Identification of Synergistic Signals Initiating Inner Ear Development

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Tissue manipulation experiments in amphibians more than 50 years ago showed that induction of the inner ear requires two signals: a mesodermal signal followed by a neural signal. However, the molecules mediating this process have remained elusive. We present evidence for mesodermal initiation of otic development in higher vertebrates and show that the mesoderm can direct terminal differentiation of the inner ear in rostral ectoderm. Furthermore, we demonstrate the synergistic interactions of the extracellular polypeptide ligands FGF-19 and Wnt-8c as mediators of mesodermal and neural signals, respectively, initiating inner ear development.

In amphibians, induction of the otic placode, which forms the inner ear, has been shown to require a mesodermal signal followed by a neural signal (*1, 2*). Neuroectodermal signals have also been shown to be involved in chick and mouse, but definitive proof for mesodermal signaling has yet to be demonstrated (*2–5*). In all systems, the signaling factors involved have remained elusive. Fibroblast growth factors (FGFs) have been implicated in otic development. In particular, FGF-3, which is essential for inner ear development, is crucial for the later stages of otic induction, acting within the otic ectoderm and for otic maintenance (*2, 6–8*). In addition to roles in otic development, FGFs are involved in caudalization of the neuroectoderm, partly by signaling through the paraxial mesoderm (*9*). Because otic development is dependent on mesodermal and neural signals, this raises the possibility that FGFs in the paraxial mesoderm may also play a role in otic induction. By low-stringency screening of a cDNA library, we identified a new chick FGF: FGF-19 (*10*). In situ expression analysis suggested a potential role for FGF-19 in otic development, which we have characterized here (*11*).

During early chick embryogenesis, *Fgf-19* is expressed transiently (stages 6 to 9) in a restricted region of paraxial mesoderm, initially underlying the neural plate (stages 6 and 7), whereas later it is in contact with neural and nonneural ectoderm (Fig. 1, A to C and F to I). From stage 9^-, *Fgf-19* is also expressed in the endoderm and transiently in the developing neural tube until stage 9^+ (Fig. 1, C to E and H to K) [Web fig. 1 (*12*)]. Fate mapping of *Fgf-19*–expressing mesoderm at stage 7 and neuroectoderm at stage 9 (*13*) showed that these regions colocalize with the presumptive otic placode along the rostro-caudal axis (Web fig. 2). The otic placode arises lateral to the neural plate and initially at stage 7 does not overlie *Fgf-19*–expressing mesoderm, but will later be in contact with or in close proximity to *Fgf-19*–expressing mesoderm as the neural groove closes (Web fig. 2).

Coincident expression of *Fgf-19* in the mesoderm and neural tube with the position of the presumptive placode made FGF-19 a candidate to direct otic development. Therefore, we tested the ability of *Fgf-19*–expressing mesoderm at stage 7 (regions a and b, Fig. 2) versus nonexpressing mesoderm (regions c and d, Fig. 2) to direct otic development in stage 5 ectoderm (Et) (*14*). Otic development was assessed using markers that are expressed at various stages of inner ear development from the presumptive placode to terminal differentiation (*15*). *Fgf-19*–expressing mesoderm induced all otic markers tested, whereas nonexpressing mesoderm did not (Fig. 2, A through D, and Web fig. 3) (*16*). At stage 9^+, the equivalent mesoderm (a′, Fig. 2), which now does not express *Fgf-19*, could not induce otic development, as assessed by *Pax-2* and *Nkx5.1* expression (Fig. 2E and Web fig. 3). Only when adjacent neuroectoderm (N, Fig. 2), which expresses *Fgf-19*, was included could otic development proceed (Fig. 2F and Web fig. 3). This supports work in amphibians demonstrating that mesodermal signaling acts over a defined period and is followed by a neural signal (*1, 2*).

Because *Fgf-19* expression correlated spatially and temporally with mesodermal and neural otic-inducing signals, we tested whether FGF-19 alone could induce expression of otic markers in the presumptive, but uncommitted, otic region (region c, Fig. 2) and nonotic regions (regions d and e and Et, Fig. 2) (*17*). FGF-19 alone did not induce any of the otic markers tested when added to the presumptive otic (c) or nonotic (e) regions but could induce otic markers in nonotic tissue if neural (d) or presumptive neural tissue (Et) was included (region c: *Dlx-5*, *Fgf-3*, *Nkx5.1*, *Pax-2*, and *SOHo-1*; region e: *Pax-2* and 0/4; Fig. 3 and Web figs. 4 and 6) (*16*). These data suggest that under certain conditions one molecule can initiate several components of the otic signaling cascade, provided that a neural signal is also present.

In *Xenopus laevis* embryos, cooperative Wnt and FGF signaling modifies cell fate, and it has been proposed that FGFs mediate neural crest production via Wnt signaling (*18–20*). Thus, we investigated the role of Wnts, which are expressed in the neuroectoderm (*21*), during early otic development. We focused on Wnt-8c because it is expressed in

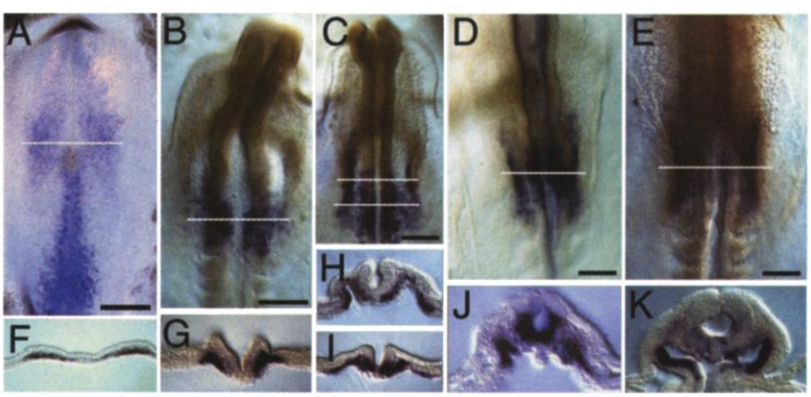

Fig. 1. *Fgf-19* expression is associated with otic development. (**A** through **K**) Whole-mount in situ hybridizations show the expression of *Fgf-19* in chick embryos at stages 6 (A and F), 8^+ (B and G), 9^- (C, H, and I), 9^+ (D and J), and 10^- (E and K). [(F) through (K)] Section analyses through the levels indicated show that *Fgf-19* is expressed in the paraxial mesoderm between stages 6 to 9 [(F) through (I)], the developing caudal hindbrain between stages 9^- to 9^+ [(I) and (J)], and endoderm from stage 9^- until at least stage 24 [(I) through (K); see Web fig. 1] (*16*). Scale bars: 500 μm (A), 250 μm [(B) and (C)], and 125 μm [(D) and (E)].

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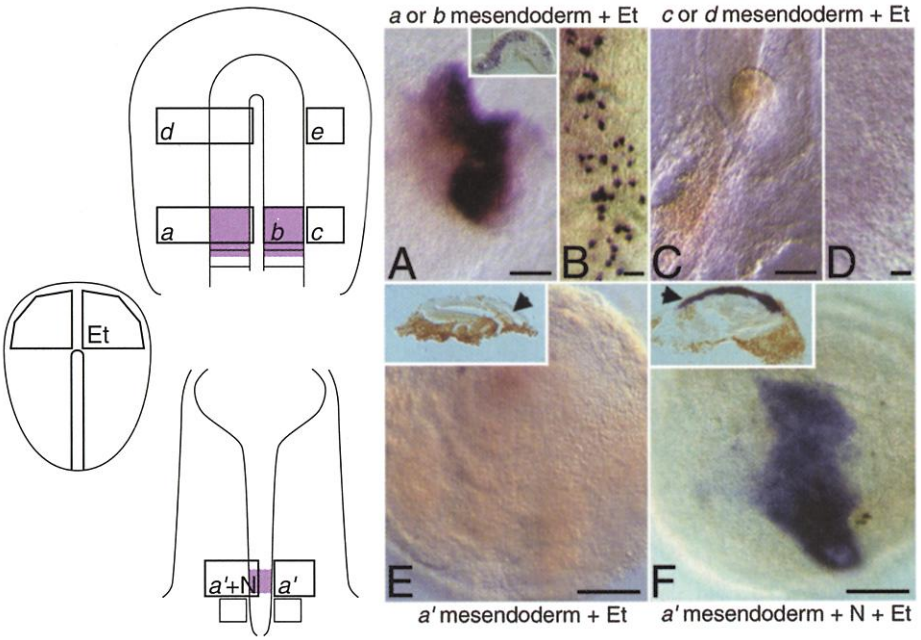

Fig. 2. *Fgf-19*–expressing mesoderm directs otic development. Diagram shows the region of stage 5 ectoderm, Et (test ectoderm) and stage 7 mesendoderm (regions a through d) or stage 9^+ mesendoderm a′, which were recombined in collagen gel culture. The *Fgf-19*–expressing regions are purple. The otic placode arises from region c. Whole-mount in situ hybridizations and antibody staining show that only regions a and b, which include *Fgf-19*–expressing mesoderm, and not regions c, d, or a′, can induce the expression of *Pax-2* (**A**, **C**, and **E**) and development of inner hair cells (**B** and **D**) (*Pax-2*, region a, $n = 5/5$; b, $n = 5/5$; c, $n = 0/5$; d, $n = 0/5$, a′, $n = 0/5$. Inner hair cells, a, $n = 4/4$; b, $n = 4/4$; c, $n = 0/4$; d, $n = 0/4$). However, a′ can induce the expression of *Pax-2* when the overlying neural tube N, which expresses *Fgf-19*, is included [(**F**), $n = 5/5$]. The inset in (A) confirms that *Pax-2* expression is confined to the ectoderm while the insets in (E) and (F) show section analysis following antibody staining to determine the position of the quail cells (stained brown). Scale bars: 100 μm (A, C, E, and F) and 10 μm (B and D).

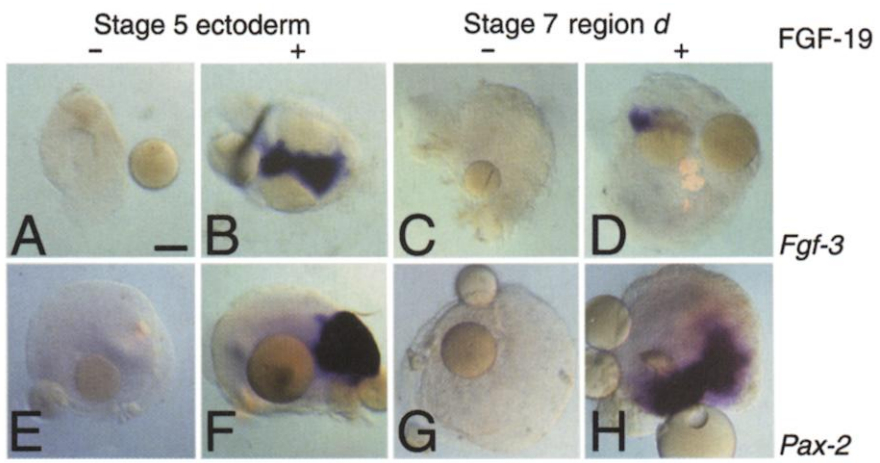

Fig. 3. FGF-19 can induce components of the otic signaling cascade. Whole-mount in situ hybridizations show that FGF-19 (**B**, **F**, **D**, and **H**) but not control (**A**, **E**, **C**, and **G**) beads can induce the expression of otic markers *Fgf-3* (A through D), *Pax-2* (D through H) in stage 5 ectoderm (A, B, E, and F); Et, Fig. 2] and in region d (Fig. 2) at stage 7 (C, D, G, and H). For additional data, see Web fig. 4. Although these genes are expressed in other ectodermal derivatives (*15*), the combinatorial expression of these genes is consistent with the induction of otic pathway components. Stage 5 ectoderm: *Fgf-3*, $n = 3/4$; *Pax-2*, $n = 4/5$. Region d: *Fgf-3*, $n = 4/10$; *Pax-2*, $n = 4/5$. Scale bar: 100 μm.

the neural plate, initially overlying *Fgf-19*–expressing mesoderm (Web fig. 5) (*22*). Later, both are coexpressed in the caudal hindbrain until stage 10, after which the otic inductive capabilities of the hindbrain are lost (Web fig. 5) (*5, 22*). Furthermore, FGF-19 can induce the expression of *Wnt-8c* in preneural stage 5 ectoderm (Fig. 4, A and B) but not in presumptive otic tissue (region c, Fig. 2; $n = 12$; 6 to 18 hours) (*16, 23*). This raised the possibility that mesodermal FGF-19 initiates and/or maintains *Wnt-8c* expression in the neural plate, acting via or in cooperation with Wnt-8c to direct otic development.

To test this, we cultured the uncommitted presumptive otic region (region c, Fig. 2) in the presence of one or both of these factors (*17, 24*). As previously determined, FGF-19 alone could not induce expression of otic markers (Fig. 4, D, H, and L, and Web fig. 6). Wnt-8c alone induced strong expression of *Fgf-3* (Fig. 4M); however, only weak or negligible expression of *Dlx-5* ($n = 1/3$), *Nkx5.1* ($n = 3/4$), and *SOHo-1* ($n = 3/5$) was observed (Web fig. 6). The combination of both FGF-19 and Wnt-8c consistently induced strong expression of *Pax-2* (Fig. 4F), *Dlx-5* (Fig. 4J), and *Nkx5.1* and *SOHo-1* (Web fig. 6). Furthermore, section analysis identified regions of thickened ectoderm characterized by otic gene expression ($n = 8/11$), and in one case, with vesicular morphology indicative of otic placode development (*16*). Otic induction does not occur through the prior induction of neural tissue because FGF-19 or Wnt-8c, together or alone, do not induce neural characteristics in the presumptive otic region (region c, Fig. 2), as assessed by lack of *Sox-2*, *Wnt-8c*, and *Fgf-19* expression following treatment (*16, 25*). Additionally, FGF-19 and Wnt-8c can cooperate to induce at least one otic marker, *Pax-2*, in non-otic ectoderm (region e, Fig. 2; $n = 3/3$) (*16*), suggesting that these molecules are sufficient to initiate otic development.

We propose that FGF-19 in the paraxial mesoderm induces and/or maintains *Wnt-8c* expression in the competent neuroectoderm (*26*). Subsequently, Wnt-8c induces *Fgf-3* in the presumptive otic placode but cooperates with FGF-19 to induce the other otic markers investigated: *Nkx5.1*, *Pax-2*, *SOHo-1*, and *Dlx-5*. Wnt-8c alone can also induce weak expression of some otic markers, either directly or possibly via the induction and subsequent synergy with *Fgf-3* (*8*). The inability to induce inner hair cell development ($n = 0/11$) may suggest that additional independent signals are required, or that the temporal expression of Wnt-8c and FGF-19 used in these studies is insufficient, or alternatively, inhibitory for differentiation.

Embryological studies in amphibians (*1*) and chicks (*5*) indicate that inner ear induction starts during or after late gastrulation, coincident with expression of *Fgf-19* in chicks. We propose FGF-19 as an inducer of otic development, acting in part by patterning the neuroectoderm. This suggests that, in general, localized expression of mesodermal signals may be the key components to pattern the tissue layers. Furthermore, we have characterized a novel function of Wnt signaling. This will facilitate the elucidation of additional signaling interac-

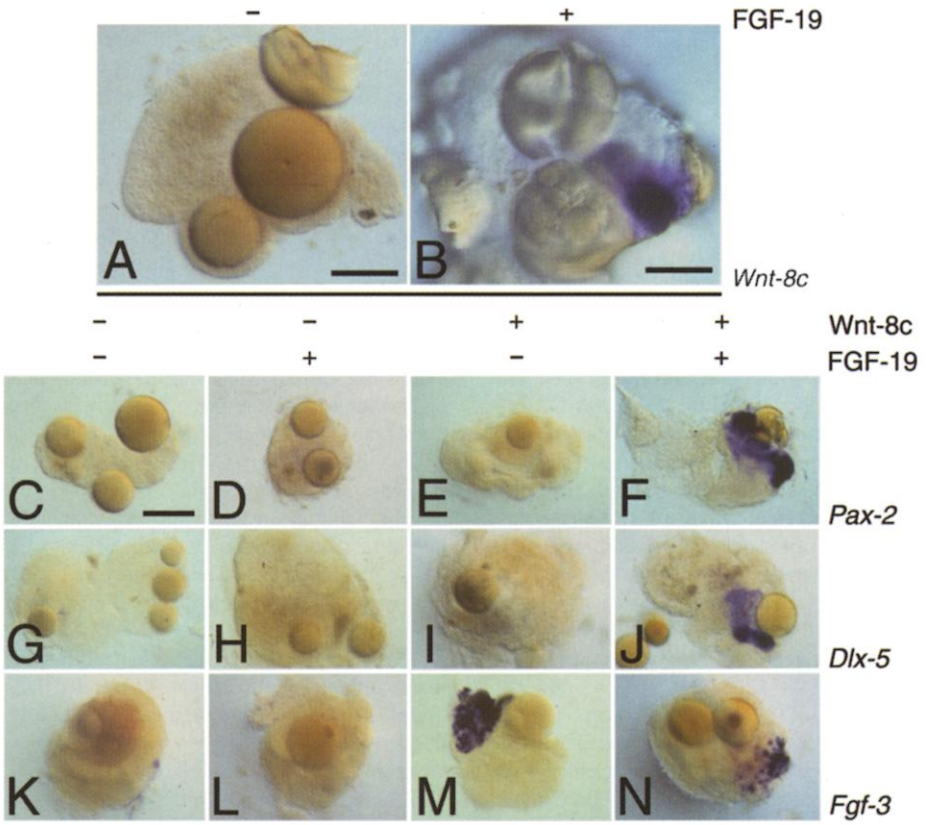

Fig. 4. FGF-19 may initiate otic development by the induction of Wnt-8c. Whole-mount in situ hybridizations show that FGF-19–soaked beads (**B**), but not control beads (**A**), induce the expression of *Wnt-8c* in rostral stage 5 ectoderm (n = 5/7; Et, Fig. 2). (**C** through **N**) Whole-mount in situ hybridizations show that Wnt-8c alone can induce *Fgf-3* expression, while FGF-19 and Wnt-8c synergize to induce expression of other otic markers. Stage 7 region c (Fig. 2) explants do not express otic markers when cultured with beads soaked in a control solution (C, G, and K) or FGF-19 (D, H, and L). Similarly, with the exception of *Fgf-3* [(M); n = 3/3], *Wnt-8c*–expressing COS cells do not strongly induce otic markers *Pax-2* (E) and *Dlx-5* (I). However, region c (Fig. 2) explants strongly express the otic markers *Pax-2* [(F); n = 4/4] and *Dlx-5* [(J); n = 3/3] when co-cultured with both Wnt-8c–expressing cells and beads soaked in FGF-19. Scale bars in (A) through (C): 200 μm. For additional information, see Web fig. 6 (*12*).

tions controlling otic development. Otic development is characterized not only by differentiation of several cell types, but also by complex morphogenetic changes.

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10. The Fgf-19 probe was obtained by screening a stage 11/12 chick λZAP II cDNA library using a mouse *Fgf-15* cDNA probe as in P. H. Francis, M. K. Richardson, P. M. Brickell, and C. Tickle [*Development* **120**, 209 (1994)]. The sequence has been deposited in GenBank (accession number AF315355).
11. Whole-mount in situ hybridization for spatiotemporal gene expression analysis was carried out using a modification of A. Myat, D. Henrique, D. Ish-Horowicz, and J. Lewis [*Dev. Biol.* **174**, 233 (1996)], such that all washes included 0.25% Triton X-100.
12. Supplementary Web figures are available at www.sciencemag.org/cgi/content/full/290/5498/1965/DC1
13. Lineage labeling was carried out as described in S. Yuan and G. C. Schoenwolf [*Development* **125**, 201 (1998)]. Embryos were subsequently sectioned at 50 μm.
14. Chick and quail embryos were staged according to V. Hamburger and H. L Hamilton [*J. Morphol.* **88**, 49 (1951)]. Chick or quail stage 7 or 9^+ mesendoderm (mesoderm and endoderm) and ectoderm were separated with 5 units/ml dispase for 3 to 5 min at room temperature. They were either heterotopically recombined or explanted alone. Quail and chick recombinations were performed where appropriate to ensure that expression of otic markers was not due to contamination of cultures with presumptive otic ectoderm. Quail cells and inner hair cells were localized using a QCPN antibody (Developmental Studies Hybridoma Bank) and hair cell antibody (gift of Guy Richardson), respectively. Whole-mount stained embryos were sectioned at 50 μm. Explants were cultured in defined media for 8 hours for analysis of *Wnt-8c* expression, 36 hours for *Dlx-5*, *Nkx5.1*, *Pax-2*, and *SOHo-1*, and 10 days for inner hair cell analysis, using a defined serum-free medium (Neurobasal with B27 supplement; Gibco Life Technologies, catalog no. 17504-010). Explants were cryosectioned at 20 μm.
15. *Dlx-5*, *Nkx5.1*, and *Pax-2* mark the presumptive otic placode; *SOHo-1* is expressed in the otic cup; the antibody to hair cell antigen labels inner hair cells, marking the terminal stages of inner ear differentiation (*5*, *27*–*30*). Note that *Dlx-5* is also expressed throughout the most rostral ectoderm; later, this is down-regulated except in the developing otic and olfactory placodes. *Pax-2* is also found in other ectodermal derivatives: the eye, the epibranchial placodes, and midbrain-hindbrain junction. *SOHo-1* is expressed in the epibranchial and otic placodes. *Nkx5.1*, in the chick, is exclusive to the otic placode, and the inner hair cells are only derived from the inner ear. Therefore, *Nkx5.1* and inner hair cells are definitive markers for inner ear development. However, because previous studies have suggested that multiple signaling pathways/interactions may control inner ear development (*5*, *30*), it is important to analyze a number of genes.
16. R. K. Ladher, K. U. Anakwe, A. L. Gurney, G. C. Schoenwolf, P. H. Francis-West, data not shown.
17. Heparin beads were washed in phosphate-buffered saline (PBS) and soaked in recombinant human FGF-19 (0.24 mg/ml; 70% amino acid similarity with chick FGF-19), or in 0.1% bovine serum albumin in PBS for 1 hour at 37°C before application to explants that were cultured as in (*14*). In these experiments, regions c, d, and e were explanted intact and therefore consisted of mesendoderm and overlying ectoderm. Induction of *Fgf-3* was also determined as its temporospatial pattern of expression suggested that it may be downstream of FGF-19 signaling [R. Mahmood, P. Kiefer, S. Guthrie, C. Dickson, I. Mason, *Development* **121**, 1399 (1995)].
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23. In region d, FGF-19 induced otic markers within 36 hours (Fig. 3) but not *Wnt-8c* at 12 hours (n = 2) or 18 hours (n = 2), possibly due to transient expression of *Wnt-8c* and/or loss of competence of the neuroectoderm to respond. Therefore, other Wnts such as *Wnt-1* and *Wnt-2*, which are expressed in this region, may substitute for the Wnt-8c signal (*16*).
24. Wnt-8c–expressing cells were obtained by transient transfection of COS cells with pMT23/Wnt-8c using Superfect (Qiagen) for 4 hours. Wnt-8c–expressing cell pellets or control cells expressing β-galactosidase were grafted into explants 24 to 48 hours later.
25. *Sox-2* expression following culture with FGF-19 alone (n = 2), Wnt-8c alone (n = 4), or FGF-19 and Wnt-8c (n = 4) was assessed after 18 hours. *Wnt-8c* expression after FGF-19 treatment of region c was assessed between 6 and 18 hours (n = 12). *Fgf-19* expression after Wnt-8c treatment of region c was assessed after 18 hours (n = 4).
26. *Pax-2* and *Dlx-5* are expressed in the presumptive otic placode before neural expression of *Fgf-19*, suggesting that mesodermal FGF-19 expression initiates otic induction. *SOHo-1* and *Nkx5.1* are expressed later during otic development, suggesting that FGF-19 signaling from the neural tube is also involved in otic development. Other studies have shown that, in vivo, FGF-3 can induce *Nkx5.1* and *Pax-2* (*8*). *Nkx5.1* was induced weakly by Wnt-8c alone, possibly via induction of *Fgf-3*. It is also possible that FGF-3 may subsequently synergize with Wnt-8c to promote otic induction.
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