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Progressive restriction of otic fate: the role of FGF and Wnt in resolving inner ear potential

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The development of the vertebrate inner ear is an emergent process. Its progression from a relatively simple disk of thickened epithelium within head ectoderm into a complex organ capable of sensing sound and balance is controlled by sequential molecular and cellular interactions. Fibroblast growth factor (FGF) and Wnt signals emanating from mesoderm and neural ectoderm have been shown to direct inner ear fate. However, the role of these multiple signals during inner ear induction is unclear. We demonstrate that the action of the FGFs and Wnts is sequential, and that their roles support a model of hierarchical fate decisions that progressively restrict the developmental potential of the ectoderm until otic commitment. We show that signalling by Fgf3 and Fgf19 is required to initiate a proliferative progenitor region that is a precursor to both the inner ear and the neurogenic epibranchial placodes. Significantly, we find that only after FGF action is attenuated can the subsequent action of Wnt signalling allow otic differentiation to proceed. In addition, gain and loss of function of Wnt-signalling components show a role for this signalling in repressing epibranchial fate. This interplay of signalling factors ensures the correct and ordered differentiation of both inner ear and epibranchial systems.

KEY WORDS: Inner ear, Epibranchial, Sensory placode, Fibroblast growth factor, Wnt

INTRODUCTION

The inner ear of all vertebrates develops adjacent to the hindbrain from a thickened disk of epithelium known as the otic placode. The otic placode forms in stereotypical locations in almost all vertebrates, forming in the non-neural ectoderm just rostral to the level of the first somite and on either side of the hindbrain. Otic induction is mediated by the localised action of signalling factors (Baker and Bronner-Fraser, 2001). In both chick and mouse, the inner ear is initiated by the action of localised fibroblast growth factor (FGF) signals (Kil et al., 2005; Ladher et al., 2005). In the case of chick, the combined actions of Fgf3 and Fgf19 acting from the mesoderm, initiate a cascade of events that ultimately result in the induction of the inner ear. One important step in this cascade is the induction in the overlying neural plate of the Wnt signalling protein Wnt8c (Ladher et al., 2000). FGF and Wnt signalling are then thought to induce otic fate in adjacent, non-neural ectoderm. However, these studies do not resolve the temporal requirements for these interactions nor do they assign individual roles to each signal.

The role of Wnt signalling in otic induction has been subject to considerable speculation. Experiments from zebrafish seem to discount a direct role for Wnt signalling in the early specification of the inner ear (Phillips et al., 2004), although data from the mouse contradict this view (Ohyama et al., 2006). Modulation of canonical Wnt signalling in the mouse suggests that this signalling actually directs a fate choice between otic and epidermis tissue within a progenitor field. This progenitor region, which was termed the 'pre-otic field', is marked by the expression of *Pax2*. However, data from both genetic labelling in the mouse and vital dye labelling in the chick show that the *Pax2* expression domain should, more properly, be considered to encompass the inner ear and the epibranchial placodes, a group of neurogenic placodes that will give rise to the geniculate, petrosal and nodose ganglia (Ohyama and Groves, 2004b; Streit, 2002). To reflect these derivatives, and to prevent any ambiguity with a definition for 'pre-otic' that could be understood as the region of the embryo rostral to the otic placode, we have termed the *Pax2*-expressing progenitor domain the otic-epibranchial progenitor domain (OEPD). Although the mechanisms of OEPD induction are not fully understood, in zebrafish an FGF signal has been proposed as being sufficient and necessary for the induction of both otic and epibranchial placodes (Nechiporuk et al., 2007; Nikaido et al., 2007; Sun et al., 2007). Such a common precursor domain may also reflect a common evolutionary relationship between the inner ear and epibranchial derivatives (Baker et al., 2008).

Inner ear cells must be specified from the precursor domain, and the view that otic fate restriction is progressive is suggested by careful examination of the timing of particular genes within the chick otic placode. *Pax2* is expressed at around the 4 somite stage (ss). However, the otic placode is not actually morphologically visible until 8/9ss. At this stage, inner ear markers, such as *Nkx5.1*, *Soho1* and *Bmp7*, begin to be expressed (Baker and Bronner-Fraser, 2001; Groves and Bronner-Fraser, 2000). Experiments that isolate the otic placode from potential extrinsic signals suggest a further dimension; the progressive restriction of inner ear fate is due to changes in either the nature or the duration of signalling interactions. Otic ectoderm isolated at 5ss and cultured for 24 hours will express *Pax2*; however, it will not express later markers, such as *Bmp7*. Only otic explants isolated after 7-8ss express both *Pax2* and *Bmp7* (Groves and Bronner-Fraser, 2000). The relevance of such a restriction is not clear, and indeed these experiments do not identify the nature of the signals or the mechanisms by which they act.

In this report, we provide definitive evidence for the progressive restriction of developmental fate in inner ear induction. Overexpression and inhibition studies allow us to determine the clear functional significance of FGF and Wnt signalling during early inner ear induction. We find that an initial pulse of FGF establishes a mitotically active progenitor domain to both the inner ear and

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epibranchial placodes, the OEPD. Subsequently, the attenuation of FGF together with the action of canonical Wnt signalling allow the medial region of the OEPD to commit to an inner ear fate. Conversely, epibranchial placode differentiation is enhanced by continued FGF signalling but inhibited by canonical Wnt signalling. Thus, the progressive restriction of inner ear and epibranchial potential results from the interplay of the FGF and Wnt signals that form the basis of signalling checkpoints that determine the correct and orderly differentiation of the inner ear and the epibranchial placodes.

MATERIALS AND METHODS

Embryos

Fertilised hens' eggs (Shiroyama Farms, Kanagawa, Japan) were incubated in a humidified chamber at 38°C. Embryos were staged by counting somites, or, if prior to somitogenesis, by using the stage series of Hamburger and Hamilton (Hamburger and Hamilton, 1992).

Embryo dissections

Embryos at 1ss/HH7-16ss/HH12 were removed and washed in Ringer's solution. Two types of explant were performed. In both cases, the inner ear region was dissected by making two transverse cuts, just rostral to the first somite and just caudal to rhombomere 3. Presumptive otic explants were dissected away from neural ectoderm and underlying paraxial mesendoderm. Presumptive otic regions are similar but were just bisected at the midline. The explanted tissue was transferred immediately to a chilled 10-μl drop of collagen. Once set, collagen drops were flooded with pre-warmed medium [DMEM+10% knockout serum replacement (KSR; Invitrogen, CA]. Otic regions were additionally incubated in media supplemented with 100 ng/ml recombinant human DKK1 (R&D Systems). Explants were grown for 1 or 7 days in a humidified CO_2 incubator, at which time they were rinsed in PBS and fixed in 4% PFA for one hour.

Collagen drops were processed for in situ hybridisation and immunohistochemistry as described previously (Wright et al., 2004).

DNA constructs and electroporation

Knockdown of *Fgf3* and *Fgf19* was performed using shRNA-encoding constructs. Briefly, putative shRNA sequences designed against 20 bases starting from +251 and +324 of *Fgf3* and *Fgf19*, respectively were inserted into pSilencer 1.0 (Ambion). In addition, a scrambled oligonucleotide based on the *Fgf19* +324 sequence was inserted in pSilencer (sh*Scrambled*) to act as a control for non-specific effects. To overexpress *Fgf3* and *Fgf19*, coding regions were cloned downstream of the Ef1α promoter. A stabilised, and thus, constitutively active β-catenin and a construct expressing mouse Dickkopf1 (Dkk1) were the kind gift of Drs Fumi Kubo and Shinichi Nakagawa (RIKEN ASI, Wako, Japan).

DNA was unilaterally co-electroporated with a tracer (mCherry) into either the anterior streak of HH4 embryos or the ectoderm of HH5 embryos that were cultured ex ovo (Uchikawa et al., 2003). The embryos were then incubated in a humidified CO_2 incubator at 37°C for 10-49 hours.

In situ hybridisation and immunochemistry

Embryos were fixed in 4% paraformaldehyde and rinsed in PBS. The following probes were used for whole-mount in situ hybridisation: *Fgf3*, *Fgf19*, *Nkx5.1*, *Pax2*, *Phox2b* and *Soho1*. These probes were as described previously (Begbie et al., 2002; Ladher et al., 2000). A probe recognising *Foxi2* was obtained through the BBSRC chick EST database (ChEST 884m4) (Boardman et al., 2002). In situ hybridisation was performed as described previously (Ladher et al., 2005). Some stained embryos were cryosectioned.

Double-fluorescent in situ hybridisation was based on published protocols (Denkers et al., 2004). Digoxigenin-labelled probes were detected using an alkaline phosphatase-conjugated antibody and revealed using VectorRed (Vector Laboratories). The second probe was labelled using fluorescein. This was detected using a peroxidase-conjugated antibody, and revealed using a fluorescein-tyramide kit (Perkin Elmer).

The following antibodies were used: anti-hair-cell-antigen (a kind gift of Prof. Guy Richardson, University of Sussex, UK), anti-phospho-histone H3 (Upstate) and anti-Ds-Red (Living Colors), which was used to detect mCherry protein.

Statistical analysis

Collagen cultures were analysed after in situ hybridisation for *Soho1* and immunohistochemistry for hair-cell antigen (HCA). Explants were grouped based on stage and the proportion of positive staining. We used statistical tests (Smith's statistical package) for two samples with two possible probabilities (positive or negative) to determine the earliest stage at which the expression of *Soho1* and the development of inner ear hair cells were autonomous.

Cell counts for phospho-histone H3 (PHH3)-positive cells were performed after imaging whole embryos, using Adobe Photoshop software. Briefly, the OEPD was first defined as being an area just rostral to the first somite adjacent to the hindbrain, and that extended rostrally for three somite lengths. All subsequent counts were made of positive cells within this area. PHH3-positive cells were counted and the normal difference between the left and right sides of 10 control embryos between 10ss and 13ss calculated. Such values provide a strong control for differences among embryos. The difference in PHH3-positive cells between left, control sides and right, electroporated sides of six pEF-*Fgf* electroporated embryos were similarly calculated. *P*-values were calculated using Student's *t*-test.

RESULTS

Otic ectoderm passes through multiple states of commitment

We have previously shown that FGF and Wnt signalling induce the inner ear; however, we were aware of a lack of temporal resolution in these studies (Ladher et al., 2000). To understand individual roles that FGF and Wnt play during inner ear development, the particular steps in the developmental program of the inner ear required clarification. Studies had hinted at multiple states during inner ear induction. Explants of presumptive otic tissue are competent to express *Pax2*, but not *Bmp7* when isolated at 5ss; explants can express both when isolated at 7-8ss (Groves and Bronner-Fraser, 2000). We hypothesised that these studies reflected a progressive commitment to otic differentiation. Thus, to assess whether presumptive otic ectoderm, isolated at 5ss, is committed to a definitive inner ear fate, we tested the ability of this tissue to express the otic marker *Soho1* and to develop inner ear hair cells, the mechanosensory receptor of the inner ear (Fig. 1A) (Bartolami et al., 1991; Deitcher et al., 1994). Few (18%) presumptive otic explants isolated at 5ss and cultured for 24 hours expressed *Soho1* (Fig. 1B,C). Similarly, few presumptive otic explants were able to undergo differentiation, as judged by immunoreactivity to HCA after seven days of culture (Fig. 1B,D). These numbers were not statistically significant. Thus, we concluded that, although 5ss explants are able to express *Pax2*, they lack the ability to develop definitive otic character. By contrast, a significant number of presumptive otic explants isolated at 7-8ss (HH9) showed expression of *Soho1* and could develop hair cells (Fig. 1B,E,F).

Attenuation of FGF expression is necessary for otic differentiation

The fibroblast growth factor family members Fgf3 and Fgf19 act from caudal cephalic paraxial mesoderm in the chick to induce otic ectoderm. We have previously shown that *Fgf19* is downregulated from the mesoderm and neuroectoderm at 8ss (Ladher et al., 2000). Soon after, at 9ss, we find that mesodermal *Fgf3* expression is also downregulated. The coincidence of the ability of presumptive otic ectoderm to differentiate with this downregulation led us to suspect that the lowering of FGF expression was a pre-requisite for otic differentiation. To test the necessity of FGF attenuation for otic commitment, we introduced constructs of *Fgf3* and *Fgf19* driven by the Ef1α promotor (pEF-*Fgf3* and pEF-*Fgf19* respectively, collectively called pEF-*Fgf*). This promotor acts constitutively to drive sustained FGF expression in the early chick embryo.

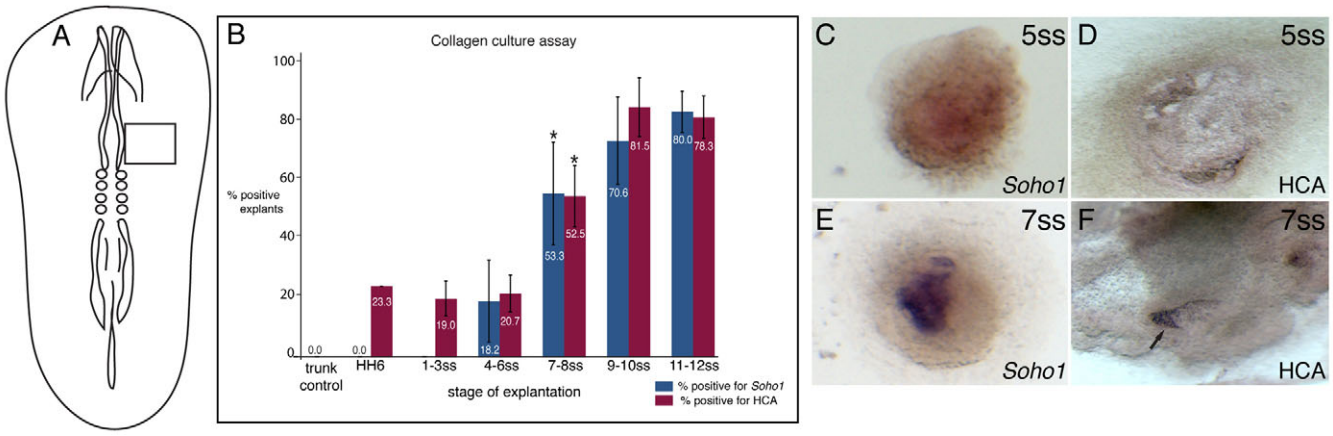

Fig. 1. Otic commitment is progressive. (**A**) The prospective otic region (boxed) was isolated over a number of stages, and cultured in collagen. (**B**) Histogram showing the number of explants positive for *Soho1* and hair-cell antogen (HCA). Greater than 50% of isolates explanted at 7-8ss showed autonomous *Soho1* expression and HCA immunoreactivity. Asterisk represents the stage at which the autonomy of differentiation can be considered statistically significant. (**C**,**D**) Explants isolated at 5ss do not express *Soho1* (C) and do not develop hair cells (D). (**E**,**F**) At 7ss, explants express *Soho1* (E) and show good hair cell differentiation (arrow, F).

We first verified the function of the pEF-*Fgf* constructs by assessing their effects on *Pax2* expression. Several groups have identified a role for FGF signalling in the induction of *Pax2* expression in the OEPD of zebrafish (Nechiporuk et al., 2005; Nikaido et al., 2007; Sun et al., 2007). We thus unilaterally introduced pEF-*Fgf3*, pEF-*Fgf19*, or both, into the anterior streak region of HH4 embryos by electroporation (Fig. 2A-C). Such electroporation targets the mesoderm as well as the neural ectoderm adjacent to the presumptive otic region (Iimura et al., 2007; Psychoyos and Stern, 1996). Overexpression of pEF-*Fgf* constructs caused expansion of the normal OEPD *Pax2*-expression domain (Fig. 2D-I). This expansion was seen as early as 8-14 hours after

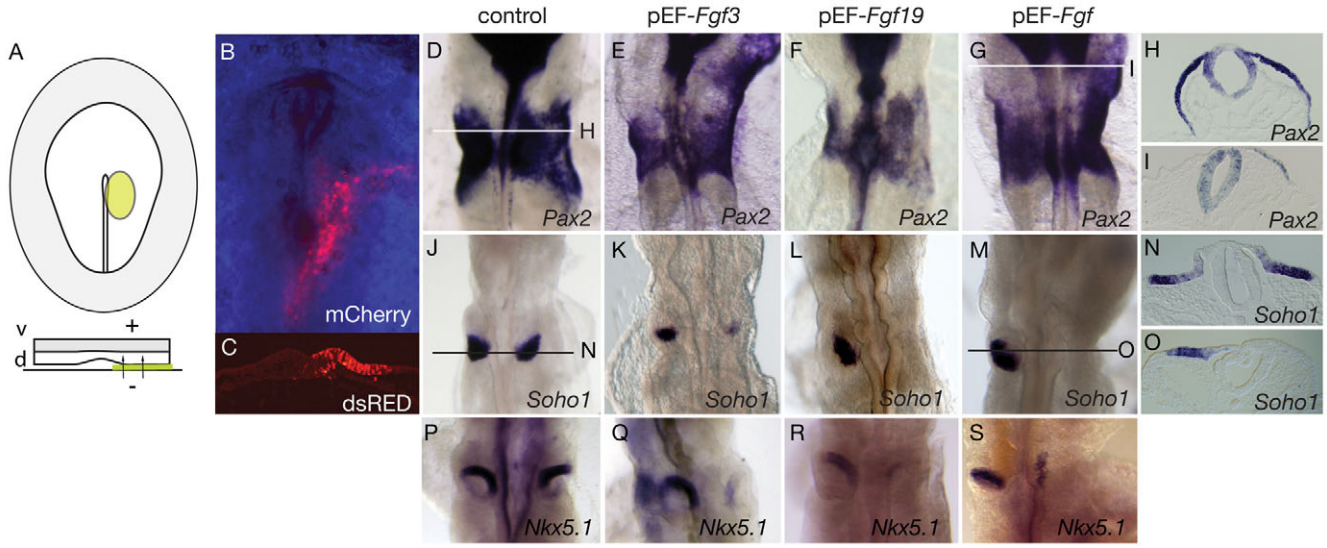

Fig. 2. Sustained FGF overexpression stimulates OEPD fate but inhibits otic differentiation. (**A**) Schematic showing the strategy for electroporation into the anterior primitive streak of HH4 ex ovo cultured chicken embryos. Cathode and anode are shown, dorsal (d) is down and ventral (v) up. (**B**,**C**) Unilateral electroporation targets the paraxial mesoderm and the overlying neural ectoderm on one side of the embryo (B). Section shows unilateral electroporation of the mCherry tracer construct revealed by a dsRed antibody (C). (**D**) Control embryos, electroporated with empty vector show normal *Pax2* expression. Line marks the axial level of the section. (**E**) pEF-*Fgf3* extends the *Pax2* expression domain (*n*=10/22). (**F**) pEF-*Fgf19* extends the *Pax2* expression domain (*n*=15/28). (**G**) Both pEF-*Fgf3* and pEF-*Fgf19* extend the *Pax2* OEPD expression domain (*n*=15/22). Line marks the axial level of the section. (**H**) Section taken through the control embryo in D, showing normal *Pax2* expression. (**I**) Section taken through the trigeminal region of the pEF-*Fgf* embryo in G, showing the unilateral extended *Pax2* expression domain. (**J**) Control embryos, electroporated with empty vector, show normal bilateral *Soho1* expression. Line marks the axial level of the section. (**K**) pEF-*Fgf3* reduces the *Soho1* domain (*n*=7/10). (**L**) pEF-*Fgf19* reduces *Soho1* expression (*n*=9/13). (**M**) Both pEF-*Fgf3* and pEF-*Fgf19* reduce *Soho1* expression (*n*=8/11). Line marks the axial level of the section. (**N**) Section taken through the control embryo in J, showing normal bilateral *Soho1* expression. (**O**) Section of the pEF-*Fgf* electroporated embryo in M, showing unilateral reduction of *Soho1* expression. (**P**) Control embryos, electroporated with empty vector, show normal bilateral *Nkx5.1* expression. (**Q**) pEF-*Fgf3* reduces the *Nkx5.1* domain (*n*=1/2). (**R**) pEF-*Fgf19* reduces *Nkx5.1* expression (*n*=5/11). (**S**) Electroporation of both pEF-*Fgf3* and pEF-*Fgf19* reduces *Nkx5.1* expression (*n*=5/5). In all cases, the electroporated side is on the right.

electroporation, when embryos were at 9-16ss. The effect of electroporating either pEF-*Fgf3* or pEF-*Fgf19* alone was indistinguishable; however, introducing both constructs caused a larger expansion and stronger expression; we therefore presumed that the two ligands are additive during OEPD induction. These results are consistent with those reported in zebrafish.

We next investigated *Soho1* expression in response to sustained FGF action. As shown in the preceding section, *Soho1* expression correlates with the ability of the otic placode to differentiate, and is thus a marker for definitive otic specification (Fig. 1B). In contrast to *Pax2* expansion, the otic expression of *Soho1* was reduced or absent in embryos electroporated with pEF-*Fgf3*, pEF-*Fgf19*, or both (Fig. 2J-O). To verify this, we tested a second marker of the inner ear, *Nkx5.1*. This gene, like *Soho1*, is expressed once the inner ear has become morphologically apparent (Adamska et al., 2001). Like *Soho1*, expression of *Nkx5.1* was similarly reduced in response to sustained FGF signalling (Fig. 2P-S). These results strongly suggest that definitive otic specification was inhibited in embryos when FGF signalling was sustained, and that, during normal development, FGF attenuation is necessary for otic differentiation.

The expansion of the OEPD and the block in otic commitment caused by sustained FGF expression could be the result of the maintenance of proliferation within the OEPD; the failure to exit cell cycle in this case may result in a block in commitment. To test this, we used an antibody to the serine-10 phospho-form of histone H3 (PHH3), which labels mitotic cells between late G2 to anaphase (Hans and Dimitrov, 2001; Hendzel et al., 1997). In normal, non-electroporated embryos, there is a slight difference in the number of PHH3-positive cells between the left and right sides of the embryo. On average there are 3% fewer PHH3-positive cells on the right side of non-electroporated embryos. By contrast, embryos unilaterally electroporated with pEF-*Fgf* constructs, have 23% more mitotic cells on the right, electroporated, side than on the left, non-electroporated side (Fig. 3). These data strongly suggest that one of the functions of FGF during early OEPD induction is to maintain the proliferation of at least a portion of these progenitors.

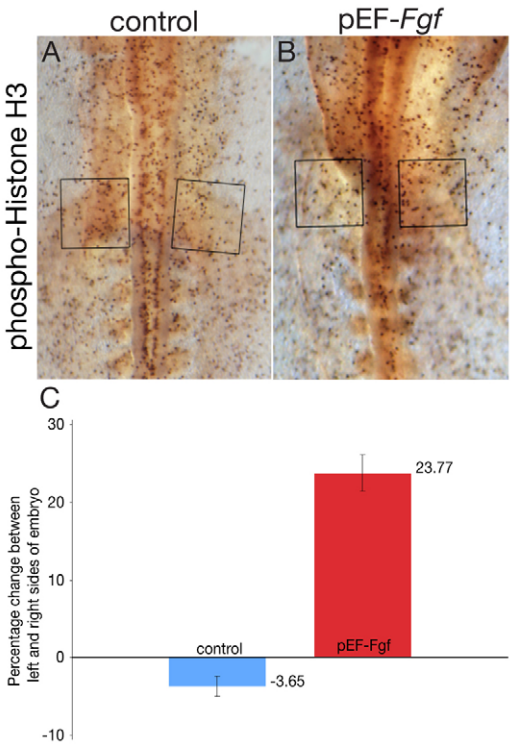

Fig. 3. Sustained FGF expression increases the number of proliferating OEPD cells. (**A**) Control embryos showing phospho-histone H3 localisation. The boxed region demarcates the approximate area of the OEPD and cells were counted in this region. (**B**) Unilaterally pEF-*Fgf* electroporated embryos transfected on the right side, showing phospho-histone H3 (PHH3) localisation. The number of PHH3-positive cells in the OEPD were counted and compared between the left, unelectroporated,side and the right, electroporated side. (**C**) Graph showing the difference in the number of PHH3-positive OEPD cells between left and right sides of control and pEF-*Fgf* electroporated embryos. In control, non-electroporated embryos, the right side of the embryo has 3% fewer PHH3-positive cells (n=565 in 10 embryos) than the left (n=575 in 10 embryos). The right side of experimental embryos, electroporated with pEF-*Fgf3* and pEF-*Fgf19*, has 24% more PHH3-positive cells (n=347 in six embryos) than the left, non-electroporated side (n=261 in six embryos). P-value is <0.001.

Fgf3 and *Fgf19* downregulation prevents otic commitment by repressing OEPD induction

Many studies have described a role for FGF signalling in otic induction (reviewed by Schimmang, 2007), thus the finding that sustained FGF signalling actually inhibited otic differentiation was surprising. In many of these studies, suppression of FGF expression was used to show necessity for inner ear development. We hypothesised that, in these studies, FGF suppression actually inhibited inner ear development by blocking OEPD formation. To confirm this, we investigated the effect of removing *Fgf3* and *Fgf19* on OEPD induction and otic commitment. Constructs encoding short-hairpin interfering RNA were designed to knockdown *Fgf3* and *Fgf19* (known as sh*Fgf3* and sh*Fgf19*, respectively, and, collectively, as sh*Fgf*). Electroporation of either sh*Fgf3* or sh*Fgf19* caused a reduction of *Fgf3* and *Fgf19* expression, respectively (Fig. 4A,B). Such electroporated embryos were assessed after 12 hours for *Pax2* expression (Fig. 4C-F) and after 24 hours for *Soho1* expression (Fig. 4G-J). Knockdown of both *Fgf3* and *Fgf19* substantially reduced the expression of both *Pax2* and *Soho1* (Fig. 4F,J). Reducing the levels of either *Fgf3* or *Fgf19* individually had some effect on OEPD induction (Fig. 4D,E) and otic development (Fig. 4H,I), indicating that during normal development the two act redundantly. These data show that FGF signalling is necessary for OEPD induction and, consequently, otic fate.

FGF signalling does not affect otic/epibranchial patterning within the OEPD

The early expression of *Pax2* defines the otic-epibranchial precursor domain (OEPD), encompassing the otic and epibranchial placodes (Ohyama and Groves, 2004b; Streit, 2002). Even though late otic markers are inhibited by sustained FGF signalling, *Pax2* is not. Thus, it is possible that sustained FGF signalling, as well as maintaining proliferation, also altered specification within the OEPD by converting the presumptive otic portion into non-otic cell types arising from the OEPD. Thus, we assessed *Foxi2* expression. In chick, as has been described in mouse (Ohyama and Groves, 2004a), *Foxi2* expression is excluded from the otic part of the OEPD (Fig. 5A). At 22ss, *Foxi2* is detected in epibranchial placodes. Thus, we hypothesised that early *Foxi2* expression overlapped with *Pax2* to label non-otic OEPD derivatives. This was confirmed using double fluorescence in situ hybridisation (Fig. 5D). Such analysis identified regions of

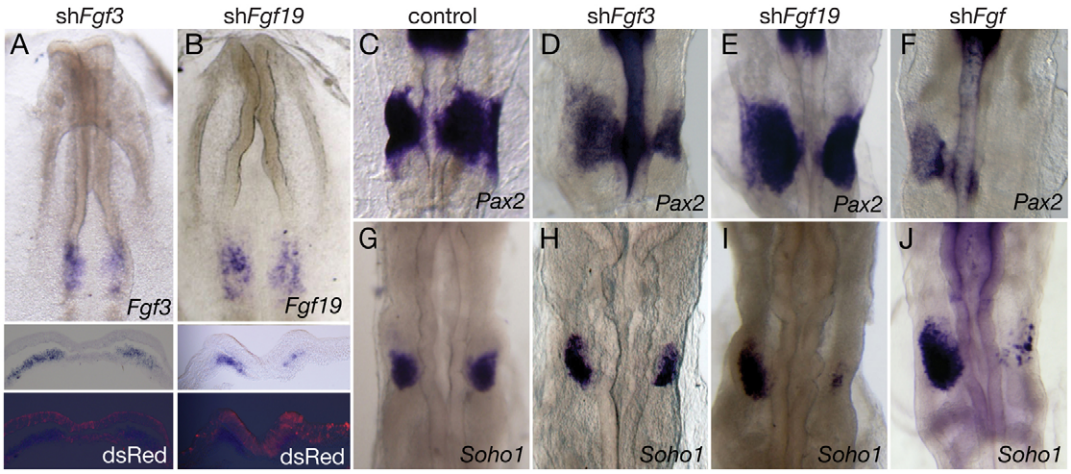

Fig. 4. Downregulation of *Fgf3* and *Fgf19* expression downregulates both early and late otic genes. (**A**) The introduction of sh*Fgf3* downregulates endogenous expression of *Fgf3* in the mesoderm of 1-4ss chick embryos. Sections show a reduction of *Fgf3* expression in regions that express the mCherry tracer. (**B**) Introduction of sh*Fgf19* reduces endogenous *Fgf19* expression in the mesoderm of 1-4ss chick embryos. Sections show a reduction of *Fgf19* expression in regions that express the mCherry tracer. (**C**) Control embryos electroporated with control sh*Scrambled* show no change in *Pax2* expression (n=19/21). (**D**) sh*Fgf3* electroporation results in a slight reduction of *Pax2* OEPD expression (n=17/38). (**E**) sh*Fgf19* causes a slight reduction in *Pax2* expression (n=15/30). (**F**) Electroporation of both sh*Fgf3* and sh*Fgf19* causes a strong reduction of the normal *Pax2* OEPD expression domain (n=13/21). (**G**) Control embryos electroporated with sh*Scrambled* show no change in *Soho1* expression (only one out of 10 showed aberrant expression). (**H**) sh*Fgf3* electroporation results in a slight reduction of *Soho1* expression (n=8/14). (**I**) sh*Fgf19* causes a slight reduction in *Soho1* expression (n=9/15). (**J**) Electroporation of both sh*Fgf3* and sh*Fgf19* causes a strong reduction of normal *Soho1* otic expression (n=12/19). In all cases, the electroporated side is on the right.

the ectoderm that are either *Pax2* or *Foxi2* positive, as well as a domain of *Pax2* and *Foxi2* co-expression at the periphery of the normal *Pax2* expression domain.

Downregulation of *Fgf3* and *Fgf19* using sh*Fgf* constructs, resulted in a diminution of *Foxi2* expression (Fig. 5B). By contrast, electroporation of pEF-*Fgf* constructs resulted in slightly stronger *Foxi2* expression (Fig. 5C), and the region of *Pax2*/*Foxi2* overlap was slightly broader (Fig. 5F). However, we noted that *Foxi2* expression was still excluded from the presumptive otic region. These data suggest that FGF signalling is required for the development of the non-otic portion of the OEPD. In addition, sustained expression does not force the presumptive otic ectoderm to adopt a non-otic fate.

Sustained FGF signalling does not repress epibranchial differentiation

We next asked whether, like the otic portion of the OEPD, sustained FGF signalling also inhibited the differentiation of the non-otic portion of the OEPD. To investigate this, we assessed the development of the epibranchial-derived neurons. At 13ss, *Pax2* expression begins to partition into separate otic and epibranchial regions (Fig. 5G). After pEF-*Fgf* electroporation, epibranchial *Pax2* expression was expanded (Fig. 5I). We note that segregation of *Pax2* into an 'otic' domain also occurred normally after pEF-*Fgf* electroporation, despite the inhibition of otic commitment. To further analyse the effect of sustained FGF signalling upon epibranchial differentiation, we investigated the expression of *Phox2b*. This homeodomain protein is expressed in the sensory neurons of the epibranchial placodes, with expression apparent in all epibranchial placodes by 23ss/HH14+ of development (Begbie et al., 2002) (Fig. 5J). In response to sustained FGF signalling, epibranchial neurogenesis, as marked by *Phox2b* expression, is increased (Fig. 5L).

To test the necessity of FGF signalling for epibranchial development, knockdown constructs were electroporated to reduce *Fgf3* and *Fgf19* expression. These resulted in a marked reduction in epibranchial placodes, as revealed by *Pax2* and *Phox2b* expression (Fig. 5H,K). Together these data suggest that, like the otic placode, FGF signalling is necessary for epibranchial development, most likely through the induction of the common progenitor domain. However, in contrast to otic development, sustained FGF signalling stimulates epibranchial neurogenesis.

Wnt signalling inhibits epibranchial fate

FGF signalling is sufficient and necessary for the early expression of *Pax2* in the OEPD, but it has profound effects on the differentiation of its derivatives, repressing *Soho1* and *Nkx5.1*, markers of otic commitment, and stimulating *Phox2b*, a marker for epibranchial neurogenesis. However, as evidenced by the continued exclusion of *Foxi2* from the presumptive otic region (Fig. 5C), sustained FGF expression does not alter the patterning of the otic-epibranchial progenitor domain. This suggests that additional signals are necessary to direct definitive otic specification.

Wnt signalling is strongly implicated in otic development (Jayasena et al., 2008; Ohyama et al., 2006; Riccomagno et al., 2005). To test whether Wnt signalling partitions the OEPD into otic and epibranchial fates, we used a stabilised mutant of β-catenin. Canonical Wnt signalling is mediated through the repression of cytoplasmic β-catenin degradation. Thus, the stabilised β-catenin acts as a constitutively active effector of canonical Wnt signalling. We introduced this construct into the ectoderm of HH5/HH6 chick embryos and then allowed these embryos to develop until 10-15ss.

Ectodermal overexpression of constitutively active (CA) β-catenin did not alter the expression of *Soho1* (Fig. 6A,B). Similarly, the dorsal expression domain of *Nkx5.1*, an additonal inner ear marker, was unchanged (Fig. 6C,D). We investigated the effect of Wnt activation on OEPD development using *Pax2* expression. At 7ss, *Pax2* expression is unaffected by the overexpression of CA-β-catenin (Fig. 6E-G); however, by 13ss, the *Pax2* expression domain was more rounded, without the typical lateral flares (Fig. 6H-J). We postulated

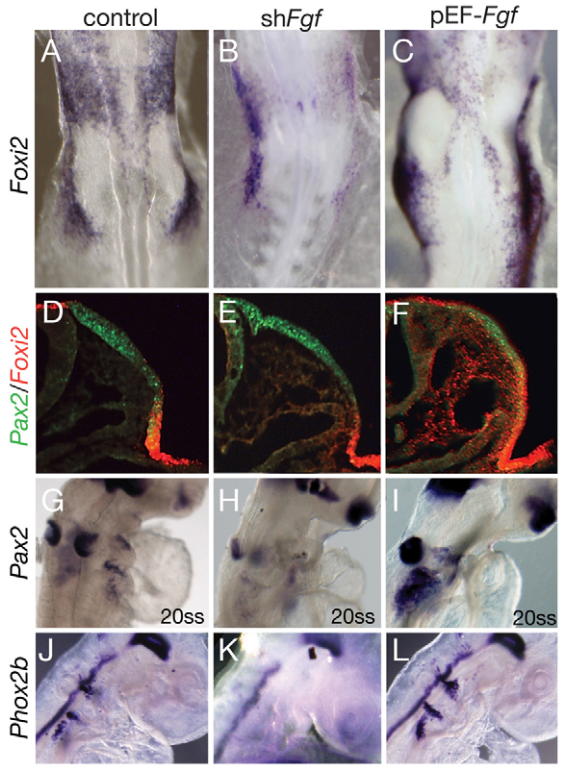

Fig. 5. Epibranchial development is modulated by FGF signalling. (**A**) Chick *Foxi2* is normally excluded from the presumptive otic region. (**B**) Downregulation of *Fgf3* and *Fgf19* expression following electroporation of sh*Fgf* causes a downregulation of *Foxi2* expression (*n*=16/29). (**C**) pEF-*Fgf* electroporation results in stronger *Foxi2* expression, although it remains excluded from the otic domain (*n*=11/25). (**D**) Frozen sections of double in situ hybridisations showing the region of overlap between *Pax2* (green) and *Foxi2* (red). (**E**) The region of overlap is reduced or absent when FGF expression is suppressed. (**F**) FGF overexpression increases the region of *Pax2*/*Foxi2* overlap. (**G**) At the 20ss of chick development, *Pax2* normally segregates into epibranchial and otic expression domains. (**H**) Downregulation of *Fgf3* and *Fgf19* results in the reduction of both otic and epibranchial *Pax2* domains at 20ss. (**I**) Sustained FGF expression causes stronger *Pax2* expression in both otic and epibranchial domains. (**J**) By 27ss, epibranchial precursors undergo neurogenesis, forming *Phox2b*-positive neuroblasts. (**K**) Epibranchial neurogenesis, as marked by *Phox2b* expression, is suppressed in sh*Fgf* electroporated embryos (*n*=9/15). (**L**) In pEF-*Fgf* electroporated embryos, the *Phox2b* expression domain is expanded (*n*=6/9). In all panels the electroporated side is to the right.

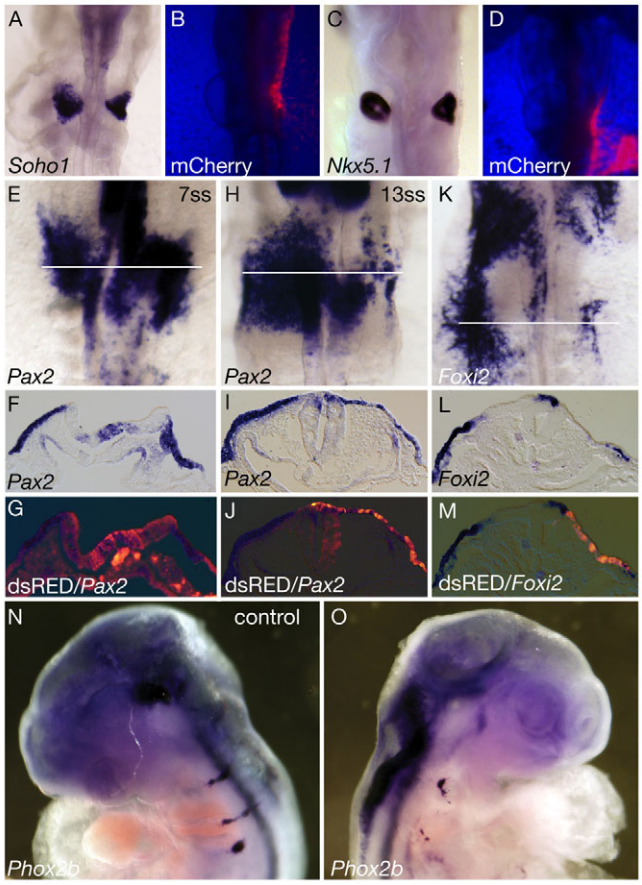

Fig. 6. Canonical Wnt signal activation inhibits epibranchial formation but does not affect OEPD formation. In all panels, embryos have been electroporated unilaterally on the right-hand side with a constitutively active (CA) β-catenin and a tracer expressing mCherry. (**A**) Electroporation of CA-β-catenin does not affect the size of the otic placode, as assessed by *Soho1* expression. (**B**) The location of the mCherry tracer in the embryo shown in A indicates widespread electroporation. (**C**) The expression of *Nkx5.1* is not altered in CA-β-catenin-expressing embryos. (**D**) The location of the mCherry tracer in the embryo shown in C indicates widespread electroporation. (**E**) Expression of *Pax2* in the OEPD is initially normal at 7ss (*n*=3/4 show no change). (**F**) Section of the embryo shown in E, showing normal ectodermal *Pax2* expression, although some ectopic expression is detected within the neural tube. (**G**) Section of the embryo shown in E, showing electroporated cells revealed by dsRed immunostaining, which cross-reacts with mCherry. (**H**) By 13ss, the lateral edge of the *Pax2* expression domain is downregulated (*n*=7/9). Line depicts the level of the section taken. (**I**) Section of the embryo shown in H, showing reduced lateral ectodermal *Pax2* expression. (**J**) Section of the embryo shown in H, showing electroporated cells revealed by dsRed immunostaining. (**K**) *Foxi2* expression is reduced in response to CA-β-catenin (*n*=5/6). Line depicts section shown (L,M). (**L**) Section of the embryo shown in K, showing reduced lateral *Foxi2* expression. (**M**) Section of the embryo in K, showing electroporated cells revealed by dsRed immunoreactivity. (**N**) Epibranchial neurogenesis, marked by *Phox2b* expression, is unaffected on the control side of electroporated embryos. (**O**) *Phox2b* expression is reduced on the electroporated side of the embryo expressing CA-β-catenin (*n*=10/12).

that this lateral domain represented the non-otic part of the OEPD. This was confirmed after observing the reduction of *Foxi2* expression in these embryos (Fig. 6K-M). The reduction of lateral *Pax2* OEPD expression and the loss of *Foxi2* expression suggests that ectopic Wnt signalling might also inhibit the differentiation of non-otic regions of the OEPD. Expression of the epibranchial-derived neuronal marker *Phox2b* was reduced 36 hours after the electroporation of constitutively active β-catenin (Fig. 6N,O).

Wnt signalling is permissive for otic differentiation

Stimulation of Wnt signalling by the introduction of activated β-catenin inhibits epibranchial placode development but has little or no effect on otic development. Although these data do not support the idea that Wnt signalling acts to apportion otic fate within OEPD, they do not rule out the possibility that Wnt acts permissively for inner ear commitment. Thus, we investigated whether Wnt signalling was necessary for inner ear commitment. Unilateral

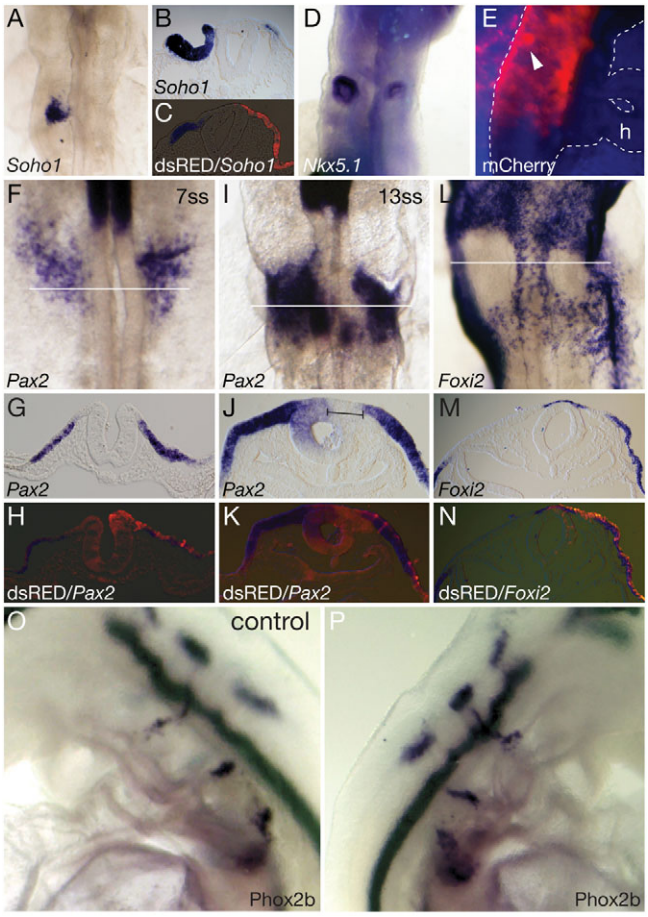

Fig. 7. Wnt is necessary for inner ear formation before 9ss of development. (**A**) *Soho1* expression is reduced as a result of Dkk1 electroporation (*n*=12/18). (**B**) Section shows a reduction of *Soho1* and thickened otic ectoderm on one side of the embryo. (**C**) mCherry tracer expression, as revealed by dsRed immunoreactivity, is unilateral and is seen throughout the ectoderm. (**D**) *Nkx5.1* is also reduced by Dkk1 overexpression. (**E**) A lateral view of the embryo shown in D, showing widespread mCherry expression throughout the ectoderm. For clarity, the outline of the embryo has been traced. The heart tube (h) can be visualised. (**F**) Despite unilateral Dkk1 electropoartion, *Pax2* expression in the OEPD is initially normal at 7ss (*n*=8/9 showing normal expression). (**G**) Sections show normal ectodermal *Pax2* expression. (**H**) Electroporated cells are revealed using dsRed immunostaining. (**I**) At 13ss, *Pax2* is downregulated in a medial portion of the *Pax2* expression domain (*n*=4/5). Line marks the axial level of the section. (**J**) Section of the embryo shown in I, showing a reduction of *Pax2* medially (marked by the bar). (**K**) Section of the embryo shown in I, showing that electroporation of Dkk1 targets a large extent of the ectoderm, as shown by dsRed immunoreactivity. (**L**) *Foxi2* expression encroaches into the otic territory (*n*=5/8). (**M**) Section through embryo shown in L. The region of *Foxi2* otic exclusion is reduced by 50%. (**N**) dsRed immunoreactivity shows the extent of electroporation. (**O**,**P**) Epibranchial neurogenesis as marked by *Phox2b* is not obviously affected as a result of Dkk1 electroporation when control (O) and electroporated (P) sides are compared.

electroporation of the Wnt inhibitor Dkk1 into the ectoderm resulted in a dramatic reduction of *Soho1* and *Nkx5.1* expression (Fig. 7A-D).

The above experiments indicated that Wnt signalling was necessary for otic commitment, but did not indicate whether Wnt signalling was necessary for OEPD induction. To verify this, we analysed *Pax2* expression. We investigated the genesis of the *Pax2* domain at several time points after Dkk1 overexpression. Nine hours after electroporation (7ss), *Pax2* expression was unchanged in response to Wnt signalling inhibition (Fig. 7F-H). However, by 12 hours after electroporation (13ss), the expression domain was altered, missing the medial-most expression (Fig. 7I-K). Consistent with the downregulation of *Soho1* and dorsal *Nkx5.1*, this medial portion is most likely to be the region fated to give rise to the inner ear.

To understand the effect of Wnt inhibition on patterning within the OEPD, we next analysed *Foxi2* expression. *Foxi2* is normally excluded from the presumptive otic region. After Dkk1 overexpression, the region of *Foxi2* exclusion was reduced, but *Foxi2* never invaded the whole of this presumptive otic portion of the OEPD (Fig. 7L-N). To determine whether the expansion of *Foxi2* expression also correlated with an expansion of epibranchial neurogenesis, we investigated the expression of *Phox2b*. We could detect no clear difference in Dkk1 overexpressing embryos, although in a few cases the domain of epibranchial neurogenesis was slightly larger (*n*=4/11) (Fig. 7P). When considered together, these data suggest that canonical Wnt signalling is not required for the initial induction of the OEPD; however, Wnt signalling is necessary for otic commitment.

DISCUSSION

The induction and specification of otic and epibranchial fates are directed by the actions of FGF and Wnt signals from adjacent mesoderm and neural ectoderm. Here, we ascribe exact roles for these factors during otic and epibranchial induction. We show that FGF provides an initial signal that induces a progenitor domain to both. The subsequent commitment to inner ear or epibranchial fates depends, in part, on the action of Wnt signals and the continued function of FGF. More specifically, we make the unexpected observation that sustained FGF signalling is inhibitory to otic differentiation. Thus, whereas otic differentiation is instructed by the combination of canonical Wnt signalling and a downregulation of FGF signalling, epibranchial differentiation is stimulated by sustained FGF signalling and inhibited by Wnt signalling. The use of these multiple signals act as checkpoints to ensure the proper co-ordination of otic and epibranchial induction, guiding the transition between progenitor and commitment states and ensuring their correct localisation within the cranial ectoderm (Fig. 8).

FGF induces and maintains inner ear progenitors

A role for FGF signalling in early inner ear development has been firmly established (Adamska et al., 2001; Alvarez et al., 2003; Hans et al., 2007; Ladher et al., 2005; Ladher et al., 2000; Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Phillips et al., 2001; Vendrell et al., 2000; Wright and Mansour, 2003; Zelarayan et al., 2007). More recent data from zebrafish suggest that FGF signalling additionally induces the epibranchial placodes (Nechiporuk et al., 2007; Nikaido et al., 2007; Sun et al., 2007). This is consistent with the data reported here, showing that FGF overexpression expands the precursor domain of both. Conversely, knockdown using a shRNA strategy results in repression of the OEPD and, consequently, a loss of both committed otic and epibranchial precursors. The idea that only early FGF signalling is sufficient for inner ear development is supported by studies using pharmacological inhibition. Here, *Pax2* expression is inhibited if the inner ear is treated with the FGF inhibitor SU5402 prior to 5ss. If the treatment is performed between 5-8ss, *Pax2* expression is unaffected (Martin and Groves, 2006).

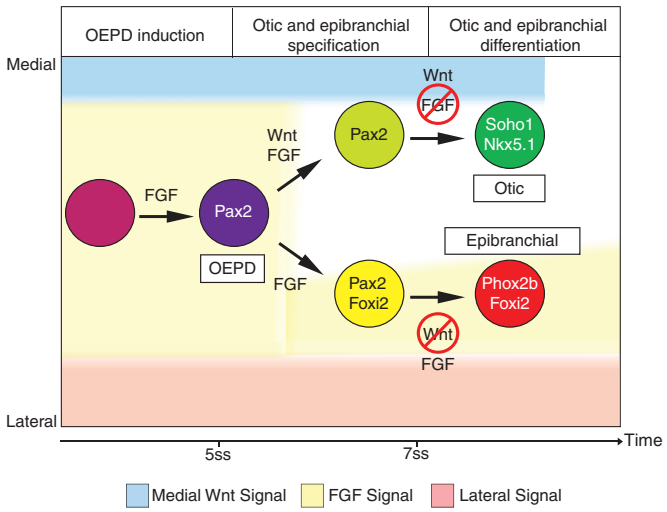

Fig. 8. Schematic model of otic and epibranchial development. Competent non-neural ectoderm is acted on by FGF signalling (yellow) to induce *Pax2*-positive OEPD ectoderm by 5ss of chick development. After 7ss, FGF expression is attenuated and medial Wnt signalling (blue) emanating from the neural tube steers juxtaposed cells to an inner ear fate. Laterally, endodermal FGF signalling together with an unknown signal (red) cause OEPD to be routed into an epibranchial lineage.

An unexpected finding is the suppression of otic commitment when FGF expression is sustained. However, FGF signalling is under tight regulation; the mesodermal expression of *Fgf3* and *Fgf19* is rapidly downregulated at 7-8ss. Furthermore, *sprouty2*, an inhibitor of FGF signalling is expressed in presumptive inner ear tissue from 8ss (Chambers and Mason, 2000). Thus, the observation that otic commitment is suppressed when FGF expression is experimentally sustained is not so surprising. When considered with the behaviour of isolates of otic ectoderm, a clearer understanding of the role of FGF signalling during inner ear development emerges. Isolates express *Pax2*, but can neither express *Soho1* nor show inner ear hair cell differentiation when removed from their embryonic environment at 5ss. During this time, FGF signalling induces the OEPD. Otic commitment only takes place at 7-8ss, concomitant with the downregulation of *Fgf3* and *Fgf19* expression, and the suppression of FGF signals by *sprouty2*.

The observation that the OEPD of FGF-expressing embryos contains a larger number of cells labelled with phospho-histone H3 suggests that mitotically active otic progenitors are maintained by FGF signalling. Thus, the attenuation of such signalling is necessary for cell cycle exit and, consequently, for the transit to a committed state that is able to differentiate definitive inner ear character. Interestingly, the downregulation of FGF expression can also be detected in the mouse. *Fgf8* and *Fgf10*, which with *Fgf3* are the putative murine inner ear inducers, are also downregulated in the peri-otic mesoderm at 8ss (Ladher et al., 2005). This raises the intriguing possibility that FGF attenuation might also be required in mice for otic commitment to take place.

A role for FGF in blocking otic commitment has not been previously described. Indeed, previous data appear to contradict this model. However, one caveat is that in many cases only early OEPD markers have been investigated (Leger and Brand, 2002; Maroon et al., 2002; Sun et al., 2007). Some experiments have investigated the effect of FGF overexpression on otic commitment and differentiation. In *Xenopus*, the implantation of Fgf2-soaked beads resulted in ectopic otocysts (Lombardo and Slack, 1998). In chick, the implantation of Fgf2-soaked beads resulted in larger otocysts (Adamska et al., 2001). Similarly, treatment of chick ectodermal explants with Fgf19-soaked beads resulted in the induction of *Soho1* expression (Ladher et al., 2000). In all cases, it is likely that the position of the beads has shifted during development, and, when considered with protein half-life, it is possible that the tissue can adopt a committed otic fate when it escapes the influence of exogenous protein. A more stable method of gene transfer was performed in chick embryos using an *Fgf3*-expressing retrovirus (Vendrell et al., 2000). In these experiments, even though viral infection was widespread, ectopic otocysts formed adjacent to only some infected cells. A similar study overexpressed *Fgf3* in the hindbrain adjacent to the neural tube. Again, ectopic otocysts appeared adjacent to the region of overexpression (Zelarayan et al., 2007). In both studies, only a single FGF was overexpressed, and it is possible, as we have shown in this study, that the sustained action of two or more Fgfs is necessary for efficient repression of otic commitment. Finally, a zebrafish transgenic line that expresses a heat-inducible Fgf8 construct showed larger, well-patterned, otocysts only when Fgf8 was overexpressed at the late gastrula stage (Hans et al., 2007). Such experiments might indicate a difference in the action of particular FGF molecules.

Wnt activity is necessary for otic differentiation

Similar to the effect of sustained FGF action, inhibition of Wnt signalling also results in the suppression of otic commitment. However, there are differences in the effect of each on *Pax2* expression. As described, FGF overexpression expands the early OEPD domain. At later stages, and despite the inhibition of otic commitment, *Pax2* expression is detected in the otic placode. By contrast, Wnt inhibition causes only a reduction of the medial portion of the *Pax2* expression domain in the OEPD after 7-8ss, at the onset of otic specification. This requirement for Wnt signalling may be transient: treatment of otic regions with recombinant DKK1 protein does not inhibit otic formation after 9/10ss (S.F. and R.K.L., unpublished). The ectopic activation of canonical Wnt signalling using a stabilised, constitutively active β-catenin does not overtly affect otic development and differentiation; however, epibranchial development is affected, again only after 7-8ss (see below). These data strongly suggest that Wnt signalling is not necessary for OEPD induction, but is required for otic specification, acting after FGF signalling has established the OEPD.

Using conditionally active or mutant lines of β-catenin, Ohyama et al. proposed a model for mouse inner ear induction that strongly suggested an involvement of Wnt signalling in OEPD patterning (Ohyama et al., 2006). Similar to our data in the chick, these authors found that the removal of canonical Wnt signalling from the OEPD resulted in the loss of the inner ear; however, the absence of a detailed time course of the changes to the OEPD meant that the possibility that the expression of *Pax2* and *Pax8* (the markers that define the mouse OEPD) was initially normal was not ruled out.

Epibranchial differentiation

As described above, sustained expression of *Fgf3* and *Fgf19* blocks otic differentiation. We suggest that otic differentiation is blocked because these cells remain in a proliferative state; however, in the absence of otic differentiation, does this region also develop a non-otic-OEPD epibranchial fate? Our analysis of the expression of *Foxi2* and *Phox2b* suggests that it does not. *Foxi2* is normally excluded from the region of the OEPD fated to

the otic lineage, and its co-expression with *Pax2* marks the non-otic OEPD region. Even when FGF signalling is sustained, and otic differentiation inhibited, this region of *Foxi2* exclusion is maintained. However, the region of overlap between *Foxi2* and *Pax2* is slightly broader, and epibranchial-derived neurogenesis (as marked by *Phox2b*) is slightly enhanced. These data suggest that early mesodermal FGF signalling is not involved in patterning the OEPD, instead a second phase of FGF signalling, probably from the endoderm, might permit epibranchial fate. This is suggested by recent data showing that endodermal *Fgf3* is involved in zebrafish epibranchial development (Nechiporuk et al., 2005). It should be noted that additional signals also play a role in the development of the epibranchial placodes. Endodermal Bmp7 has been shown to stimulate neurogenesis of these precursors (Begbie et al., 1999).

Our data also show a negative role for Wnt signalling in the development of the epibranchial placodes. Constitutively active β-catenin overexpression results in the repression of epibranchial derivatives. Ohyama and colleagues have touched on the role of Wnt signalling in epibranchial development in the mouse (Ohyama et al., 2006). Similar to our data in chick, the stimulation of canonical Wnt signalling in mouse results in a reduction of epibranchial-derived precursors. However, the result of removing β-catenin in mouse is somewhat confusing: here it results in a reduction of epibranchial development. It is possible that the mouse and chick use different mechanisms for epibranchial development, and that a low basal level of Wnt signalling may be required for mouse epibranchial development. However, a TOP-gal reporter that detects canonical Wnt signalling was not active within epibranchial precursors (Ohyama et al., 2006; Riccomagno et al., 2005). This argues against canonical Wnt signalling acting within epibranchial placodes. Alternatively, the known participation of β-catenin in tight junctions could suggest that mutants in this gene have impaired epithelial integrity. In such a situation, the epibranchial placodes might be induced normally in mouse embryos lacking β-catenin; however, continued development and morphogenesis might be aberrant.

Patterning the OEPD

Our data suggest a sequential role for first FGF signalling and then Wnt signalling in the development of the inner ear. In this revised model, we propose that FGF signalling, from subjacent mesoderm and adjacent neural ectoderm, establishes a mitotically active progenitor domain, the OEPD. The OEPD is then influenced by Wnt signals from the neural tube. By acting on the OEPD, Wnt enables otic differentiation of a subset of OEPD cells, while repressing epibranchial development in others. These data point to a crucial role for canonical Wnt signalling in the lineage choice between otic and non-otic portions of the OEPD. However, an important question is whether other signals are also involved. The failure of constitutively active β-catenin to expand the otic domain could suggest a lateral signal that antagonises the pro-otic Wnt signal. Similarly, the failure of Wnt inhibition to cause the complete conversion of putative otic precursors to non-otic *Foxi2*-expressing cells, despite the downregulation of otic *Pax2*, *Nkx5.1* and *Soho1*, may suggest that the lateral 'anti-otic' signal regulates the expression of *Foxi2*, but that its limited range renders it insufficient to cause complete conversion of the OEPD to *Foxi2*-positive precursors. Thus, the progenitor region is the site of competing signals: a medial otic-promoting/epibranchial-repressing Wnt signal and a lateral, as yet unidentified, signal that promotes epibranchial development and is likely to repress otic development (Fig. 8). An important feature of this model is that the inner ear and epibranchial placodes can be positioned only at sites of intersecting permissive interactions. Furthermore, by controlling the timing of these interactions, the transition between cell states can be controlled, ensuring the correct balance between progenitor proliferation and cell-type differentiation.

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