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Identification of differentially expressed genes in early inner ear development

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Abstract

To understand the etiology of congenital hearing loss, a comprehensive understanding of the molecular genetic mechanisms underlying normal ear development is required. We are identifying genes involved in otogenesis, with the longer term goal of studying their mechanisms of action, leading to inner ear induction and patterning. Using Agilent microarrays, we compared the differential expression of a test domain (which consisted of the pre-otic placodal ectoderm with the adjacent hindbrain ectoderm and the underlying mesendodermal tissues) with a rostral control domain (which included tissue that is competent, but not specified, to express inner ear markers in explant assays). We identified 1,261 transcripts differentially expressed between the two domains at a 2-fold or greater change: 463 were upregulated and 798 were downregulated in the test domain. We validated the differential expression of several signaling molecules and transcription factors identified in this array using *in situ* hybridization. Furthermore, the expression patterns of the validated group of genes from the test domain were explored in detail to determine how the timing of their expression relates to specific events of otic induction and development. In conclusion, we identified a number of novel candidate genes for otic placode induction.

Keywords

Otic induction; inner ear; Wnt; Fgf; Notch; Hox; otic placode; microarray; gene expression screen

1. RESULTS AND DISCUSSION

Congenital hearing loss is among the most common birth defects, affecting approximately 3 out of every 1000 infants born each year in the United States. Genetic causes account for about 50% of all congenital hearing loss, but the roles of only a small number of genes directly linked to hearing loss are known. To better understand the mechanisms underlying congenital hearing

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loss, a clearer understanding of ear development and the genes required for otogenesis are needed.

Through candidate gene approaches and subsequent gene mis-expression experiments, a small number of secreted proteins have been identified in the initiation and early patterning of the otic placode and vesicle, such that a rudimentary signaling network is beginning to be established. The signaling proteins thus far identified in this cascade belong mainly to the FGF, WNT, SHH, and Notch protein families (Ladher et al., 2000, 2005; Vendrell et al., 2000; Adamska et al., 2001; Phillips et al., 2001, 2004; Leger and Brand, 2002; Maroon et al., 2002; Liu et al., 2002, 2003; Riccomagno et al., 2002, 2005; Alvarez et al., 2003; Wright and Mansour, 2003; Ohyama et al., 2006; Sun et al., 2007; Zelarayan et al., 2007; Jayasena et al., 2008; Park and Saint-Jeannet, 2008). Inductive signals from all three germ layers of the gastrulating/neurulating embryo play roles in otic induction. For example, in chick, Ladher and colleagues (2000, 2005) have shown that endodermal FGF8 induces the expression of mesodermal *Fgf19*. Subsequently, FGF19 induces *Wnt8a* (formerly called *Wnt8c*) expression in the hindbrain ectoderm. FGF19 and WNT8A then work together to signal the adjoining pre-placodal ectoderm to form otic tissue. Other studies have shown that Notch signaling also cooperates with the WNT pathway, in this case specifying the size of the otic placode (Jayasena et al., 2008).

Although some signals have been identified in early induction and patterning of the otic epithelium, it is unclear how these signals work in concert to induce the otic placode or what additional signals may exist. For example, FGF19 (from the mesoderm) and WNT8A (from the hindbrain) are both required for otic induction in the chick (Ladher et al., 2000). In contrast, Phillips and colleagues (2004) reported that in zebrafish, hindbrain WNT8 is dispensable in otic induction, but it plays a role in regulating the timing of hindbrain FGF3 expression, a factor required for otic induction in fish. Wright and Mansour (2003) showed that the mesodermal signal in mouse consists of both FGF3 and 10, in contrast to chick, but that the mesodermal inducing signals in both species are themselves induced by FGF8 (Ladher et al., 2005). Other studies have indicated that FGF-signaling is necessary to establish an otic precursor field, but must be subsequently attenuated for otic progression to occur (Freter et al., 2008). It is likely that the induction of the otic placode and its subsequent development involve additional signals and downstream effectors that remain to be identified, and that the exact players differ among species. At present, the candidate gene approach has largely exhausted known candidates. To better elucidate the molecular mechanisms of otogenesis, new candidate genes involved in the initiation and early patterning process need to be identified. To address this goal, we have performed a microarray analysis comparing a test domain (in which both the inducing and responding tissues for otic induction are localized) to a more rostral control domain that includes tissue (surface or non-neural ectoderm) that is competent, but not specified, to form otic tissue in explant assays.

1.1 Differential Expression by Microarray

To identify genes that are potentially involved in otic placode induction, we used microarray hybridization techniques to compare the test and control domains. To eliminate or at least to reduce the potential of identifying general placodal competency factors and to restrict our findings to genes that are specific to the ear-forming region, we selected a control domain, in Hamburger and Hamilton (1951) stage 6–7 chick embryos, that includes surface or non-neural ectoderm that is competent, but not specified, to form otic placode in explant assays (Fig. 1A: b; Ladher et al., 2000: area “d”). Using Agilent chick genome microarray chips, we directly compared the test and control domains (Fig. 1A: a, b, respectively), identifying 1,261 transcripts differentially expressed at a change of 2-fold or greater: 463 transcripts were

upregulated and 798 transcripts were downregulated in the test domain as compared to the control domain.

Several genes known from previous studies to be involved in otic placode induction and/or early development of the inner ear were upregulated, as expected, in the test domain, including *Fgf19*, *Fgf3*, *Gbx2*, *Hoxa1*, and *Hoxb1*, demonstrating the validity of our microarray approach for identifying candidate genes for inner ear development. Also as expected, two additional inducers of otic placode, *Fgf8* and *Wnt8a*, were *not* differentially expressed in our microarray. Differential expression of *Fgf8* was not expected, because previous *in situ* hybridization studies (Ladher et al., 2005) showed that *Fgf8* is expressed in *both* the test and the control domains. Moreover, we did not expect *Wnt8a* to be differentially expressed, because our previous *in situ* hybridization studies (Ladher et al., 2000) showed that it is not expressed in the test domain until stage 8, just after the stages used to collect tissues for microarray analysis.

1.2 In Situ Hybridization Validation of Microarray Differential Expression

As a control, we took a subset of embryos in which the test and control domains were removed on one side and processed them for *in situ* hybridization with *Fgf19* (Fig. 1B). To identify the test domain during our dissections of living embryos, we used the first somite as a landmark for the pre-otic caudal boundary, and we micro-dissected a piece of tissue just rostral to the first somite that included the pre-placodal region, adjacent hindbrain, and underlying mesendoderm. The sizes of the test and control isolates were based on our earlier explant study (respectively called area “a” and area “d” in Ladher et al., 2000). Loss of most of the *Fgf19* expression domain, after micro-dissection and subsequent *in situ* hybridization, demonstrated that the correct tissue for the test domain was reliably collected.

Thirty genes with differential expression of 2-fold or greater were selected for validation. Expression patterns were visualized first by *in situ* hybridization from pre-placode development—or during the stages of otic induction (stages 4–9)—through otic placode formation (stages 10–12). Those genes selected for validation consisted of signaling molecules and transcription factors. We focused on these because the majority of genes involved in otic induction reported in the literature belong to these two classes. Several signaling molecules were differentially expressed, including members of the FGF-, WNT-, and Notch-signaling pathways (Table 1). In addition, a number of transcription factors, including several Hox genes, were also differentially expressed.

1.2.1 FGF-Signaling Pathway—In our microarray, three members of the FGF family were upregulated in the test domain: *Fgfs3*, 4, and 19. One FGF receptor, *Fgfr3*, was down regulated. *Fgf3* was expressed at 4-fold higher, *Fgf4* at 2.5-fold higher, and *Fgf19* at 55-fold higher levels in the test domain as compared to the control domain. Conversely, *Fgfr3* was expressed 4-fold higher in the control domain.

FGF-signaling plays a well-documented role in inner ear development and disease. FGF3 (Wright and Mansour, 2003; Phillips et al., 2004) and FGF19 (Ladher et al., 2000) are known inducers of the otic placode; mutations in *Fgf3* cause congenital hearing loss and inner ear agenesis (Tekin et al., 2007, 2008; Alsmadi et al., 2009). Although the various skeletal dysplasia syndromes associated with FGF-receptor mutations are usually associated with conductive hearing loss, Kallmann syndrome 2 is associated with sensorineural hearing loss when caused by mutations in *FGFR1* (Dode et al., 2003). Kallmann syndrome with hearing loss has also been reported in association with mutations in *FGF8* (Falardeau et al., 2008). Moreover, a specific mutation in *FGFR3* causes Muenke syndrome, consisting of coronal craniosynostosis and congenital sensorineural hearing loss (Muenke et al, 1997; Holloway et al., 1998; Lowry et al., 2001). Mutations in the tyrosine kinase domain of *FGFR2* and 3 cause

lacrimoauriculodentodigital syndrome (LADD), a component finding of which is mixed hearing loss (Milunsky et al., 2006; Rohmann et al., 2006).

Expression patterns of these genes, visualized by *in situ* hybridization, validated the specificity and sensitivity of our microarray results (Fig. 2A–P). During placode induction stages, *Fgfs3*, *4*, and *19* were all expressed as early as stage 4 in the node and primitive streak (Fig. 2A–C), and expression of all three occurred in the test domain at stages 6–7 (Fig. 2E–G). The first expression of *Fgf19* occurred in the mesoderm (Fig. 2G'), whereas *Fgf3* and *Fgf4* were first expressed in both the mesoderm and the overlying neuroectoderm of the developing caudal hindbrain (Fig. 2E', F'). Expression of *Fgf4* in the test domain was transient and disappeared by stage 9. Shortly after, however (~stage 11), *Fgf4* was expressed in the endoderm of the second pharyngeal pouch subjacent to the developing otic placode. Expression of all three FGF ligands persisted at the ear-forming region through stages of early placode formation, and the primitive streak expression of these genes became consolidated into the developing tail bud (Fig. 2M–O). Although *Fgf3* and *19* have described roles in otic formation, *Fgf4* has not been identified previously as a potential candidate and studies of its potential role in ear induction are underway (Urness et al., in preparation).

In contrast to the three FGF ligands that were upregulated, the expression of *Fgfr3* was downregulated in the test domain as compared to the control domain. *Fgfr3* was the only FGF receptor that was identified in our microarray as differentially expressed between the test and control domains at stages 6–7. *Fgfr3* was expressed only in the rostral half of the embryo at stages 4–5 (Fig. 2D), and it persisted in the most rostral portion of the embryo throughout stages 6–7 (Fig. 2H). From stages 6–9, *Fgfr3* was expressed throughout the neural plate, extending to the level of the developing first somite. *Fgfr3* expression was much weaker just rostral to the first somite, namely, in the region of the test domain, than in the adjoining regions (Fig. 2H, L, arrows). During stages of early placode formation, *Fgfr3* was broadly expressed in the neural tube adjacent to the ear-forming region, extending both rostrally and caudally (Fig. 2P). Hindbrain expression of *Fgfr3* at late otic placode induction and early placode formation stages could play a role in the induction and/or maintenance of the otic placode via potential effects on hindbrain specification. As discussed earlier, the hindbrain secretes growth factors, such as *Wnt8a*, that are important for otic induction, in responds to FGF signaling by the adjacent head mesoderm of the ear-forming region.

1.2.2 WNT-Signaling Pathway—In our microarray, several members of the Wnt pathway were upregulated in the test domain, including both ligands and receptors. Two ligands, *Wnt5b* and *Ndp*—the latter, a non-traditional Wnt-signaling molecule—and one Wnt receptor, Frizzled (*Fzd10*), were upregulated in the test domain; another Wnt receptor, *Fzd8*, was downregulated. *Wnt5b* was expressed at 3.5-fold higher, *Ndp* was expressed at 19-fold higher, and *Fzd10* was expressed at 5-fold higher in the test domain, whereas *Fzd8* was expressed at 11.5-fold higher in the control domain. Like the FGF pathway just described, the WNT-signaling pathway is also involved in otic placode induction and patterning of the inner ear (Ladher et al., 2000; Ohshima et al., 2006). *Ndp* is the gene responsible for Norrie disease (oculoacousticocerebral dysplasia), a syndrome that includes progressive hearing loss of cochlear origin with onset in late childhood as a component finding (Warburg, 1966; Berger, 1992).

In situ hybridization of these genes validated our microarray results (Fig. 3A–T). At stages 4–5, *Wnt5b* was expressed throughout the primitive streak and extended laterally in ingressing cells (Fig. 3A). Through stages 6–7, expression was maintained in the caudal two-thirds of the embryo, with its rostral boundary located near the level of the test domain (Fig. 3F). By stage 8, discrete expression occurred in bilateral patches (Fig. 3K, arrows) just rostral to the first somite, corresponding to the test domain. In addition, expression of *Wnt5b* persisted throughout

the regressing primitive streak and in the segmented somites (Fig. 3K). By placodal stages, *Wnt5b* expression was restricted to the caudal-most portion of the embryo in the forming tail bud and flanking mesoderm (Fig. 3P).

At stages 4–5 *Ndp* was expressed in the caudal half of the primitive streak (Fig. 3B). By stages 6–7, *Ndp* was expressed in discrete bilateral patches of the neural plate ectoderm in the test domain, extending caudally to overlie the forming somites (Fig. 3G, G'). As development progressed, this expression extended caudally throughout the area of the neural plate overlying the segmenting somites (Fig. 3L). By placodal stages, *Ndp* was expressed throughout the neural tube medial to the somites, and in rhombomere 4, adjacent to the otic placode (Fig. 3Q: arrowheads).

Ndp expression was also studied at post-placodal (i.e., otocyst) stages to ask whether its pattern of expression might suggest a potential role in inner ear development. In whole mounts, *Ndp* appeared to be expressed at these stages throughout most of the length of the neural tube, as well as in the otic vesicle (Fig. 4A–C). However, sections revealed that much of the neural tube staining was trapping, except for localized regions of weak expression in the neuroepithelium, presumably marking cell columns (Fig. 4C': arrows), and ventral neural tube around the floor plate (Fig. 4C', C''). Similarly, staining in the otocyst largely consisted of trapping (Fig. 4C'). By stage 22, however, *Ndp* was robustly expressed in the dorsal region of the developing mesonephric kidneys (Fig. 4C'').

Although *Fzd4* was not differentially expressed in our microarray, we looked at its expression pattern as well, because it is reported to be the only receptor that binds with *Ndp* (Smallwood et al., 2007). Furthermore, progressive hearing loss was observed in a *Fzd4* null mouse (Wang et al., 2001). *Fzd4* was expressed, although faintly, in bilateral mesodermal patches within the test domain (Fig. 3H, H', M). The rostral end of the embryo, corresponding to our control domain, also lightly expressed *Fzd4*, which accounts for it not being detected as differentially expressed in our microarray. In placodal stages, *Fzd4* was expressed throughout the head, including the level of the ear-forming region (Fig. 3R). Thus, within the ear-forming region there is overlap in the pattern of expression of both the Wnt ligand and its receptor (cf., Figs. 3Q, R), suggesting a potential role NDP signaling in early otic development.

Fzd10 was expressed at stages 4–5 throughout the primitive streak including Hensen's node; to a lesser degree, expression of *Fzd10* in ingressing cells also flanked the primitive streak laterally (Fig. 3D). By stages 6–7, *Fzd10* was also expressed slightly more rostrally, extending up into the region of the test domain (Fig. 3I). Sections revealed that expression of *Fzd10* was restricted to the epiblast (Fig. 3I'). Slightly later, at stage 8, *Fzd10* was expressed mainly in the caudal portion of the embryo. However, discrete bilateral patches of expression occurred in the hindbrain portion of the test domain (Fig. 3N: arrows). The expression of *Fzd10* persisted in the hindbrain into the placodal stages of development, but also extended rostrally into the developing midbrain, and caudally throughout the length of the spinal cord and into the tail bud and flanking mesoderm (Fig. 3S).

Expression of *Fzd10* in the neural tube persisted through post-placodal stages and extended rostrally from the tail bud and flanking mesoderm, and throughout the spinal cord, up to the caudal limit of the forebrain (Fig. 4D, E). With formation of the hindlimb buds, expression extended into the hindlimb mesenchyme (Fig. 4E). Sections revealed that *Fzd10* was expressed in the dorsal two-thirds of the neural tube and was not expressed in the otic vesicle (Fig. 4E').

For comparison we visualized the expression of *Fzd8*, which was downregulated in our microarrays in the test domain as compared to the control domain. Consistent with our microarray results, *Fzd8* was strongly expressed in the rostral midline of the embryo as early as stage 5, but was completely absent more caudally during the stages we used for our

microarrays (Fig. 3E, J). Expression persisted in the developing head fold at stages 8–9 and extended into the forebrain, midbrain, and hindbrain regions (Fig. 3O). *Fzd8* was expressed throughout the brain at the placode stage, but expression was considerably weaker than at earlier stages (Fig. 3T).

1.2.3 Notch-Signaling Pathway—In our microarray, three genes of the Notch-signaling pathway were differentially expressed in the test domain. All three were expressed throughout stages of induction. One of these, *Jagged1* (*Jag1*), is a ligand of the Notch receptor and was expressed 2-fold higher in the test domain as compared to the control domain. Mutations in *JAG1* cause Alagille syndrome, but have also been associated with nonsyndromic familial deafness and congenital heart defects (Le Caignec et al., 2002). *JAG1* is required for normal sensory development of the inner ear (Kiernan et al., 2001). The other two Notch-signaling genes identified in this study were *Hairy* and *Enhancer-of-Split* (*Hes*)5, expressed at 9-fold higher, and *Hes6*, expressed at 19-fold higher, in the test domain as compared to the control domain. The *Hes* transcription factors are downstream targets of Notch-signaling. *Hes5* and *Hes6* have both been implicated in sensory development of the inner ear at later stages in otogenesis: *Hes5* is required for inner hair cell formation (Zine et al., 2001), whereas *Hes6* is an inner ear marker that delineates the inner hair cells (Qian et al., 2006), although its role in their development is unknown.

In situ hybridization analysis of these genes validated our microarray results (Fig. 5A–L). At stages 4–5, *Jag1* was expressed throughout the primitive streak and adjacent areas (Fig. 5A). At stages 6–7, *Jag1* was expressed bilaterally in the test domain and extended caudally to either side of Hensen's node, where it was expressed in both neuroectoderm and more lateral mesoderm (Fig. 5D, D'). Two additional bilateral patches of expression occurred in the developing heart field (Fig. 5D, G). During later otic induction and placodal stages, *Jag1* was restricted mainly to the developing heart tube (Fig. 5G, J).

Jag1 expression was maintained at post-placodal stages. At stage 14, *Jag1* was expressed in many other areas including the atria and ventricles of the heart, lens vesicles, mesonephric kidneys, spinal ganglia, mesencephalon, hindbrain, rostral spinal cord, and otic vesicles (Fig. 5M, M'). At stage 14, *Jag1* was expressed throughout the entire extent of each otic vesicle, whereas by stage 22 its expression was restricted to patchy ventral domains marking the vesicle's rostral, caudal, medial, and lateral walls (Fig. 5N, N'). Moreover, expression in the neural tube at most rostrocaudal levels was restricted to discrete cell columns (Fig. 5N, N', N'').

Hes5 was not expressed at stage 4, but *Hes6* was expressed at this time in the rostral half of the primitive streak (Fig. 5B, C). However, by stages 6–7, *Hes5* and *Hes6* were both expressed in bilateral patches in the test domain and in Hensen's node (Fig. 5E, F). In the test domain, expression of both was restricted to the neuroectoderm (Fig. 5E', F'). At placodal stages, *Hes5* was expressed throughout a large rostrocaudal extent of the neural tube, extending rostrally throughout the developing cephalic region (Fig. 5K). This expression extended throughout the developing floor plate but was excluded from the developing roof plate (Fig. 5K'). In addition to neural tube expression, *Hes5* was expressed in bilateral patches near the otic placodes (Fig. 5K: arrows). Sections revealed that *Hes5* was expressed in the pharyngeal pouch endoderm subjacent to the otic placodes (Fig. 5K'). *Hes6* expression was more restricted, with *Hes6* being expressed medial to the somites, in the caudal hindbrain/rostral spinal cord, and in the metencephalon up to the midbrain boundary (Fig. 5L). To date there is no known role for *Hes5* or 6 in otic induction.

1.2.4 Transcription Factors

1.2.4a Hox genes: In our microarray, several of the Hox genes, particularly the Hoxa gene family, were upregulated in the test domain. Four Hoxa genes (*Hoxa1–4*) were identified in

our microarray data, along with *Hoxb1*. In the test domain, *Hoxa1* was expressed at 6-fold higher, *Hoxa2* was expressed at 8.5-fold higher, *Hoxa3* at 3.5-fold higher, *Hoxa4* at 3-fold higher, and *Hoxb1* at 36-fold higher than in the control domain.

In situ hybridization of these genes validated our microarray data (Fig. 6A–P). At stages 4–5, *Hoxa1–3*, and *Hoxb1* were all expressed in the primitive streak, with *Hoxa* members being restricted to the caudal half or less of the primitive streak (Fig. 6A–D; *Hoxa4* was not visualized by *in situ* hybridization in this study). At stages 6–7, *Hoxa1* was expressed more rostrally than the other *Hoxa* genes, completely overlapping the test domain, whereas *Hoxa2* and *3* overlapped approximately the caudal half of the test region (Fig. 6E–G). *Hoxa1* and *Hoxb1* were expressed only in the neuroectoderm, whereas *Hoxa2* and *3* were expressed in the both the neuroectoderm and underlying mesoderm (Fig. 6E'–H'). *Hoxb1* is of particular interest because it is expressed in two discrete patches (Fig. 6H, arrow) in the test region that are separated from the broad caudal expression that extends only as far rostrally as the caudal border of the first somites. Furthermore, Gavalas and colleagues (1998) demonstrated that *Hoxa1* and *Hoxb1* act synergistically in hindbrain formation, which subsequently provides signals for inner ear development, as we discussed above. They found that *Hoxa1* null mice had cochlear and vestibular malformations, whereas in double mutant mice in which both *Hoxa1* and *Hoxb1* were disrupted the entire inner ear was absent. Recessive loss of human *HOXA1* causes Athabaskan brainstem dysgenesis syndrome with associated sensorineural deafness due to inner ear malformations, including bilateral absence of the cochlea (Erickson 1999; Tischfield et al., 2005). Mutations in human *HOXA2* cause microtia, mixed hearing loss, and cleft palate (Alasti et al., 2008). From stages 8–12, all 4 *Hox* genes studied here were expressed throughout the ear-forming level of the neuraxis (Fig. 6I–P).

1.2.4b Additional transcription factors: Six other transcription factors differentially expressed in our microarrays were chosen for *in situ* hybridization studies. Five of those (*Gbx2*, *Meox1*, *Nkx6.2*, *Tbx22*, and *Tshz3*) were upregulated in the test domain, and one (*Dlx5*) was downregulated, as compared to the control domain. *Gbx2* was expressed in the test domain at 5-fold higher, *Meox1* at 23-fold higher, *Nkx6.2* at 13-fold higher, *Tbx22* at 2.5-fold higher, *Tshz3* at 5.5-fold higher, and *Dlx5* at 3-fold higher than in the control domain.

In situ hybridization of these genes validated our microarray data (Fig. 7A–Y). With the exception of *Tbx22* and *Tshz3* at stages 4–5 (Fig. D, E), all of the other transcription factors were expressed during all placode induction (Fig. 7A–C, F–R) and placodal (Fig. 7S–Y) stages.

Gbx2 expression has previously been described in early chick development (Shamim and Mason, 1998). In summary, *Gbx2* was expressed in the test domain neuroectoderm and adjacent medial non-neural ectoderm (Fig. 7G') and along the cardiac crescent and subsequent heart tube during the stages of induction (Fig. 7G, M). *Gbx2* expression persisted at the level of the otic placode (Fig. 7S; Shamim and Mason, 1998). *Gbx2* expression is required for normal endolymphatic duct formation in both chick (Miyazaki et al., 2006) and mouse (Lin et al., 2005). Whether the otic placode itself was abnormal at earlier stages in mutants was not reported.

Meox1, originally called *Gmox1*, was expressed bilateral to the node as early as stages 4–5 (Fig. 7B). At stages 6–7, the expression of *Meox1* extended caudally from the test domain through the first somite (Fig. 7H). At this time, expression in the test domain was restricted to the mesoderm (Fig. 7H'). As development proceeded, *Meox1* expression extended caudally down the length of the embryo, persisting in the developing somites (Fig. 7N, T).

Nkx6.2 was expressed in faint bilateral patches flanking the node as early as stage 5 (Fig. 7C). At stages 6–7, its expression extended through the test domain from the developing first somite

to about the level of the midbrain (Fig. 7I). Expression within the test domain was restricted to the neuroectoderm (Fig. 7I'). By stages 8–9, and into placodal stages, *Nkx6.2* expression extended more rostrally along the rostrocaudal axis and was found mainly in the developing mid- and hindbrain regions (Fig. 7O, U), and transiently in the rostral spinal cord (Fig. 7O).

The expression of *Nkx6.2* throughout the rostrocaudal extent of the neural tube persisted into post-placodal stages of development (Fig. 8A, B). At stage 15, *Nkx6.2* expression was restricted to the ventral midline of the neural tube, from the spinal cord extending rostrally to the isthmus at the juncture of the metencephalon and mesencephalon (Fig. 8A, A', A''). This expression pattern persisted through stage 18 (Fig. 8B). *Nkx6.2* was not expressed in the otic vesicles, although *Nkx6.2* was expressed in the ventral portion of the hindbrain adjacent to the vesicles (Fig. 8A'').

Tbx22, a member of the T-box family of transcription factors, was first expressed at stages 6–7, bilaterally in small faint patches within the test domain (Fig. 7J). Sections revealed that this expression was restricted to the mesoderm (Fig. 7J'). At stages 8–9, expression was more pronounced and expanded rostrally and caudally, extending throughout the caudal forebrain and mid- and hindbrain regions, and into the spinal cord, as well as being expressed in the somites (Fig. 7P). Later, at stages 10–11, expression persisted in the somites, but was absent from the mid- and hindbrain regions (Fig. 7V). However, *Tbx22* was expressed in midline rostral head mesenchyme at stage 10, just rostral to the tip of the forebrain (Fig. 7V; inset).

During post-placodal stages, expression of *Tbx22* became restricted to two areas: the somites and bilateral patches of ventral head mesenchyme (Fig. 8C–D). At stage 14, *Tbx22*-expressing cells flanked the floor of the developing midbrain as two distinct stripes (Fig. 8C–C'). By stage 16, *Tbx22*-expressing cells were more broadly distributed ventral and lateral to the mesencephalon and diencephalon, surrounding the developing pituitary rudiments (Fig. 8D, inset). *Tbx22* expression persisted in the developing somites throughout post-placodal stages, at least until stage 22 (not shown), the latest stage studied.

Loss of function mutations in *TBX22* cause X-linked submucous cleft palate with associated conductive hearing loss (Braybrook et al., 2001), but defects of the inner ear have not been reported.

The Teashirt zinc-finger 3 gene (*Tshz3*) was first expressed at stages 6–7 (Fig. 7K) in the primitive streak and in discrete bilateral patches in the neuroectoderm of the test domain (Fig. 7K'). By stages 8–9, *Tshz3* ectodermal expression was restricted largely to the neural folds, except for a broader area of neuroectodermal expression of the level of the developing hindbrain/rostral spinal cord (Fig. 7Q). At stage 12 *Tshz3* was expressed in the neural tube adjacent to the somites, with a bilateral patch of expression in rhombomere 4 (Fig. 7W; arrowheads).

Tshz3 expression persisted into post-placodal stages. At stage 17, *Tshz3* was expressed in a striped pattern along the anterior-posterior axis of the body. *Tshz3* was expressed throughout the length of the neural tube, extending rostrally up into the caudal hindbrain, adjacent to the otic vesicles (Fig. 8E, inset). Laterally and parallel to the neural tube, *Tshz3* was expressed in two bands, one on each side of the embryo, in the mesoderm flanking the developing gut tube (Fig. 8E'). By stage 21, *Tshz3* was expressed throughout much of the embryo, including the limb buds and neural tube (Fig. 8F). Sections through the otic vesicles revealed that *Tshz3* was expressed in the anterior-most portion of the otic vesicle (Fig. 8F') and in localized regions in the ventral neural tube where neurites accumulate. The region of expression in the otocyst likely coincides with the position of the developing statoacoustic ganglion and its projections.

Tshz3 is a good candidate for a gene involved in otic induction and patterning. *Tshz3* is a known down-stream target of FGF8 signaling in tendon development (Manfroid et al., 2006). This is of interest because FGF8 is a known inducer of otic development (Ladher et al., 2005), suggesting that *Tshz3* may also be a target of FGF8 signaling during otic induction by endodermal FGF8.

Shown for comparison, *Dlx5* was downregulated in the test domain as compared to the control domain. *Dlx5* was expressed as early as stages 4–5 in the rostral portion of the developing embryo (Fig. 7F). At stages 6–7, *Dlx5* was expressed in an oval-shaped region overlying the lateral plate mesoderm, just lateral to the developing neural plate and primitive streak (Fig. 7L). At this time throughout the head region, including within the test domain, *Dlx5* was expressed at the lateral edge of the neural plate and in the adjacent non-neural ectoderm, as well as in the underlying mesoderm (Fig. 7L'). Based on its early ectodermal expression, *Dlx5* (and other *Dlx* members) have been used as markers of the common pre-placodal domain (e.g., Brugmann and Moody, 2005; Streit, 2007). Although *Dlx5* was downregulated in the test domain as compared to the control domain, it was still expressed there, where it likely plays a role in otic development. By stage 8–9, *Dlx5* was expressed in the cranial portion of the embryo, in the heart tube, and in the developing tail bud and flanking mesoderm (Fig. 7R). Subsequently, it was expressed in several areas, most notably for the topic of this paper, in the developing eyes and otocyst (Fig. 7Y: arrowheads; Brown et al., 2005). Targeted disruption of *Dlx5* in mice resulted in craniofacial and inner ear defects (Acampora et al., 1999; Depew et al., 1999). In addition, disruption of both *Dlx5* and *Dlx6* also resulted in limb malformations that phenocopied Split hand/foot and mouth syndrome (SHFM) in humans (Robledo et al., 2002). The *Dlx5* and *6* genes are located in the SHFM type 1 locus on human 7q21, and approximately 35% of those with SHFM1 also have sensorineural, or mixed hearing loss (Elliott and Evans, 2006; Saitsu et al., 2009).

In conclusion, we have identified a number of novel candidate genes for otic placode induction. Although several of these candidate genes have roles at later stages of inner ear development, to our knowledge, this is the first time that the expression of most of them has been reported prior to otic placode formation. Several of the genes reported here had discrete bilateral patches of expression specific to the test domain and some surrounding tissues, indicative of potential roles in otic placode induction. Furthermore, a number of these genes have been associated with human or murine inner ear malformations and hearing loss.

2. EXPERIMENTAL PROCEDURES

2.1. Chick Embryos

Fertilized White Leghorn chicken eggs were obtained from Utah State University (Logan, UT, USA). They were incubated in humidified chambers at 38°C until embryos reached the desired stages, using the criteria of Hamburger and Hamilton (1951).

2.2. RNA Isolation, cDNA Preparation, Labeling, and Microarray Hybridization

For differential gene expression analysis by microarray, we collected from stage 6–7 chick embryos two domains, each consisting of all three germ layers: a test domain, and a more rostral control domain (Fig. 1A). The test domain, located just rostral to the first somite, consisted of the pre-otic placodal ectoderm with the adjacent hindbrain ectoderm and the underlying mesendodermal tissues. The control domain, located near the rostral end of the embryo, included tissue (surface or non-neural ectoderm) that is competent but not specified to express inner ear markers in explant assays (e.g., Ladher et al., 2000). Tissue isolates consisted of all three germ layers because previous studies showed that signals for otic placode induction arise from the endoderm, mesoderm, and hindbrain neuroectoderm of the ear-forming region

(Ladher et al., 2000,2005). Bilateral dissections from seven embryos were pooled for each (of four) biological replicates. RNA was isolated as described by Eisenhoffer et al. (2008) for micro-amounts of tissue. After microdissection of the test and control domains in a subset of embryos, we visualized *Fgf19* expression, one of the earliest expressed otic-inducing genes, to confirm our ability to reliably collect the test domain.

Cy-3 and Cy-5 fluorescently labeled cRNA was synthesized and microarray hybridizations were performed at the University of Utah Microarray Core Facility. Labeled cRNA was generated using the Agilent Two-Color Low RNA Input Linear Amplification Kit according to the manufacturer's protocol. Briefly, cDNA was synthesized from total RNA with MMLV-RT and dT/T7 RNA polymerase promoter oligonucleotide sequences. Labeled cRNA was then generated using T7 RNA polymerase and dye-labeled nucleotides. The cRNA was purified using the Qiagen RNeasy mini kit. The microarray analysis was performed using the Agilent 4×44k chicken genome array according to microarray methods established by Agilent. The raw data files were uploaded to the GEO Database and are accessible through GEO Series accession number GSE16918

(<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16918>).

2.3. Data Analysis

Microarray data was analyzed with the web-based GeneSifter® Analysis Edition (www.genesifter.net/web/) analysis software. Using this software, we performed a basic t-test that included: a quality cutoff value of 1, correction according to Benjamini and Hochberg, a minimum threshold limit of a 2-fold, and log2 transformation of the data. Our results included are presented based on the adjusted p-value assigned by the Benjamini and Hochberg correction.

2.4. In situ Hybridization

We performed whole mount *in situ* hybridization on stage 4–22 chick embryos as previously described (Chapman et al., 2002) using the following primers for digoxigenin probe design:

Ndp (cggatagtgcctgagagag-3'; 5'-gcacattgctgctgcttag-3')

Fz10 (5-agaagcacccaagtgagcat-3; 5-accacgacacagtaaagag-3)

Nkx6.2 (5-tcggacaatgaggatgatga-3; 5-aagtctttccaagcccgtgtg-3')

HoxB1 (5-cctcgagacggctaaaacag-3; 5-accacaggctttcaggatg-3)

Tshz3 (5-cgacactttgcaggaactca-3; 5-cccgtttccacaggttcta-3)

Hes5 (5-ccagagacaccaaccaact-3; 5-agatgtaccctgcaacctc-3)

Hes6 (5-ctaccggcacaaatccatct-3; 5-accacagctctcctctgt-3)

Gbx2 (5-ggtgcaggtgaagatctgtg-3'; 5-attcacaagacgggagttgg -3')

Meox1 (5-gtcccaaatgctcagaaag-3; 5-atgggttgagaggaccagtg-3)

Tbx22 (5-ggcctttaatttggggtaa-3; 5-gctcaatcctcatcctctgc-3)

Jag1 (5-cctgtggtcatggaggaact-3; 5-ggtgccagtgaatcctttgt-3)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Design strategy for microarray experiments. (A) Cartoon of a stage 7 chick embryo showing the test domain (a) and the more rostral control domain (b). The test domain is marked on its caudal boundary by the first somite (pink ovals) and includes all-three germ layers in the mediolateral plane from the pre-placodal non-neural ectoderm (blue circles) to the midline of the neural plate of the future caudal hindbrain. The neural plate is colored red, the floor plate orange, and the primitive streak green. (B) *In situ* hybridization (ISH) showing *Fgf19* expression in an embryo in which the test and control domains were removed prior to fixation and processing for ISH. The loss of most tissue expressing *Fgf19* in the extirpated area confirms that the test domain was correctly removed for the microarray study.

Fig. 2. FGF-signaling pathway. Members of the FGF-signaling pathway identified in our microarray study as differentially expressed were visualized by *in situ* hybridization at induction (A–L) and placodal (M–P) stages of development. *Fgf3*, *4*, and *19* are all expressed in bilateral patches of the test domain (E–G). In contrast, *Fgfr3* expression is reduced in the test domain as compared to the control domain (H and L, arrows). Stage 6–7 embryos (E–H) were sectioned through the test domain as indicated (black lines). The sections (E'–H') provide details on the germ layer in which each gene is expressed.

Fig. 3.

WNT-signaling pathway. Members of the WNT-signaling pathway identified in our microarray study as differentially expressed were visualized by *in situ* hybridization at induction (A–O) and placodal stages of development (P–T). At stages 4–5, *Wnt5b*, *Ndp*, and *Fzd10* are all expressed in the primitive streak, whereas *Fzd4* is not expressed at all, and *Fzd8* is restricted to the most rostral portion of the embryo, rostral to the node (A–E). *Wnt5b* and *Fzd10* are expressed throughout the entire length of the primitive streak (A, D) and *Ndp* is expressed only in the caudal half of the streak (B). By stages 6–7, *Wnt5b* and *Fzd10* have extended their streak-associated expression rostromedially into the test domain (F, I). At this time, *Ndp* and *Fzd4* are also expressed in the test domain (G, H): *Ndp* is expressed robustly in

bilateral patches within the test domain, with additional expression extending caudally into the developing somites, but its primitive streak expression is fading; and *Fzd4* is also expressed in bilateral patches in the test domain, but faintly, as well as in the cardiac crescent. Stage 6–7 embryos (F–J) were sectioned through the test domain as indicated (black lines). The sections provide details on the germ layer in which each gene is expressed (F'–J'). By stages 8–9 (K–O), all 5 of these WNT-family genes are expressed to some degree in bilateral patches in the test domain (arrows). Expression of *Wnt5b* extends caudally from the test domain (K, arrows) throughout the developing somite region and persists in the caudal portion of the streak, *Ndp* expression (L) extends into the area of the developing somites, *Fzd4* expression (M) persists in the test domain and in the developing heart tubes, and *Fzd10* (N) expression continues robustly in the primitive streak region. At this stage, *Fzd10* is faintly expressed in distinct patches in the test domain (N, arrows). Like *Fzd10*, *Fzd8* is only faintly expressed bilaterally in the test domain (O, arrows), but in contrast to the other WNT-family members, *Fzd8* is also expressed robustly in the most rostral portion of the embryo throughout the placode induction stages. At placodal stages (P–T), the expression of *Wnt5b* (P) is restricted to the tail bud and flanking mesoderm, *Ndp* is expressed throughout the spinal cord level of the neural tube (Q) and adjacent to the otic placode in rhombomere 4 of the hindbrain (Q, arrowheads), *Fzd4* is expressed throughout the heart and optic vesicles (R), *Fzd10* persists in the tail bud and surrounding mesoderm, extending rostrally throughout the spinal cord level of the neural tube and hindbrain (S), and *Fzd8* is expressed weakly throughout the brain level of the neural tube (T).

Fig. 4.

Expression of *Ndp* (A–C) and *Fzd10* (D–E) at otocyst stages. At stages 15–19, *Ndp* is expressed (A, B) throughout most of the length of the neural tube from the spinal cord to the mid-diencephalon region. By stage 22, *Ndp* expression in the neural tube is faint and restricted to specific patches (C, C', arrows); staining observed in the otic vesicle and other parts of the head in whole mounts was due to trapping. *Ndp* is also expressed at the dorsal region of the developing mesonephric kidneys (C'', arrowheads). *Fzd10* was also expressed along the rostrocaudal extent of the neural tube in a pattern very similar to *Ndp* at stages 14 (D) and 19 (E). However in sections, *Fzd10* expression within the neural tube was broader and restricted to its dorsal two-thirds (E'). ov, otic vesicle; nt, neural tube; lb, limb bud.

Fig. 5.

Notch-signaling pathway. Members of the Notch-signaling pathway identified in our microarray study as differentially expressed were visualized by *in situ* hybridization at induction (A-I), placodal (J-L), and post-placodal (M-N) stages of development. All three genes from the Notch-signaling pathway identified in our microarray are expressed in bilateral patches in the test domain beginning at stages 6-7 (D-F). *Hes5* (E) and *Hes6* (F) are also expressed in the node, whereas *Jag1* (D) is also expressed in the developing heart field. Stage 6-7 embryos (D-F) were sectioned through the test domain as indicated (black lines). The sections provide details on the germ layer in which each gene is expressed (D'-F'). At placodal stages, *Hes5* (K) and *Hes6* (L) are expressed in the hindbrain neural tube. In addition, *Hes5* is

expressed near the otic placodes in the pharyngeal pouch endoderm (K: arrowheads, K'). *Jag1* (J) expression at this time is concentrated in the developing heart. *Jag1* expression is more prominent in the post-placodal stages of otic development, with persistent expression in the heart and new areas of expression in the lens, mesonephric kidneys, and otic vesicles at both stage 15 (M) and stage 19 (N). Dorsal view (N') and sections at both stages 15 (M') and 19 (N'') show *Jag1* expression in the otic vesicle. op, otic placode; ov, otic vesicle; nt, neural tube; nc, notochord; mn, mesonephros; ht, heart.

Fig. 6.

Hox genes. Members of the Hox gene family of transcription factors identified in our microarray study as differentially expressed were visualized by *in situ* hybridization at induction (A–L) and placodal (M–P) stages of development. *Hoxa1–4* (*Hoxa1–3* shown here) and *Hoxb1* genes are all differentially upregulated in the test domain. Each of the Hox genes is expressed in the primitive streak at stage 4 (A–D). Expression in the primitive streak persists throughout placode induction stages, overlapping the test domain (E–P). The expression of *Hoxb1* is unique among the Hox genes identified in our microarray in that at stages 6–7 it occurs in bilateral patches in the test domain, separated from the broader expression domain along the caudal half of the embryo (H, arrow). Stage 6–7 embryos (E–H) were sectioned

through the test domain as indicated (black lines). The sections provide details on the germ layer in which each gene is expressed (E'–H').

Fig. 7.

Other transcription factors. A subset of other transcription factors identified in our microarray study as differentially expressed was visualized by *in situ* hybridization at induction (A–R) and placodal (S–Y) stages of development. Five other transcription factors chosen from our microarray data for further study are upregulated in the test domain, and all five are expressed in the bilateral patches in the test domain at stages 6–7 (G–L). Stage 6–7 embryos (G–L) were sectioned through the test domain as indicated (black lines). The sections provide details on the germ layer in which each gene is expressed (G'–L'). *Gbx2* expression spans the test domain patches and extends into the developing cardiac crescent (G). *Meox1* expression extends caudally from the test domain patches into the area of the developing somites (H, N, T), whereas

Nkx6.2 extends slightly rostrally from the test domain into the hindbrain (I, O, U). *Tbx22* is initially expressed in two small patches within the test domain (J) and later expression extends rostrally into the mid- and hindbrain and caudally into the forming somites (P). At placodal stages, *Tbx22* expression persists in the somites and in undefined head mesenchyme (V, inset; inset sectioned at level indicated by the black line in V). *Tshz3* is first expressed at stage 6 throughout the test domain, and in the regressing streak (K). Expression extends caudally throughout the middle portion of the embryo in the developing neural tube (Q). In addition to persistent expression of *Tshz3* through the mid-level of the neural tube, *Tshz3* is also expressed in rhombomere 4, adjacent to the otic placode (W, arrowheads). *Dlx5* is expressed in the pan-placodal region from stage 4 (F) through stages 6–7 (L). By stage 9 *Dlx5* is expressed in the heart tube and prosencephalon (R). At the placodal stages, *Dlx5* is expressed within the otic placode (Y, arrowheads).

Fig. 8.

Expression of selected transcription factors at otocyst stages. *Nkx6.2* expression (A–B). At stage 15, *Nkx6.2* is expressed in the neural tube from the spinal cord to the mid- and hindbrain regions (A). Sections revealed that *Nkx6.2* expression is restricted to the ventral midline of the neural tube, extending up into the isthmus at the juncture between the metencephalon and mesencephalon (A', A''). Expression persists along the neural tube and in the mid- and hindbrain regions through stage 18 (B). *Tbx22* expression (C–D). During otocyst stages *Tbx22* is expressed in the anteroventral portion of the developing somites (C–D). At stage 14, *Tbx22*-expressing mesenchymal cells flank the floor of the developing midbrain as two columns of cells (C, C'). By stage 16, these columns of mesenchymal cells expand to surround

the developing pituitary gland (D, inset). *Tshz3* expression (E–F). At stage 17, *Tshz3* is expressed in a striped pattern along the anterior-posterior axis of the body. *Tshz3* is expressed throughout the neural tube extending rostrally from the spinal cord into the posterior region of the hindbrain, adjacent to the otic vesicles (E, inset). Laterally and parallel to the neural tube, *Tshz3* is expressed in two bands on either side of the embryo flanking the developing gut tube (E'). By stage 21, *Tshz3* is expressed throughout much of the embryo, including the limb buds and neural tube (F). Sections through the otic vesicles revealed that *Tshz3* is expressed in its anterior-most portion (F'), as well as in the marginal zone of the neural tube. e, eye; ov, otic vesicle; nt, neural tube; st, stomach.

Table 1

Select genes differentially expressed in the pre-otic placode and adjacent signaling tissues

Gene Symbol	Gene Identifier	Gene Name	Ratio	adj. p-value *
FGF-Signaling				
FGF3	NM_205327	Fibroblast growth factor 3	4.0	0.0117
FGF4	NM_001031546	Fibroblast growth factor 4	2.6	0.0093
FGF19	NM_204674	Fibroblast growth factor 19	55.4	0.0023
FGFR3	NM_205509	Fibroblast growth factor receptor 3	-4.1	0.0080
Wnt-Signaling				
FZD10	NM_204098	Frizzled homolog 10	4.9	0.0129
FZD8	AF224318	Frizzled homolog 8	-11.3	0.0034
NDP	XM_416765	Norrie disease (pseudoglioma)	18.9	0.0031
WNT5B	NM_001037269	Wingless-type family, member 5B	3.5	0.0107
Notch-Signaling				
HES5	NM_001012695	Hairy and enhancer of split 5	9.2	0.0036
HES6	XM_422641	Hairy and enhancer of split 6	19.1	0.0023
JAG1	BX935649	Jagged 1 (Alagille syndrome)	2.2	0.0340
HOX Transcription Factors				
HOXA1	AY188521	Homeobox A1	5.9	0.0136
HOXA2	NM_205150	Homeobox A2	8.4	0.0071
HOXA3	NM_204548	Homeobox A3	3.7	0.0294
HOXB1	CR390382	Finished cDNA, clone ChEST424c1	35.7	0.0023
Other Transcription Factors				
GBX2	NM_205068	Gastrulation brain homeobox 2	5.2	0.0042
MEOX1	NM_204765	Mesenchyme homeobox 1	23.4	0.0023
NKX6-2	AF189767	NK6 homeobox 2	13.2	0.0100
TBX22	NM_204106	T-box 22	2.4	0.0228
TSHZ3	CR386769	Teashirt family zinc finger 3	5.6	0.0073
DLX5	NM_204159	Distal-less homeobox 5	-2.9	0.0060

* Indicates the adjusted p-value calculated by the GeneSifter™ software based on Benjamini and Hochberg correction. Genes were included in the dataset based on a p-value < 0.05.