FGF signaling regulates otic placode induction and refinement by controlling both ectodermal target genes and hindbrain Wnt8α

Lisa D. Urness a, Christian N. Paxton b, Xiaofen Wang a, Gary C. Schoenwolf b, Suzanne L. Mansour a,b,*

a Department of Human Genetics, University of Utah, 15 N 2030 E, RM 2100, Salt Lake City, UT 84112-5330, USA
b Department of Neurobiology and Anatomy, University of Utah, 30 N 1900 E, RM 2R066 SOM, Salt Lake City, UT 84132-3401, USA

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The inner ear epithelium, with its complex array of sensory, non-sensory, and neuronal cell types necessary for hearing and balance, is derived from a thickened patch of head ectoderm called the otic placode. Mouse embryos lacking both Fgf3 and Fgf10 fail to initiate inner ear development because appropriate patterns of gene expression fail to be specified within the pre-otic field. To understand the transcriptional “blueprint” initiating inner ear development, we used microarray analysis to identify prospective placode genes that were differentially expressed in control and Fgf3−/−;Fgf10−/− embryos. Several genes in the down-regulated class, including Hmx3, Hmx2, Foxg1, Sox9, Has2, and Scl26a9 were validated by in situ hybridization. We also assayed candidate target genes suggested by other studies of otic induction. Two placode markers, Fgf4 and Foxi3, were down-regulated in Fgf3−/−;Fgf10−/− embryos, whereas Foxi2, a cranial epidermis marker, was expanded in double mutants, similar to its behavior when WNT responses are blocked in the otic placode. Assays of hindbrain Wnt genes revealed that only Wnt8α was reduced or absent in FGF-deficient embryos, and that even some Fgf3−/−;Fgf10−/− and Fgf3−/− embryos failed to express Wnt8a, suggesting a key role for Fgf3, and a secondary role for Fgf10, in Wnt8α expression. Chick explant assays showed that Fgf3 or Fgf4, but not Fgf10, were sufficient to induce Wnt8α. Collectively, our results suggest that Wnt8α provides the link between FGF-induced formation of the pre-otic field and restriction of the otic placode to ectoderm adjacent to the hindbrain.

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Introduction

The inner ear is one of the most sophisticated vertebrate sensory organs, relaying both acoustical and motion/balance information to the brain. The entire structure, its intricate auditory and vestibular compartments, and innervating neurons, develops from the otic placode, a localized thickening of the caudalmost cranial ectoderm. All vertebrate cranial sensory organs derive from ectodermal placodes. Evidence suggests that these placodes emerge from a common ‘pan-placodal field’ that, shortly after gastrulation, surrounds the developing cranial neural plate in a narrow, horseshoe-shaped band. This field is defined as a distinct entity based on a characteristic set of expressed genes, including members of the Dkk, Eya, and Six gene families (Ohyama, 2009; Ohyama et al., 2007; Schlosser, 2006; Streit, 2007).

To form specific sensory placodes, the ectoderm must first acquire competence to respond to inductive signals (Groves and Bronner-Fraser, 2000). Many of the genes expressed in the pan-placodal field are transcription factors that may act individually or combinatorially as competence factors, preparing the ectoderm to receive subsequent inductive signals from the surrounding tissues (Martin and Groves, 2006; Schlosser and Ahrens, 2004). After acquiring competence, a second step in placode development is a differential response of competent pre-placodal ectoderm to a unique code of local inducing signals that specify particular placode identity along the anteroposterior axis of the head. Thus, placode induction consists of a series of consecutive inductive events rather than binary fate assignments (Jacobson, 1966; McCabe and Bronner-Fraser, 2009; Ohyama, 2009; Ohyama et al., 2007; Schlosser, 2006). Much effort is focused on elucidating the nature of these signals and the transcriptional effectors they, in turn, activate within the target ectoderm to induce the subsequent development of a given placode.

Fibroblast growth factor (FGF) family members play pivotal roles in inner ear induction in several species, although the identity and sources of the FGFs often differ among species (Ohyama et al., 2007; Schimmang, 2007; Wright and Mansour, 2003b). Our studies and others showed that FGF3 and FGF10 are the most proximal signals that induce otic placodal fate in mice. Both factors are expressed near the pre-otic field (in the hindbrain and mesencephyme, respectively) at the time of otic placode induction, and mice lacking both Fgf3 and Fgf10 either fail to form otic vesicles altogether, or form microvessicles that do not differentiate into ear structures (Alvarez et al., 2003; Wright and Mansour, 2003a). Similarly, chick Fgf3 and Fgf19 are required for expression of otic placode marker genes (Freret et al.,...
control and induction. Molecule(s) are required in vivo, and whether the FGF and WNT signaling modulates the otic placode mediated inhibition of ectodermal WNT signaling, or expression of activated β-catenin, caused an expansion of the otic domain at ectoderm. In contrast, constitutive activation of WNT signaling using soaked beads resulted in the expression of several otic markers, in chick otic placode signaling, in addition to FGF signaling, in chick otic placode development at inductive or later stages. We also assayed candidate genes suggested by other studies of otic induction, including those described above. Two placode markers, Fgf4 and Foxi3, were down-regulated in FGf3−/−;Fgf10−/− embryos, whereas Foxi2, a cranial epidermis marker, was expanded in the double mutants, similar to its behavior when WNT signaling is blocked in the otic placode. Assays of hindbrain Wnt genes revealed that only Wnt8a was reduced or absent in FGF-deficient embryos, and that even some Fgf3−/−;Fgf10−/− and Fgf3−/− embryos failed to express Wnt8a, suggesting a key role for Fgf3, and a secondary role for Fgf10, in maintaining Wnt8a. Moreover, chick explant assays showed that Fgf3 and Fgf4, but not Fgf10, were sufficient to induce Wnt8a. Taken together, our results show that FGF signaling is both necessary and sufficient to induce Wnt8a, and suggest that Wnt8a expression links FGF-induced formation of the pre-otic field with restriction of the otic placode to the ectoderm adjacent to the hindbrain.

Materials and methods

All mouse studies complied with protocols approved by the University of Utah Institutional Animal Care and Use Committee.

Generation of Fgf3 and Fgf10 conditional alleles

Fgf3- and Fgf10-genomic DNA-containing plagues were isolated by recombinase cloning from the KO2 library (Zhang et al., 2002). One phage bore an approximate 9.0 kb fragment of Fgf3, with a loxP site located in the intron 3.8 kb downstream of exon 2, and the other carried an approximate 10.0 kb fragment Fgf10, with a loxP site located in the intron 0.85 kb downstream of exon 2. After recovery of the phage inserts, we generated targeting vectors, each of which had a tetracycline response element (TRE) with or without an antisense CMV promoter element, followed by loxP and FRT sites, and Neo6 and Kan4 expression cassettes inserted in an intron 0.58 and 0.30 kb upstream of Fgf3 and Fgf10 exon 2, respectively. Each of the four targeting vectors was linearized and electroporated into R1–45 ES cells, which were selected in G-418 and ganciclovir as described (Li et al., 2007). Correct homologous recombination between the vectors and the genome was determined by Southern blot hybridization using 5′ and 3′ probes flanking the targeting sequences. Following introduction into C57Bl/6 embryos, correctly targeted cell lines generated high chimeric males that transmitted the targeted alleles to offspring. Phenotypic observations of targeted homozygotes showed that the initially targeted alleles were hypomorphic (data not shown). To remove the intronic selection cassettes, heterozygotes were crossed to FLPe-expressing mice (Gt(Rosa)26Sto/m1(Mis)Jml) [The Jackson Laboratory (JAX) strain #003946] (Farley et al., 2000). The resulting conditional strains were verified by Southern blot hybridization and PCR analyses (not shown) and have an intronic TRE with or without an antisense CMV promoter, and exon 2, comprised in both cases of 104 bp, is flanked by loxP sites. These alleles are designated c (no CMV promoter) or P (with the intronic CMV promoter). Each of the four homozygous conditional strains had normal viability, but Fgf3Pc or Fgf10Pc mice invariably showed slight tail kinks—a mild version of the Fgf3 null short-tail phenotype (Alvarez et al., 2003; Hatch et al., 2007; Mansour et al., 1993). All experiments reported here involved the “c” alleles. For global removal of exon 2, hypomorphic mice were crossed to the Cre deleter strain, BALB/c-Tg(CMV-cre)1Cgn/J (JAX stock #003465) (Schwenk et al., 1995). Mice/embryos homozygous for the exon 2 deletions (designated Δ2 alleles) or compound homozygotes with established null alleles (Fgf3+/+ or Fgf10+/+) (Mansour et al., 1993; Min et al., 1998) were indistinguishable from the established null homozygotes (data not shown).

Generation and genotyping of Fgf3/Fgf10 global null embryos

Global deletion of both conditional alleles was accomplished using Hprt1m1(cre)Mnn (Tang et al., 2002), kindly supplied by M. Capocci. Standard genetic crosses were used to generate Fgf3Δ2;Fgf10Δ2/+;HprtCre/+ females, which were crossed to Fgf3Δ2;Fgf10Δ2/c males. Allele-specific PCR assays for genotyping included the following primers: Hprt: SLIM10 (5′-GCCGTCTTCGCGAATGTTGG-3′), SLIM44 (5′-CCGGTTTATTTCTATAGCAGGATTTAC-3′), and SLIM545 (5′-TTGCTGACACTCTGGATCT-3′). The Jackson Laboratory (JAX) strain #003465 (Schwenk et al., 1995). Mice/embryos homozygous for the exon 2 deletions (designated Δ2 alleles) or compound homozygotes with established null alleles (Fgf3+/+ or Fgf10+/+) (Mansour et al., 1993; Min et al., 1998) were indistinguishable from the established null homozygotes (data not shown).
it is inadvisable to attempt to generate Fgf3Δ+/−:Fgf10Δ+/−;HprtCre+/− females from such males.

Otic placode RNA isolation and microarray analysis

Timed matings between Fgf3Δ−/−:Fgf10Δ−/−;HprtCre females and Fgf3−/−:Fgf10−/−;HprtCre males were initiated, and embryos were harvested on embryonic day (E)8.5. The yolk sac was collected for genotyping. The otic region, including the prospective placodal ectoderm, neural ectoderm, and underlying mesendoderm was dissected from 4–8 somite embryos using tungsten knives. Isolation of placodal ectoderm was accomplished by modification (Y. Saijoh, personal communication) of the enzymatic procedure described by Hogan et al. (1994). Briefly, the bilateral “tri-layer” otic region fragments were rinsed in ice-cold PBS and moved to 50 μl of PT solution (25 mg/ml pancreatic [Sigma], 5 mg/ml trypsin [Sigma], and 5 mg/ml polyvinylpyrrolidone MW360 [Sigma] in Tyrode’s solution) for 5 min on ice to promote germ layer separation. The fragments were transferred to ice-cold HEPES-buffered DMEM with 10% FBS for 2–5 min, then the placodal ectoderm and associated neural ectoderm were teased away from the mesendoderm with tungsten needles, and the placodal ectoderm was freed by resection of the attached neural ectoderm with a tungsten knife. The two placodal ectoderm fragments from each embryo (~100 μm each) were aspirated into 30 μl RLT buffer (Qiagen Micro-RNA Easy kit), vortexed for 1 min, and stored at ~70 °C. All tissue isolates were stored separately prior to genotyping.

RNA was extracted (Qiagen MicroRNA Easy kit) from three biological replica pools; each comprising 10 Fgf3Δ−/−:Fgf10Δ−/− or 10 Fgf3Δ+/−:Fgf10Δ+/− placentas. After elution, RNA integrity was determined on a Bioanalyzer (Agilent). Recovery was ~100 ng/pool. RNA was subjected to a single linear amplification labeling reaction (Fgf3Δ−/−:Fgf10Δ−/− control with Cy3, and Fgf3Δ+/−:Fgf10Δ+/− mutant with Cy5) and hybridized to microarray with whole genome microarray slides (Agilent GPL1344) using the standard Agilent 2-color gene expression hybridization protocol. Slides were scanned (Agilent G2505B) at 5 μm resolution using an extended dynamic range protocol, and images were processed with Agilent Feature Extraction software 9.5.1.1. The Lowess-normalized log2 ratio of Cy3/Cy5 was calculated. Genes significantly up- or down-regulated in all three mutant pools were identified by rank products analysis (Bretling et al., 2004). The full dataset was deposited with GEO (GSE18702).

Riboprobe preparation and in situ hybridization

Digoxigenin-labeled antisense cRNA probes were generated from plasmids carrying cDNA fragments according to standard procedures, or following direct PCR amplification of 3’ UTRs from genomic DNA (adapted from Ambion Technical Bulletin 154). For the latter, a 28-base T7 RNA polymerase promoter (5′-GACTCACTATAGGGAG-3′) was incorporated at the 5′ end of the reverse primer.

Cloned mouse cDNAs used to prepare riboprobes included Erm (Li et al., 2007), Wnt8a (Bouillet et al., 1996), Wnt1, Wnt3a, and Hmx3 (Hatch et al., 2007), Spy1 (Minowada et al., 1999), Fgf4 (Wright and Mansour, 2003b), Sox9 (Wright et al., 1995), and Wnt6 (Gavin et al., 1990) as described in the cited publications. An 886 bp Has2 3’ UTR clone was purchased from OpenBiosystems (GenBank accession AI592649, clone ID 633823).

3′ coding region probes for Foxg1 and Cldn4 were generated by cloning PCR-amplified DNA fragments into pCRII-TOPO (Invitrogen). The amplicons included residues 2615–2976 of Foxg1 (GenBank accession NM_001160112) and residues 392–749 of Cldn4 (GenBank accession NM_009903).

Probes for the 3′ UTRs of Foxi3, Foxi2, and Hmx2 were PCR-amplified from genomic DNA using T7 promoter-tagged reverse primers. The primer sequences were: Foxi3 forward primer SL6M56: 5′-ACTAACCCCTTTTCTGTTGC-3′, reverse primer SL6M55: 5′-T7-CGACATTTGCTACAGTAACAGC-3′; Foxi2 forward primer SL6M48: 5′-GCTTTGGTTGGCTACTTGC-3′, reverse primer SL6M47: 5′-T7-CAGCACACCGATGGAAACACAC-3′; Hmx2 forward primer SL6M91: 5′-TGAATCCGACCCAAAGTCTG-3′, reverse primer SL6M95: 5′-T7-GAGAGCTCCCCCTCTCAAATACT-3′; Slc26a9 forward primer SL6M61: 5′-TTCCTTGGCTTCTTACTTGC-3′, reverse primer SL6M62: 5′-T7-CCAGTGGCGGATTACATTAG-3′. PCR products of 530, 594, 534, and 554 bp respectively, were directly transcribed using T7 RNA polymerase.

Whole-mount in situ hybridization was performed on embryos isolated from timed pregnancies essentially as described (Wilkinson, 1992). Stained embryos were post-fixed in 4% paraformaldehyde, cryoprotected in 15% sucrose/7.5% gelatin, and cryosectioned at 14 μm (Sechrist et al., 1995).

Collagen gel cultures

The collagen gel matrix for tissue explant culture was made by mixing on ice, 25 μl of 10× DMEM, 40 μl of 7.5% sodium bicarbonate, 5 μl 1 M HEPES buffer (Invitrogen), and 200 μl of 2.5 mg/ml rat collagen (Roche). The matrix was gently stirred until it reached a pale pink color and was kept on ice until used.

Hamburger and Hamilton (HH) stage 4–5 ectoderm, located rostral to Hensen’s node, and consisting of both neural and non-neural ectoderm, was used as the test tissue (region “E”, Ladher et al., 2000, Fig. 2). Rostral ectodermal isolates were recombined with 0.1% BSA/PBS control or 0.25 μM recombinant human FGF (R&D Biosystems) coated Affi-blue gel beads (Bio-Rad Laboratories) in a 10 μl drop of collagen gel matrix, which was incubated at 37 °C until the matrix gelled. Tissue/head recombinants were cultured overnight at 37 °C in a humidified chamber (5% CO2/95% air) in medium consisting of Neurobasal Media (Invitrogen) supplemented with B27 serum-free supplement (Invitrogen), 200 mM L-Glutamine, and 1:1000 penicillin and streptomycin and then fixed in 4% PFA at 4 °C overnight. Explants were analyzed by in situ hybridization for expression of chick Wnt8a (Hume and Dodd, 1993).

Results

Conditional alleles enable efficient generation of Fgf3/Fgf10 double null embryos for isolation and microarray analysis of prospective otic ectodermal RNA

To enable conditional or global deletion of Fgf3 and/or Fgf10, we generated targeted alleles in which the 104 bp exon 2 of each gene was flanked by loxP sites (Fig. 1A; “c” alleles). Single or double homozygous conditional animals were normal (data not shown). In each case, germline deletion of exon 2 yielded a null allele (designated Δ2 or –, for brevity), and the gross phenotypes of homzygous null animals were indistinguishable from those generated by other means (Abler et al., 2009; data not shown; Hatch et al., 2007). For efficient generation of double mutants we crossed Fgf3Δ−/−:Fgf10Δ−/−;HprtCre+/− females with Fgf3Δ−/−:Fgf10Δ−/− males. Since CRE was deposited into oocytes, the “c” alleles were deleted to “Δ2” alleles in all offspring, regardless of whether they inherited Hprt Cre+/−. Thus, one quarter of the E10.5 embryos were double heterozygotes (Fgf3Δ−/−:Fgf10Δ−/−) that had a normal phenotype (Fig. 1B), and one quarter were double homozygotes (Fgf3Δ−/−:Fgf10Δ−/−) that, as expected, had shortened tails, lacked limbs, and had little or no otic vesicle development (Fig. 1C). To evaluate the efficacy of FGF signaling disruption at the otic placode stage, we stained E8.5 (7–8 somites) control and double mutant embryos with FGF signaling target genes, Erm and Spy1. Both genes were normally expressed in a domain that includes, but is larger than, the prospective otic placode (Fig. 1D,E,E1), and were strongly
in dorsal ectoderm of Fgf3/Fgf10 double null embryos generated from standard double heterozygous intercrosses (Alvarez et al., 2003; Wright and Mansour, 2003a). To enable a more comprehensive assessment of FGF-regulated otic ectodermal genes we compared gene expression profiles of three pools each of prospective otic ectoderm microdissected from 4–8 somite Fgf3Δ2/−;Fgf10Δ2/− and Fgf3Δ2/−;Fgf10Δ2/Δ2 embryos (Fig. 2A,B). Rank products analysis yielded a list of 64 unique transcripts significantly up-regulated and 28 down-regulated by at least 1.5-fold in the FGF-deficient ectodermal RNA pools (Table S1). Technical difficulties in perfecting embryo staging and tissue isolation are likely to account for the high degree of reduced in the dorsal (presumptive otic) portion of double mutant ectoderm (Fig. 1D,D2,E,E2), demonstrating successful disruption of FGF signaling in the prospective otic domain. Retention of Ern and Spry1 in the ventral ectoderm of double mutants suggests that this region continued to receive FGF signals, which could originate from the ventral ectoderm itself (e.g. Fgf4, see Fig. 3,B,B1, below) or from the pharyngeal endoderm (e.g. Fgf8, Ladher et al., 2005; Fgf15, Wright et al., 2004; or Fgf4, see Fig. 3,B,B1, below).

**Microarray analysis reveals candidate targets of FGF signaling**

Previous studies showed that expression of several otic placode marker genes, including Pax2, Gbx2, Dlx5, and Pax8, was not detected

![Fig. 1](Image 44x368 to 275x741)

**Fig. 1.** Use of Fgf3 and fgf10 conditional alleles to produce double null mutants that lack otic vesicles at E10.5 and FGF/MAPK signaling markers at E8.5. (A) Each targeted conditional allele has loxP sites (red arrows) flanking exon 2. An FRT site (black arrow) remains from FLP-mediated removal of a Neo expression cassette. A tetracycline response element (T) is located upstream of exon 2. CRE-mediated deletion of the 104 bp exon 2 from the conditional (c) alleles generates the null alleles (designated Δ2). Thick boxes indicate the sequences included within the targeting vectors; exons are indicated in green; 5’ and 3’ UTRs in red; introns in black; recombinase recognition sequences in pink; and the T in blue. The cross generating double mutants at a frequency of 25% is indicated below the allele diagrams. (B) E10.5 Fgf3Δ2/−;Fgf10Δ2/− embryo shows a normal phenotype. (C) E10.5 pharyngeal endoderm (e.g. the corresponding (thin) ectoderm in double mutants (D2, E2, arrows). Expression (arrows) of each gene in double heterozygotes and loss of gene expression in the corresponding (thin) ectoderm below each embryo. (D1, E1) Transverse sections taken through the placodal region were probed with FGF signaling indicators, tail (t), and otic vesicle (ov) where present. Whole-mount E8.5 (7-somite) embryos vesicles, and has a shortened tail. Dashed lines demark limbs (planes numbered and indicated with dashed lines in panels C–G) show otic placode (op) expression of each gene in double heterozygotes (C1–G1, black arrows) and loss of gene expression in the corresponding (thin) ectoderm in double mutants (C2–G2, red arrows). Carets and arrowheads indicate Fgf-independent expression in pharyngeal endoderm, neural tube (nt), and migrating neural crest (mnc), respectively.

![Fig. 2](Image 312x203 to 543x595)

**Fig. 2.** Isolation of tissue and validation by in situ hybridization of microarray candidate genes down-regulated in placodal ectoderm of Fgf3/Fgf10-deficient embryos. (A) Schematic depiction of microdissection of the placodal region of an E8.5 5–8 somite embryo. Embryos were first bisected along the midline (vertical dashed line). The dorsal, presumptive otic region, was isolated from the ventral aspect with a second cut (horizontal dashed line), generating two fragments containing neural tube (nt), placodal ectoderm (ec), and mesendoderm (m, en). (B) Protease treatment of each hemisected otic region released the mesendoderm from the neural tube and attached placodal ectoderm, which was then severed from the nt and recovered for RNA extraction. (C–G) 7–8 somite Fgf3Δ2/−;Fgf10Δ2/− (3−/−;10−/−) and Fgf3Δ2/−;Fgf10Δ2/Δ2 (3−/−;10−/−) embryos hybridized with riboprobes for Hmx3 (C), Foxg1 (D), Sox9 (E), Has2 (F), and Slc26a9 (G). Anterior is to the top. Transverse sections taken through the placodal region (planes numbered and indicated with dashed lines in panels C–G) show otic placode (op) expression of each gene in double heterozygotes (C1–G1, black arrows) and loss of gene expression in the corresponding (thin) ectoderm in double mutants (C2–G2, red arrows). Carets and arrowheads indicate Fgf-independent expression in pharyngeal endoderm, neural tube (nt), and migrating neural crest (mnc), respectively.
variability between transcript levels detected in pools of the same genotype, which in turn may account for the small number of genes ultimately identified as significantly regulated in all three comparisons.

**Validation of FGF-dependent ectodermal target genes**

To validate candidate down-regulated transcripts we compared gene expression in Fgfg3Δ/+;Fgf10Δ/− and Fgfg3Δ/−;Fgf10Δ/+ 7–8 somite embryos by whole mount in situ hybridization. Hmx3, Foxg1, Sox9, Has2, and Scc26a9 were all clearly expressed in control otic ectoderm (Fig. 2C,C1,D,D1,E,E1,G,G1), but were absent from Fgfg3/Fgf10-deficient dorsal ectoderm (Fig. 2C2,C2,D,D2,E,E2,G,G2) which, unlike control dorsal ectoderm, failed to thicken. Retention of Foxg1, Sox9, and Has2 in double mutant ventral ectoderm (Fig. 2D1,D2,E2,F2) was consistent with retention of FGF signaling indicators in this region. Other sites of gene expression (e.g. pharyngeal endodermal Hmx3, Foxg1, and Has2) were unaffected in double mutants (Fig. 2C1,C2,D1,D2,E1,E2,F1,F2). Indeed, expression of Sox9 in the neural tube and migrating neural crest was unaffected in double mutants (Fig. 2E1,E2), indicative of the high degree of ectodermal target tissue purification achieved by the dissections. We also tested Hmx2 expression and found that it, too, was absent from dorsal double mutant ectoderm (data not shown). In contrast, two placode-expressed genes (Cldn4 and Zic4) and one ubiquitously expressed gene (Dusp16) were unchanged in double mutants (data not shown). Several other ubiquitously or relatively non-specifically expressed genes (Runx2, Sbn, Yawae, and Gpr83) were not examined in double mutants. Finally, Kifc1 and Dcc may represent false positives, as neither gene was expressed in control placodes, but could be detected elsewhere. Taken together, these results indicate that our strategy of gene expression profiling successfully enriched for otic genes that are directly or indirectly dependent on FGF signaling, revealing at least six genes not previously associated with FGF responses in the mouse otic placode.

**Other ectodermal genes regulated by FGF signaling include Fgf4 and Foxi genes**

We next examined the expression of several other potential targets of FGF signaling in otic placode induction, including Fgf4, which previous studies showed is expressed in a restricted portion of the prospective placode in 4–11 somite embryos (Fig. 3A,A1 and Wright et al., 2003) and Foxi3 and Foxi2, which mark the prospective otic and non-otic ectoderm, respectively (Fig. 3C,C1,E,E1) (Ohyama and Groves, 2004). Like the down-regulated candidates identified in the microarray screen, Fgf4 expression was absent from the dorsal ectoderm of 7–8 somite double mutants (Fig. 3B,B1). Interestingly, although Fgf4 expression was unchanged in ventral double mutant ectoderm, it appeared up-regulated in pharyngeal endoderm (Fig. 3B,B1). As endodermal Fgf4 is normally strongly up-regulated at the 9-somite stage, it is unclear whether this finding is significant, or merely a result of somite counting ambiguities. Foxi3 expression was also eliminated from dorsal ectoderm in all five 4–7 somite stage double mutant embryos (Fig. 3D,D1), but was only slightly reduced in the two 8–9 somite stage double mutants examined (Fig. 3D2). In contrast, Foxi2 expression was expanded in double mutant ectoderm in 7–8 somite embryos (Fig. 3F,F1,F1,F2). Furthermore, by the 11–12 somite stage, when Foxi2 expression was excluded from the otic cup of control embryos (Fig. 3G,G1), it was expressed throughout an inappropriately enlarged ectodermal domain in double mutant embryos (Fig. 3H,G2). Even when a small ventrally localized otic cup-like structure formed in double mutants (Fig. 3H1), some Foxi2 expression encroached into the thickened region, suggesting that it did not have a true otic character, much like the microvesicles found previously in older double mutants (Wright and Mansour, 2003a).

**FGF signaling is required for hindbrain Wnt8a expression**

The expansion of Foxi2 in Fgfg3Δ/−;Fgf10Δ/− ectoderm is similar to the response of head ectoderm when WNT signaling to the Pax2 lineages is blocked by Tg(Pax2-cre) otic conditional ablation of β-catenin (Ohyama et al., 2006). Hindbrain WNT signals are implicated in otic induction (Freter et al., 2008; Ladher et al., 2000; Ohyama et al., 2006; Park and Saint-Jeannet, 2008). To determine whether FGF signals function upstream of hindbrain WNT signals during otic induction, we assayed hindbrain Wnt genes in control and FGF-deficient mutants. Wnt6 (Fig. 4A) and Wnt3a (Fig. 4C) were expressed as early as the 7-somite stage, and Wnt1 (data not shown) was expressed from the 12-somite stage in the dorsal neural ectoderm, from which the neural crest emigrates; however, expression of these three Wnt genes was unaffected in Fgfg3Δ/−;Fgf10Δ/− embryos (Fig. 4B,B1,B2; Wnt1 data not shown). In contrast, Wnt8a, which is expressed in hindbrain rhombomere (r)4 at least as early as the 4-somite stage and diminished dramatically by the 9-somite stage (Fig. 4E; Bouillet et al., 1996 and L.D.U. and S.L.M., unpublished), was significantly reduced (Fig. 4F, n = 2), or in some cases absent (n = 2), in the double mutants (Table 1). Hindbrain Wnt8a was also absent from some Fgfg3Δ/−;Fgf10Δ/− (Fig. 4H, n = 3) and Fgfg3Δ/− embryos (Fig. 4J, n = 2). However, Wnt8a expression was normal in all but one Fgfg3Δ/−;Fgf10Δ/− embryos (Fig. 4G, n = 4) and in all Fgfg3Δ/− embryos (Fig. 4I, n = 2), suggesting that FGF signaling is directly or indirectly required for hindbrain Wnt8a expression, and that Fgfg3 plays a more significant role in this process than does Fgf10.

To determine whether WNT receptors are expressed in the prospective otic placode, we surveyed expression of several Fzd genes and found that Fzd1 is most specific to the placode (Fig. 4K), but expression of Fzd8 could also be detected (Fig. 4L).

**FGF signaling is sufficient to induce Wnt8a in a chick ectodermal explant assay**

Human FGF19 induces chick Wnt8a in ectodermal explants that include neural tissue (Ladher et al., 2000). To determine whether additional otic region FGFs are sufficient to induce Wnt8a expression, we combined rostral, ectodermal isolates from HH stage 4–5 chick embryos with FGF protein-coated beads in collagen gel culture. The explants were cultured overnight and then assayed for Wnt8a expression via in situ hybridization (Table 2, Fig. 5). Weak Wnt8a expression was detected in only 1 of 10 explants exposed to control beads. However, both Fgf4- and, to a lesser extent, Fgf3-coated beads, induced expression of Wnt8a (Fig. 5A–C). FGF4 strongly induced Wnt8a nearly all of the time (11 of 12 explants), whereas Fgf3 induced a weak signal less frequently (4 of 12 explants). In contrast, FGF10 almost never induced Wnt8a (1 out 12 explants, Fig. 5D).

**Discussion**

Induction of the mouse otic placode from pre-otic ectoderm located adjacent to the hindbrain requires Fgf3 and Fgf10, which are expressed in the hindbrain and head mesenchyme, respectively. In the absence of these signals, the otic vesicle either does not form, or only a small, rudimentary vesicle is induced (Alvarez et al., 2003; Wright and Mansour, 2003a). Although in some circumstances FGFs modulate target tissue proliferation and/or survival, in the case of Fgfg3/Fgf10-deficient embryos, these processes are unperturbed in the prospective otic territory (Wright and Mansour, 2003a). Instead, FGF signaling controls gene expression within the pre-otic ectoderm, and the small set of known FGF target genes, identified serendipitously, are presumably contained within a larger set of genes that serve as a molecular blueprint directing the subsequent differentiation of the otic anlage. Here we identified and validated additional FGF targets that are new candidates for genes involved in otic placode induction
and/or subsequent FGF-regulated processes in ear development. In addition, we uncovered a mechanism linking FGF and WNT signaling in series in the refinement of the otic territory (Fig. 6).

Microarray analysis reveals FGF target genes important for inner ear development

Microarray-based comparison of control and FGF3/10-deficient ectoderms revealed a relatively small set of up- and down-regulated FGF target gene candidates, with theoretical changes in expression of between 1.5- and 4-fold. We attribute the small set size to several factors, including small differences in staging between embryos that were subsequently pooled, the variation inherent in the use of microdissection and enzymatic digestions for isolating small tissue samples, and stochastic differences amplified in extremely small RNA sets.

Consequently, it is not surprising that previously identified FGF-responsive placodal transcription factor genes (including *Pax2*, *Pax8*, *Gbx2*, and *Dlx5*) did not appear on the list, which likely represents a mere "tip of the iceberg". Given these considerations, it is striking that six of the nine down-regulated candidate genes evaluated by in situ hybridization in both genotypes were strongly reduced or completely absent specifically from double mutant ectoderm. It remains to be determined, of course, whether these genes are directly or indirectly regulated by the FGFs, but their differential expression cannot be a trivial consequence of the absence of an otic placode in double mutants, given that they are expressed (and were evaluated) prior to placode-thickening.

Three transcription factor genes (*Hmx3*, *Hmx2*, and *Foxg1*) shown previously to be required for otic morphogenesis are FGF dependent and fall within this class. Targeted single and double mutagenesis of the tandemly duplicated *Hmx3* and *Hmx2* genes revealed that they are required for normal vestibular development in mice (Hadrys et al., 1998; Mennerich et al., 1999; Wang et al., 2001, 2004; Wang and Lufkin, 2005; Wang et al., 1998). In addition, some patients with hemizygous deletions encompassing *HMX2* and *HMX3* present with vestibular dysfunction, congenital sensorineural hearing loss, and inner ear malformations, implicating these genes in human, as well as mouse, inner ear development (Miller et al., 2009). However, the abnormalities described are more consistent with roles in otic vesicle morphogenesis than in otic placode induction. A third member of this gene family, *Hmx1*, is expressed in the developing otic vesicle (Munroe et al., 2009; Yoshiura et al., 1998), but apparently has no

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**Fig. 3.** Other placode-expressed genes are differentially affected in Fgf3/Fgf10-deficient ectoderm. Somite-matched E8.5 Fgf3^{−/−};Fgf10^{−/−} (3^{−/−};10^{−/−}) embryos were hybridized with probes for Fgf4 (A,B), Foxi3 (C,D), and Foxi2 (E–H). Anterior is to the left in A–D and to the right in E–H. Transverse sections taken through the otic region (planes numbered and indicated with dashed lines in panels A–H) show otic placode (op) expression of each gene in double heterozygotes (A1,C1,E1, black arrows; red caret indicates lack of expression in pharyngeal endoderm). Fgf4 was absent from dorsal double mutant ectoderm (B1, red arrow), but up-regulated in pharyngeal endoderm (B1, caret) and Foxi3 was absent from dorsal ectoderm in double mutants (D1, red arrow). Foxi2 expression was expanded both anteriorly (F1) and posteriorly (F3) in 7–8 somite double mutants, and overall expression in the placodal region was more intense (F2). In 11–12 somite embryos, Foxi2 expression was restricted from the otic cup (oc) in control embryos (G1,G2,G3), whereas Foxi2 was present throughout the dorsal ectoderm (ec) in double mutants (H1,H2,H3). The ventrally localized cup-like structure (“oc”), which may represent the precursor to one of the microvesicles occasionally seen in double mutants, showed incomplete clearing of Foxi2 expression (H2).
Fig. 4. Wnt8a is the only hindbrain Wnt that is dependent on Fgf3 and Fgf10 expression, and the otic field expresses at least two WNT receptor (Fzd) genes. Somite-matched E8.5 control Fgf3+/−; Fgf10+/−; (+/−; 10−/−; A,CE) and Fgf3+/−; Fgf10+/−; (+/−; 10−/−; B,DF) embryos were hybridized with probes for Wnt6 (A,B), Wnt3a (C,D), and Wnt8a (E,F). Wnts and Wnt3a were expressed in control neural plates (A,C), and expression was unchanged in double mutant embryos (B,D). r4 expression of Wnt8a (E) was reduced or absent (F) in all four double mutants. Fgf3+/−; Fgf10+/−; (+/−; 10−/−; C) and Fgf3+/−; Fgf10+/−; (+/−; 10−/−; I) embryos showed normal levels of Wnt8a in 4/5 and 4/4 embryos respectively. In contrast, Fgf3+/−; Fgf10+/−; (+/−; 10−/−; H) and Fgf3+/−; Fgf10+/−; (+/−; J) showed reduced Wnt8a expression in 3/6 and 2/4 embryos respectively. WNT receptor genes Fzd1 (K) and Fzd8 (L) were expressed in the otic placode (op).

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wnt8a expression in r4</th>
<th>5−8 somite mouse embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>3+/−/+</td>
<td>10−/+</td>
<td>Reduced: 0/2/0/1/0/0/0</td>
</tr>
<tr>
<td>3−/−/+</td>
<td>10−/+</td>
<td>Absent: 0/2/3/0/0/2/0/0</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Beads</th>
<th>Wnt8a pos./total</th>
<th>Wnt8a ++</th>
<th>Wnt8a +</th>
<th>Wnt8a −</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1/10</td>
<td>0</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>FGF4</td>
<td>11/12</td>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>FGF3</td>
<td>4/12</td>
<td>0</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>FGF10</td>
<td>1/0</td>
<td>0</td>
<td>1</td>
<td>11</td>
</tr>
</tbody>
</table>

++ = strong expression.
+ = weak expression.
− = no expression.

Fig. 5. FGF4 or FGF3, but not FGF10, induces Wnt8a in chick ectodermal explants. HH stage 4−5 ectodermal explants were cultured with control or FGF protein-coated beads, and Wnt8a expression was assessed by in situ hybridization. Control beads (A) were unable to induce Wnt8a, whereas FGF4 induced robust expression of Wnt8a (B, arrows) and FGF3 induced weak Wnt8a expression in approximately 1/3 of the explant cultures (C, arrow). In contrast, FGF10 did not induce Wnt8a (D). See Table 2 for quantification of the results.

et al., 2000) and otocyst-expressed Hmx2 and Hmx3 are down-regulated in SU-5402-treated zebrafish embryos (Feng and Xu, 2010).

Fox1, another otic placode–expressed transcription factor gene identified in our microarray study, is also expressed in and required for normal morphogenesis, as well as for innervation of the vestibular system (Hwang et al., 2009; Pauley et al., 2006). The Fox1+/−/− abnormalities are related to, but much milder than the Fgf10+/−/− vestibular defects, suggesting that Fox1 may be directly or indirectly regulated by FGF signaling during otic vesicle morphogenesis, similar to its behavior during placode induction.

Sox9, which encodes an SRY-box transcription factor, was also strongly down-regulated in Fgf3−/−; Fgf10−/− mutants. Sox9 is required for otic placode specification in Xenopus and zebrafish (Liu et al., 2003a; Saint-Cermain et al., 2004) and its expression is clearly dependent upon FGF signaling; it is down-regulated both in Fgf3 and Fgf8-deficient zebrafish otic ectoderm (Liu et al., 2003a) and Fgf3 and Fgf10-deficient mouse otic ectoderm (this study). Importantly, Sox9 otic conditional mutants show normal otic placode induction, but the next step of otic development, namely, placode invagination to form a cup, fails (Barrionuevo et al., 2008). Thus, our differential expression screen is clearly capable of identifying FGF-regulated genes that are critical for both otic placode and vesicle morphogenesis.

Only two validated target genes, Has2 and Sloc26a9, do not encode transcription factors. Has2 encodes hyaluronan synthase-2, which catalyzes the synthesis of hyaluronan, an extracellular glycosaminoglycan. In Xenopus inner ears, hyaluronan is found in the space between the mesenchyme and the axial protrusions of otic epithelium that eventually fuse to form the semicircular canals, where it serves as a propellant in the fusion process (Haddon and Lewis, 1991). Although the mechanism of semicircular canal formation differs...
somewhat between frogs and mice, hyaluronan and Has2 expression are also found in the developing mouse inner ear (McPhee et al., 1987; Tien and Spicer, 2005). Unfortunately, global Has2−/− mice die too early to address its roles in semicircular canal formation (Camenisch et al., 2000). It will be interesting to study semicircular canal morphogenesis using the recently reported Has2 conditional strain (Matsumoto et al., 2009). Has2−/− embryos do form an otic vesicle; therefore, Has2 does not seem to be required for placode induction. A role for Has2 in otic induction could be masked, however, by redundancy with other gene family members (Tien and Spicer, 2005).

Slc26a9, which encodes a chloride channel required for gastric acid secretion (Dorwart et al., 2008; Xu et al., 2008), has not been associated previously with otic expression or development. However, its relatives in the SLC26 family of solute carriers—Slc26a4, encoding Pendrin, and Slc26a5, encoding Prestin—play critical roles in mouse ear development and function (Everett et al., 2001; Liberman et al., 2002) and are mutated in human hearing loss subjects (Everett et al., 1997; Li et al., 1998; Liu et al., 2003b). Studies of auditory function in Slc26a9−/− mice are underway.

Expression of MAPK pathway targets, Fgf4, and Foxi genes is altered in Fgf3−/−;Fgf10+/− mutants

Concomitant with the microarray analysis, we also determined the FGF responsiveness of pre-otic field genes identified in other studies. Not surprisingly, common transcriptional targets of the MAPK pathway mediating FGF signals, Erm and Spry1, were absent from dorsal double mutant ectoderm. Similarly, Foxi4, which is the only Foxi other than Foxi2 itself, expressed in the mouse otic placode (McKay et al., 1996; Wright et al., 2003; Wright and Mansour, 2003a) was also lost from dorsal ectoderm. This suggests that FGF4 might be required downstream of FGF3 and FGF10 to maintain or augment the placodal response to FGF3 and FGF10. Mice lacking Fgf4 die during pre-implantation (Feldman et al., 1995), but Tg(Pax2 cre)-mediated otic conditional ablation of Fgf4 did not reveal a unique role for this FGF in otic induction or subsequent function of the inner ear (S. L. Mansour and E. P. Hatch, unpublished). Therefore, to determine the role of Fgf4 in the otic placode it will be necessary first to generate a conditional Fgf4−/− allele, as these Fgf4 genes are located within 20 kb of one another.

Foxi1 is a master regulator of otic development in zebrafish, and appears to function in a pathway parallel to that of FGF signaling (Hans et al., 2007; Solomon et al., 2004). Mouse Foxi1 is expressed and required at a later stage of inner ear development (Hulander et al., 2003), however, the expression patterns of mouse Foxi family members, Foxi3 and Foxi2, coincide spatiotemporally with the period of otic induction. Foxi3 is expressed in a broad ectodermal region beginning at E6.5, well before otic induction, and is maintained until approximately the 8-somite stage, when it starts to be down-regulated in the placode (Ohyama and Groves, 2004). Foxi3 was virtually absent from the dorsal ectoderm of 4–7 somite Fgf3−/−;Fgf10+/− embryos, but could be detected in 8–9 somite double mutants, suggesting that in the mouse, there are both FGF-dependent and independent pathways of Foxi3 activation. It will be interesting to learn whether mouse Foxi3 is the functional ortholog of zebrafish Foxi1 in otic placode induction, but even if so, there are clearly differences between the two species with respect to the relationship between Foxi1/3 genes and FGF signaling.

FGF signaling is required not only to specify the pre-otic domain, but also to regulate the WNT signaling that restricts the otic placode to dorsal ectoderm

Foxi2 is initially expressed throughout the cranial ectoderm, including in the presumptive placode and more ventral ectoderm that is fated to become epibranchial placode or epidermis, but is subsequently excluded from the dorsal ectoderm as otic placode fate is specified. Exclusion is particularly evident by 11-somites as the placode thickens in preparation for invagination (Ohyama and Groves, 2004). The expanded domain of ectodermal Foxi2 expression we found in 7–8 somite Fgf3−/−;Fgf10+/− mutants, and the failure of the hindbrain proximal ectoderm to extinguish Foxi2 expression at 11–12 somites is similar to the behavior of Foxi2 when WNT signal reception in the ectoderm is blocked by Tg(Pax2 cre)-mediated otic conditional ablation of β-catenin (Ohyama et al., 2006). Since WNT signals, presumably emanating from the hindbrain, have long been implicated in otic induction (Freter et al., 2008; Ladher et al., 2000; Ohyama et al., 2006; Park and Saint-Jeannet, 2008), this result suggested that FGF signaling might control the WNT signal that restricts the otic domain. Indeed, we found that FGF signaling is required for r4 expression of Wnt8a, the only hindbrain-expressed gene affected among those tested here and previously (Wright and Mansour, 2003a), suggesting that Wnt8a is the signal, or a component of the WNT signal, that restricts the otic placode to the hindbrain proximal portion of the Pax2-positive pre-otic field. We also found that Wnt8a expression reduced or absent in some Fgf3−/−;Fgf10+/−
and Fgf3/−/− embryos, but not in Fgf3−/−;Fgf10+/− or Fgf10−/− embryos, suggesting that Fgf3 may be the main regulator of Wnt8a (Fig. 6). Furthermore, our chick explant studies showed that FGF3 and, more potently, FGF4, were able to induce Wnt8a expression, whereas FGF10 was not. Taken together with the mouse loss of function data, these results show that FGF3 is both necessary and sufficient for Wnt8a expression, whereas FGF4 is sufficient but not uniquely necessary, and FGF10 is necessary but not sufficient. Preliminary data suggest that this FGF/WNT regulatory pathway is unidirectional, as R5→R6 expression of Fgf3 was unperturbed in embryos with Tg[Wnt1-cr]-mediated conditional deletion of β-catenin (S. L. Mansour and E. P. Hatch, unpublished).

Canonical WNT/β-catenin signaling relies on activation of Frizzled (Fzd) receptors (Angers and Moon, 2009; MacDonald et al., 2009; van Amerongen and Nusse, 2009) and consistent with this expectation we found two Fzd genes that are expressed in the prospective otic territory. Within the caudal cranial region, Fzd1 was exclusive to the otic placode, whereas Fzd8 was more widely expressed but was strongest within the otic domain. In chick, Fzd1, Fzd2, and Fzd7 are expressed at otic placode and otic vesicle stages (Sienknecht and Fekete, 2009; Stark et al., 2000), suggesting roles, at least for Fzd1, in otic development. Genetic loss-of-function analyses will be necessary to test the roles of Wnt8a and the Fzd genes in the restriction of the otic placode.

FGFs and WNTs have interactive roles at multiple stages of inner ear development. Otic induction is initiated by FGF signaling, with WNT limiting the final otic domain (Freter et al., 2008; Ladher et al., 2000; Ohyama et al., 2006; Park and Saint-Jeannet, 2008; this study; Whittaker et al., 1999). At otic placode and otic vesicle stages (Sienknecht and Fekete, 2009; Stark et al., 2000), suggesting roles, at least for Fzd1, in otic development. Genetic loss-of-function analyses will be necessary to test the roles of Wnt8a and the Fzd genes in the restriction of the otic placode.

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