dorsal neural tube and throughout the mesenchyme adjacent to the developing inner ear, but not in the otic epithelium. Each *Zic* gene had a unique pattern of expression at each developmental stage examined, but localized overlapping expression of multiple *Zic* genes often occurred. The timing of expression also differed, as the expression of *Zic1–5* in mouse and *Zic1–3* in chick was detected at the earliest time points analyzed in this study (E9.5 in mouse, HH stage 13 in chick), but weak expression of chick *Zic4* was not found until HH stage 18, with stronger expression seen by HH stage 24. At E9.5, expression of *Zic1* and *Zic2* in mouse was seen in both the dorsal neural tube and in the mesenchyme surrounding the otic epithelium, while mesenchymal expression of *Zic3–5 in* mouse and *Zic1–3 in* chick was found only after the onset of expression in the neuroepithelium.

The differences in spatiotemporal expression of the *Zic* genes suggest that each *Zic* gene may be playing a different role during inner ear development. *Zic2*, which is expressed in the mesenchyme surrounding the entire otic epithelium in both mouse and chick, may be important for the overall growth and shaping of the entire developing inner ear. The other *Zic* genes, which are expressed in different regions of the periotic mesenchyme, may be responsible for growth and morphological changes associated with specific inner ear structures.

Identity and Possible Roles of *Zic*-Positive Cells in Critical Tissues During Inner Ear Development

Neural crest

Our analyses identified *Zic*-positive cells outside of the neural tube, adjacent to the otic epithelium. We considered the possibility that these cells were migrating cranial neural crest cells. However, several lines of evidence argue against this. Previous work in the chick demonstrated that *Zic1*-positive cells in the trunk outside of the neural tube were not labeled with HNK-1, a marker of migratory neural crest cells (Sun Rhodes and Merzdorf, 2006). Additionally, the timing, location, and neural crest marker expression of these cells does not correlate with that of migrating cranial neural crest cells. In the mouse, cranial neural crest cells form and begin migrating at the 5-somite stage (Chan and Tam, 1988) and finish migrating by the 14-somite stage (Serbedzija et al., 1992), while in the chick, neural crest cells migrate from HH stage 9+ to HH stage 11 (Tosney, 1982). The Zic^{+} cells we detected in the mesenchyme were seen in much older embryos: 22–24 somite stage (E9.5) and older in the mouse, and HH stage 18 and older in the chick. Second, DiI labeling studies in chick embryos and lineage tracing studies in mouse embryos indicate that neural crest cells from rhombomere 5 migrate rostrally and caudally around the otocyst and arrive in the second and third branchial arches, while neural crest cells from rhombomere 6 migrate caudally around the otocyst before arriving in the third branchial arch (Sechrist et al., 1993; Trainor et al., 2002). The sections in which Zic^{+} cells were detected in the mesenchyme were taken midway through the otocyst in a region where neural crest cells do not migrate. Finally, we used *Sox10* expression as a marker for migrating neural crest cells in the mouse (Bronner, 2012). *Sox10* expression was not detected outside of the otic epithelium in sections through the medial portion of the otocyst at E10.5, but was found in sections at the most anterior or posterior levels of the otocyst. $Zic1–4^{+}$ cells at E10.5 were detected outside of the neural tube in sections through the medial portions of the otocyst, suggesting that these *Zic*-expressing cells were not of neural crest origin.

Periotic mesenchyme

We examined the expression of *Brn4* and *Tbx1*, markers of condensing mesenchyme and periotic mesenchyme, in the regions where we found *Zic*-expressing cells. The region of *Tbx1* expression overlapped with the regions of *Zic1* and *Zic2* expression, but the region of *Brn4* expression only overlapped slightly with the regions of *Zic1* and *Zic2* expression. This suggests that the majority of $Zic1^{+}$ and $Zic2^{+}$ cells in the ventral mesenchyme are $Tbx1^{+}$ periotic mesenchyme cells, and a few of these cells are $Brn4^{+}$ cells of the condensing mesenchyme. Expression of *Tbx1* and *Brn4* is regulated by SHH signaling (Riccomagno et al., 2002), and *Zic* genes regulate SHH signaling in vitro (Koyabu et al., 2001; Mizugishi et al., 2001; Pan et al., 2011), so the expression of *Zic* genes in the same regions where *Tbx1* and *Brn4* are expressed could mean that the *Zic* genes are involved in the SHH-dependent expression of *Tbx1* and *Brn4*. Alternatively, the *Tbx1* and *Brn4* expression could be independent of *Zic* expression, suggesting a different role for the *Zic* genes. *Zic* genes, like *Tbx1* and *Brn4*, could be required for proper epithelial-mesenchymal signaling. Loss of either *Brn4* ($Brn4^{-/-}$) or *Brn4* and a single allele of *Tbx1* ($Brn4^{-/-};Tbx1^{+/-}$) in the periotic mesenchyme in mice results in improper coiling of the cochlea (Braunstein et al., 2008). Loss of one or multiple *Zic* genes in the periotic mesenchyme could result in similar defects in inner ear structures, with the specific structures affected depending on which *Zic* gene is lost.

We also examined *Axin2* expression in the mesenchyme. The dorsal-most expression of *Axin2* overlapped with the expression of *Zic1–4*, while the ventral expression overlapped with the expression of *Zic1* and *Zic2*. *Axin2* expression induced by WNT signaling acts as part of a negative feedback loop to limit the extent and duration of WNT signaling (Jho et al., 2002). WNT signaling from the dorsal hindbrain and SHH signaling from the notochord and floor plate pattern the otocyst along its dorsal-ventral axis and are critical for the development of the dorsal (WNT) and ventral (SHH) structures of the inner ear (Riccomagno et al., 2002, 2005). In vitro, ZIC2 binding to TCF4 inhibits WNT signaling (Pourebrahim et al., 2011), so the combination of *Axin2* expression and *Zic* expression may selectively modulate WNT signals from the dorsal hindbrain to the otocyst as well as restricting ventral WNT signals closer to the source of SHH. Additional experiments are needed to determine this, as well as co-labeling experiments to determine whether the Zic^{+} mesenchymal cells are $Axin2^{+}/Tbx1^{+}/Brn4^{+}/Sox10^{-}$ as would be expected.

Neural tube

In both chick and mouse, all *Zic* genes (chick *Zic1–4*, mouse *Zic1–5*) were expressed in the dorsal neural tube. The inner ear lies adjacent to the neural tube, and alterations in specification and patterning of the neural tube are known to affect inner ear development. Signaling from the hindbrain, especially rhombomeres 5 and 6, is critical for proper patterning and development of the inner ear, as well as development of the cochleovestibular ganglion that innervates the ear (Bok et al., 2005, 2007; Kil et al., 2005; Choo, 2007; Vazquez-Echeverria et al., 2008; Liang et al., 2010). Neural tube closure defects, including exencephaly, in or near the hindbrain, are seen in *Zic* mutant mice examined to date, including $Zic2^{kd/kd}$

(Nagai et al., 2000), *Zic3*^{Bn} (Klootwijk et al., 2000), and *Zic5*$^{-/-}$ (Inoue et al., 2004) mutant mice. In *Zic2*$^{Ku/Ku}$ mutants, rhombomeres 3 and 5 are smaller than those in wild type embryos, and ectopic *follistatin* expression is detected in these rhombomeres (Elms et al., 2003). In addition to effects on hindbrain development, mutations in *Zic* genes affect signaling from the hindbrain to the inner ear. Expression of *Wnt3a*, a signaling molecule from the hindbrain important for inner ear development (Riccomagno et al., 2005), is delayed in the dorsal hindbrain of both *Zic2*$^{kd/kd}$ (Nagai et al., 2000) and *Zic5*$^{-/-}$ (Inoue et al., 2004) mutant mice. *Zic* genes expressed in the neural tube could effect inner ear development in multiple ways, both directly and indirectly. ZIC proteins could directly interact with WNT or BMP pathway components in the neural tube to modulate WNT or BMP signaling to the developing inner ear, or ZIC proteins could promote/inhibit the expression of other factors in the dorsal neural tube. Indirectly, loss of specific Zic genes in the neural tube leads to neural tube defects (such as exencephaly), which would alter the position of the neural tube relative to the inner ear. This change in position of the neural tube relative to the developing inner ear would alter the position of specific regions of the otic epithelium relative to sources of BMP, WNT, and SHH signaling, potentially leading to malformations of the inner ear.

Investigating the Function of *Zic* Genes During Inner Ear Development

To date, the involvement of *Zic* genes in inner ear development has only been examined at the level of gene expression (this study; Warner et al., 2003). Further functional studies are needed to investigate the role of the *Zic* genes during inner ear development. Mice with mutations in each of the *Zic* genes have been generated (Ali et al., 2012), but no ear phenotypes have been reported to date. One reason for this could be that multiple severe defects in the postnatal animals mask any ear phenotypes. Both *Zic1* and *Zic5* homozygous mutants display abnormal gait and posture characteristic of defects in the vestibular region of the inner ear, but these abnormalities were attributed to cerebellar defects (*Zic1*$^{-/-}$) or hydrocephalus (*Zic5*$^{-/-}$) (Aruga et al., 1998; Inoue et al., 2004). However, the majority of *Zic5* mutants did not have any noticeable changes in brain morphology, so the cause of the abnormal gait and posture was never fully investigated (Inoue et al., 2004). Another reason an inner ear phenotype has not been characterized is that significant numbers of mutants die, either during embryonic stages (all *Zic2*$^{Ku/Ku}$, ~24% of *Zic3*^{Bn}) or shortly after birth (50% of *Zic1*$^{-/-}$, all *Zic2*$^{kd/kd}$, some *Zic5*$^{-/-}$) (Aruga et al., 1998; Klootwijk et al., 2000; Nagai et al., 2000; Elms et al., 2003; Inoue et al., 2004), making it impossible to detect signs of inner ear defects that affect the mature function of this sensory organ, such as changes in gait or posture, abnormal behaviors such as circling, and changes in the acoustic startle response or auditory brainstem responses (ABR) (Saul et al., 2008). Studies using these mice, including recording of ABRs in any animals including heterozygotes that live to postnatal stages, as we have done for other mouse mutations (Bank et al., 2012), could provide insights into how individual *Zic* genes function during inner ear development. Further, the generation of compound *Zic* mutants would be useful to address questions of redundancy among the *Zic* genes during inner ear development, as we have shown that the *Zic* genes are expressed in overlapping regions of the periotic mesenchyme in both chick and mouse.

Attempts to Reconcile the Disparate Results of Earlier *Zic* Gene Expression Studies

Because the results found by McMahon and Merzdorf (McMahon and Merzdorf, 2010) were found to be distinctly different from those of our earlier report (Warner et al., 2003), particularly for the expression of *Zic1*, we repeated and expanded the expression study of the *Zic* genes in the developing chick inner ear. A fourth *Zic* gene, *Zic4*, has since been identified in the chicken (McMahon and Merzdorf, 2010) and its expression pattern was also examined. In addition, we wanted to compare the expression of the *Zic* genes in the otic region of the chicken to the expression pattern of the *Zic* genes in the otic region of the mouse, which had not previously been reported. To our surprise, the expression pattern of *Zic1* in the developing chick inner ear we report here is not consistent with our earlier study; however, the expression patterns of *Zic2* and *Zic3* reported here are consistent with our earlier study (Warner et al., 2003). Our findings for the expression patterns of *Zic1–4* in the neural tube of the developing chick in the present study are consistent with an earlier study examining the expression of *Zic1–4* in the developing chick embryo up to HH stage 18 (McMahon and Merzdorf, 2010). However, we found expression of both *Zic1* and *Zic2* in the periotic mesenchyme at HH stage 18, whereas McMahon and Merzdorf only saw *Zic2* expression in the periotic mesenchyme (McMahon and Merzdorf, 2010). An explanation for this is that at HH stage 18, we detected periotic mesenchyme expression in sections taken through the ear, which allowed us to examine expression patterns in locations that would not have been discernable in the whole mounts used in the other study (McMahon and Merzdorf, 2010). This leaves us to question the differences in *Zic1* expression in the developing chick inner ear that were seen in this study as contrasted with our previous study (Warner et al., 2003). All probes (this study and the previous study; Warner et al., 2003) were sequenced to confirm their identity. In this study, the probes were designed to target sequences outside of the zinc finger region where there is low homology among the *Zic* genes (Fig. 3). Both the chick *Zic2* and *Zic3* probes used in this study target similar regions as the *Zic2* and *Zic3* probes from our previous study (Warner et al., 2003). The *Zic2* probes each target a different part of the 3′ untranslated region (UTR) of the mRNA. Similarly, the *Zic3* probes target an overlapping region of the 3′ UTR, with the probe used in this study also extending both into a

portion of the 3′ coding region of the mRNA and further into the 3′ UTR. The *Zic1* probe used in our earlier study targeted the zinc finger region of *Zic1*, and this likely underscores the differences with the earlier study. The probe used in this study targets the 3′ UTR of *Zic1* and excludes this zinc finger region.

We believe this more detailed and thorough study now resolves the differences between the two earlier studies, confirming the Merzdorf lab's findings of the lack of *Zic1* expression in the developing chick embryonic inner ear, confirming the expression patterns we found in the developing chick inner ear for *Zic2* and *Zic3*, and extending the study of the chick *Zic* gene expression in the region of the developing otic region and the neural tube to characterize the expression pattern of *Zic4*. We have further extended the study of otic/periotic/neural tube expression of the *Zic* genes to the mouse, as a prelude to examining such expression in *Zic* mutant embryos and animals.

EXPERIMENTAL PROCEDURES

Mouse Embryos

Wild type Balb/c mice were used to set up timed matings. Noon on the day on which a vaginal plug was detected was designated as E0.5. Pregnant females were sacrificed by cervical dislocation. All experiments using mice were approved by the Animal Use and Care committee of the University of Michigan and conform to all guidelines of the Unit for Laboratory Animal Medicine and those of the National Institutes of Health. The uterine horns containing the embryos were dissected out and placed in PBS with 10% FBS. Embryos were then dissected out of the surrounding maternal tissues and Reichert's membrane was removed. Embryos were fixed in 4% paraformaldehyde overnight, washed in 1× PBS (3 × 5 min), and then transferred to 30% sucrose in 1× PBS and rocked at 4°C overnight.

Chick Embryos

Fertilized White Leghorn chicken eggs were obtained from the Michigan State University Poultry Farm (East Lansing, MI), and placed in a humidified incubator at 37°C. Eggs were windowed and the embryos were dissected and placed into PBS with 10% FBS. The vitelline membrane was removed and embryos were dissected out of the amnion. Embryos were fixed in 4% paraformaldehyde (PFA) overnight, washed in 1× PBS (3 × 5 min), and then transferred to 30% sucrose in 1× PBS and rocked at 4°C overnight.

Embedding and Cryosectioning

Embryos were transferred through three progressive changes of OCT (TissueTek) to remove excess sucrose. The embryos were then transferred to embedding molds, covered with OCT, and oriented such that the anterior portion of the ear pointed down. The molds were then frozen on dry ice and stored at −80°C until sectioning. Twelve- or 25-μm transverse sections through the ear were cut using a Microm HM500M cryostat and collected on SuperFrost Plus slides (Fisher, Pittsburgh, PA). Sections were air-dried on the slides at room temperature for at least 30 min, and then stored at −80°C.

In Situ Hybridization

In situ hybridization using digoxigenin-labeled antisense probes was performed on sections using a protocol adapted from Wilkinson and Nieto (Wilkinson and Nieto, 1993). The prehybridization, hybridization, and post-hybridization steps were performed essentially as described, except MBST (100 mM Maleic Acid, 150 mM NaCl, 0.1% Tween20) was used for the post-hybridization washes and the blocking solution was MBST containing 10% heat-inactivated sheep serum and 2% Blocking Reagent (Roche, Indianapolis, IN). For the color reaction, BM Purple (Roche) was used instead of NBT/BCIP. Following the color reaction, slides were washed three times in PBST, pH 4.5 (PBS with 0.1% Tween20), fixed (4% PFA/0.2% gluteraldehyde), washed three times in PBS, dehydrated in 70% ethanol, and dried on a slide warmer set at 60°C. Sections were then mounted under a coverslip with Glycergel Mounting Media (Dako, Carpinteria, CA). A minimum of 4 embryos was analyzed for each probe. Antisense probes were generated by digesting plasmids containing the cDNA sequences of either the mouse or chick genes and then transcribing with the appropriate RNA polymerase. Probes for chick *Zic3* and chick *Zic4* were prepared by PCR as previously described (McMahon and Merzdorf, 2010). Both the mouse and chick *Zic* probes were designed to target sequences outside of the zinc finger region where there is lower homology among the *Zic* genes (Fig. 3). Images were acquired with an Olympus BX51 microscope equipped with an Olympus camera.

Phylogenetic Tree and Sequence Analysis

The complete amino acid sequence of the *Zic* genes from human, mouse, chick, zebrafish, and frog were aligned with the amino acid sequence of the ancestral gene *odd-paired* (*opa*) from *Drosophila* using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). The alignment was then converted into a phylogenetic tree using Tree Vector (http://supfam.cs.bris.ac.uk/TreeVector). Pairwise comparisons of the amino acid sequences of human, mouse, and chick *Zic* genes was performed using EMBOSS Stretcher (http://www.ebi.ac.uk/Tools/psa/emboss_stretcher).

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