Wnt signals mediate a fate decision between otic placode and epidermis

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The otic placode, the anlagen of the inner ear, develops from an ectodermal field characterized by expression of the transcription factor Pax2. Previous fate mapping studies suggest that these Pax2+ cells will give rise to both otic placode tissue and epidermis, but the signals that divide the Pax2+ field into placodal and epidermal territories are unknown. We report that Wnt signaling is normally activated in a subset of Pax2+ cells, and that conditional inactivation of β-catenin in these cells causes an expansion of epidermal markers at the expense of the otic placode. Conversely, conditional activation of β-catenin in Pax2+ cells causes an expansion of the otic placode at the expense of epidermis, and the resulting otic tissue expresses exclusively dorsal otocyst markers. Together, these results suggest that Wnt signaling acts instructively to direct Pax2+ cells to an otic placodal, rather than an epidermal, fate and promotes dorsal cell identities in the otocyst.

KEY WORDS: Mouse, Otic Placode, Wnt

INTRODUCTION

One of the central questions in developmental biology is how a homogeneous field of cells undergoes patterning to give distinct territories or cell types. Embryos use a variety of strategies to establish such inductive interactions. In some cases, a field of cells responds to a gradient of inducing signals, with different cell types differentiating according to their position relative to the morphogen gradient (Briscoe et al., 1999; De Robertis and Kuroda, 2004; Lander et al., 2002; Mizutani et al., 2005; Stamataki et al., 2005). In other cases, inducing signals act over very short distances to produce an essentially all-or-none response (Bier, 2000; Shilo, 2003). Fine-grained patterning at the cellular level can also emerge in a cell field by cell-cell interactions such as lateral inhibition (Artavanis-Tsakonas et al., 1999; Lai, 2004) or gap junctional communication (Levin, 2002). Despite the great progress in understanding these different signaling strategies, much still remains to be learned about how they are orchestrated in the induction and development of particular organs.

The inner ear is an increasingly well-characterized example of embryonic induction. It derives from a simple patch of thickened ectoderm, the otic placode, that arises next to the posterior hindbrain (Barald and Kelley, 2004; Brown et al., 2003; Groves, 2005; Kiernan et al., 2002; Riley and Phillips, 2003; Torres and Giraldez, 1998). Studies in different species have suggested roles for both the hindbrain and cranial paraxial mesoderm in otic placode induction, and whereas the relative contribution of these two tissues to the induction process in different species is still uncertain, it is clear that members of the fibroblast growth factor (Fgf) family play a central and crucial role in this induction in fish, amphibians, birds and mammals (Ladher et al., 2000; Ladher et al., 2005; Leger and Brand, 2002; Liu et al., 2003; Lombardo et al., 1998; Maroon et al., 2002; Phillips et al., 2001; Phillips et al., 2004; Wright and Mansour, 2003). Fgf signaling induces the expression of a variety of molecular markers (such as the transcription factors Pax2 and Pax8) in presumptive placodal ectoderm before the placode becomes morphologically distinct (Alvarez et al., 2003; Ladher et al., 2005; Wright and Mansour, 2003).

The induction of Pax2 in cranial ectoderm is commonly thought to be synonymous with the induction of the otic placode. However, several lines of evidence suggest that many Pax2+ ectodermal cells that are initially induced by Fgf signaling will not contribute to the otic placode or the inner ear. Fate-mapping studies in chicken show that cells lying within the Pax2 domain can give rise to structures other than the otocyst, such as the epidermis or epibranchial placodes (Streit, 2002). Genetic fate mapping of Pax2+ ectoderm using Pax2-Cre mice also shows that many Pax2+ cells in the presumptive placodal region ultimately give rise to epidermis and possibly to epibranchial placodes (Ohyama and Groves, 2004b). In light of these observations, what are the mechanisms by which a field of Pax2+ precursor cells is sub-divided into otic placode and epidermis?

Activation of the Wnt signaling pathway by Wnt8 family members has been proposed to participate in otic placode induction (Ladher et al., 2000). However, more recent studies in zebrafish suggest that otic placode induction can proceed in the absence of Wnt8 expression in the hindbrain, although the otocysts of such embryos were usually of reduced size (Phillips et al., 2004). It is possible that Wnt signaling is not necessary for the induction of Pax2+ precursor cells, but instead determines the size of the otic placode by instructing these precursor cells to differentiate into placodal tissue, rather than epidermis. In the present study, we show that the canonical Wnt signaling pathway is activated in a subset of Pax2+ cells during early development. Disruption of the canonical Wnt signaling pathway in Pax2+ cells by conditional deletion of the β-catenin gene leads to an expansion of cranial epidermis at the expense of the otic placode and otocyst. Conversely, constitutive activation of the canonical Wnt signaling pathway by stabilization of β-catenin in Pax2+ cells causes an expansion of the otic placode at the expense of epidermis. Our results suggest that Wnt signaling mediates a placode-epidermis fate decision by acting instructively on a field of Pax2+ precursors to direct them to an otic placode fate.

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MATERIALS AND METHODS

Genetically modified mice

Pax2-Cre transgenic mice were generated in our laboratory (Ohayma and Groves, 2004b) and will be distributed by the Mutant Mouse Regional Resource Centers (Stock number 010569-UNC). TCF/Lef Wnt reporter mice and the conditionally active β-catenin (Ctnnb1tm2Kem) have been described elsewhere (Harada et al., 1999; Mohamed et al., 2004). The following mouse lines were purchased from the Jackson Laboratory: CMV-Cre mice [BALB/c-Tg(CMV-cre)1Cgn/J], stock number 003465 (Schwenk et al., 1995); conditional β-cateninlox/lox allele (B6.129.Ctg(1tub2)Kith2/J), stock number 004152 (Brault et al., 2001); R26R Cre reporter mice (129S-Gt(Rosa)26Sortm1Sor/J), stock number 00310 (Soriano, 1999); Z/EGR Cre reporter mice (B6.Cg-Tg(ACI-TB-Bgeo/GFP)21Lbe/J) stock number 004178 (Novak et al., 2000).

A conditional deletion of β-catenin in Pax2+ cells was generated by the following mating procedure. First, mice carrying a full deletion of β-catenin (β-catenin−/−) were generated by crossing β-cateninlox/lox mice with a CMV-Cre line (Schwenk et al., 1995) and the β-cateninlox line was maintained by breeding heterozygotes. β-catenin−/− mice were then crossed with Pax2-Cre mice to generate a Pax2-Cre: β-catenin−/− line. This line was then crossed with a homozygous β-cateninlox/lox line to generate β-cateninlox/lox; Pax2-Cre embryos (CKO) for analysis. To activate β-catenin in Pax2+ cells and their descendants, Ctnnb1lox/lox mice were crossed with Pax2-Cre mice. Genetic marking of Pax2+ cells was performed by crossing Pax2-Cre mice with either R26R or Z/EG reporter mice as described previously (Ohayma and Groves, 2004b). In all cases, 3-10 of both mutant and control embryos were examined for all markers described in the text.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed by modifications of the protocol of Stern and colleagues (Stern, 1998) as described previously (Kil et al., 2005). Detailed protocols are available upon request. Probes used in this study were Fox22 (Ohayma and Groves, 2004a), Lfg, Tbx1, Gbx2, Mx1 (provided by Doris Wu), Pax2 (provided by Greg Dressler), Pax8 (provided by Meinrad Busslinger), Krox20, Hoxb1 and EphA4 (provided by David Wilkinson), Fgf3 (provided by Suzi Mansour), Dlx5 and Hmx3 (provided by Thomas Luftin) and rat Shh (provided by Henk Roelen). A partial exon 6 fragment (0.6 kb) of mouse Wnt8a was amplified from mouse genomic DNA with PCR primers (forward: AGTGGCTTGTAACACCACAC; reverse: GTGTGACACTTGTG-CCTG). The PCR product was subcloned into pGEM-T Easy (Promega) for production of in situ probes.

Immunostaining and detection of β-galactosidase

Embryos were fixed in 4% paraformaldehyde, washed in PBS and embedded in 7.5% gelatin and 15% sucrose solution, and 10 μm frozen sections were collected as described previously (Ohayma and Groves, 2004a). The slides were washed with PBS at 50°C and blocked in PBS, containing 0.1% Triton-X100 and 10% goat serum. Primary antibodies were obtained as follows: Pax2 (Zymed), β-galactosidase (Promega), green fluorescent protein (Molecular Probes), β-catenin (Zymed, Sigma), α-catenin and γ-catenin (BD Transduction Laboratories), E-cadherin (Sigma), Phospho-Histone H3 (Upstate), activated caspase 3 (R&D Systems) and βIII tubulin (TuJ1; Covance). Alexa 488- or 594-conjugated secondary antibodies for the appropriate subtype (Molecular Probes) were used for detection together with DAPI (Molecular Probes) for nuclear staining. In some images the blue DAPI staining was converted to magenta in Adobe Photoshop for ease of viewing.

For whole-mount detection of apoptotic cells, embryos were fixed and blocked with PBS containing 0.1% Triton-X100 and 20% goat serum. Anti-activated caspase 3 was used for the primary antibody, together with a horseradish peroxidase-conjugated secondary antibody (Molecular Probes). Color reaction was performed with a DAB development kit (Vector). Detailed protocols are available upon request. X-gal staining of the TCF/Lef reporter mice was performed by a standard protocol (Mohamed et al., 2004). For the immunohistochemical detection of β-galactosidase, dissected embryos were fixed with 4% paraformaldehyde and processed for cryosectioning.

Normalization of proliferating cells in normal and mutant mice

Serial sections of normal and mutant embryos were stained with antibodies to phospho-histone H3 (pH3) and β-catenin and then stained with DAPI to reveal cell nuclei. Images of sections were captured with a Zeiss AxioImager digital camera and an Axioshot2 microscope. The number of pH3+ cells in a 150 μm length of otic placode from each control section was counted and compared with pH3+ cell counts in each section taken from an equivalent medial-lateral length of ectoderm from β-catenin CKO embryos. Cell counts were displayed graphically as the number of pH3+ cells per section.

In the case of the conditionally active β-catenin allele (cAct) where the otic placode was greatly enlarged compared with controls, the length of the apical surface of the thickened placode was measured in cAct and control embryos using Adobe Photoshop software. The number of pH3+ cells in the placode was counted in each section and the counts from cAct and control embryos were normalized per 150 μm length of placode.

RESULTS

A TCF/Lef reporter mouse reveals Wnt signaling activity in a subset of Pax2+ precursors

In the canonical Wnt signaling pathway, stabilization of β-catenin allows its translocation to the nucleus, where it binds to Lef or TCF family proteins and activates transcription from TCF/Lef DNA-binding sites (Logan and Nusse, 2004). To determine if the canonical Wnt pathway was activated in Pax2+ precursor cells, we used a reporter transgenic mouse line in which six TCF/Lef DNA-binding sites are coupled to a minimal promoter and the β-galactosidase reporter gene (Mohamed et al., 2004). We examined expression of the β-galactosidase reporter in the presumptive otic region of these mice between embryonic day (E) 8.0 and 9.5. At E8.0, no reporter activity was detected in presumptive otic ectoderm, although this ectoderm already expressed Pax2 (Fig. 1A). By E8.25, reporter activity was present in the presumptive otic ectoderm, overlapping with the most medial part of the Pax2 expression domain (Fig. 1B).

We observed strong reporter activity in presumptive otic ectoderm at E8.5 (Fig. 1C). Double immunostaining for β-galactosidase and Pax2 showed that β-galactosidase is expressed in a medial subset of the Pax2 domain adjacent to the neural tube, but not in more lateral Pax2+ cells (Fig. 1C). As the invaginating otic placode becomes morphologically distinct at E8.75, reporter activity is seen in most regions of the otic placode, with the exception of the most posterolateral regions (Fig. 1D). At this time, we did not observe reporter activity in ectoderm lateral to the thickened placode. By E9.5, reporter activity is still observed mainly in the anterior-medial region of the otic vesicle, with activity in the posterolateral region of the otic vesicle being either weak or negative. One possible candidate for the source of Wnt signaling is Wnt5a, which is expressed in rhombomere 4 between E8.0 and E8.5 (Fig. 1A,B).

These results suggest that the most lateral population of Pax2+ cells exhibit little or no activation of the canonical Wnt signaling pathway. As this lateral population of cells is likely to give rise to cranial epidermis, we hypothesized that Wnt signaling might direct Pax2+ cells to an otic placodal, rather than an epidermal, cell fate.

Conditional inactivation of β-catenin in Pax2+ precursors causes an expansion of epidermis at the expense of the otic placode

We used conditional gene targeting to inactivate β-catenin in all Pax2+ cells by crossing a conditional allele of β-catenin (Brault et al., 2001) with Pax2-Cre transgenic mice (Ohayma and Groves, 2004b). These mice drive Cre recombinase expression in the entire ectodermal Pax2 domain adjacent to the hindbrain. In addition, these mice also express Cre in the mid-hindbrain boundary and the pronephric tubules (Fig. 2A). Pax2 is initially expressed in a broad
region of cranial ectoderm extending from rhombomere 3 to rhombomere 6 at E8.5 (Ohyama and Groves, 2004b). It then becomes localized to the invaginating otic placode at E8.75 and is restricted to the ventromedial region of the otocyst at E9.5. Pax2-Cre mice therefore drive Cre-mediated recombination in the descendants of a wide region of cranial ectoderm, even though endogenous Pax2 expression becomes restricted to the otocyst by E9.0-9.5 (Ohyama and Groves, 2004b).

In contrast to β-catenin-null mice, which die during gastrulation (Haegel et al., 1995), Pax2-Cre/β-catenin conditional knockout (CKO) embryos survived until at least at E10.5. At E10.5, the midhindbrain region of CKO mice is entirely missing, a similar result to that seen in Wnt1-Cre/β-catenin mutant mice (Brault et al., 2001). The size of the otic vesicle was significantly reduced to less than 20% of its normal size in CKO embryos (Fig. 2A). Both phenotypes were consistently observed in all embryos examined, suggesting a high degree of penetrance. We verified that the hindbrains of β-catenin CKO mice were patterned normally by examining Krox20 expression as a marker of rhombomeres 3 and 5 (Fig. 2B) and Fgf3 (rhombomeres 5 and 6), Eph4 (rhombomeres 3 and 5) and Hoxb1 (rhombomere 4; see Fig. S1A in the supplementary material). To determine whether the small otocyst was due to a reduction in the size of the otic placode, we examined expression of Pax2, Pax8 and Dlx5 at E8.5. All three markers were strongly downregulated in CKO embryos compared with controls (Fig. 2B). To test whether the loss of otic tissue was accompanied by an expansion of epidermal tissue, we examined expression of Foxi2, a forkhead transcription factor expressed in cranial ectoderm immediately surrounding, but excluding the otic placode (Ohyama and Groves, 2004a). Foxi2 thus gives an estimation of the size of both the Foxi2 domain and also of the size of the otic placode, which is Foxi2–. CKO embryos had a significantly expanded region of Foxi2+ epidermal cells, and a correspondingly reduced domain of Foxi2– otic placode cells (Fig. 2B).

### Conditional inactivation of β-catenin alters both cell proliferation and survival at later stages in the otic placode

Our results suggested the expansion of Foxi2+ ectoderm in Pax2-Cre/β-catenin conditional mutants occurred at the expense of otic placode tissue. However, it is also possible that the reduction in the size of the otic placode might be due to a decrease in proliferation or survival of Pax2+ cells. We assayed cell proliferation and cell survival with antibodies to the M-phase marker phospho-histone H3 (pH3) and activated caspase 3 as a marker of apoptosis in normal and CKO embryos between E8.5 and E9.0. We only observed apoptotic cells in the otic region of CKO embryos after E8.5. By this time, the otic placode in CKO embryos is already greatly reduced in size relative to controls, as revealed by the downregulation of Pax2, Pax8 and Dlx5, and the expansion of Foxi2 (Fig. 2B). After E8.5, the
Cadherin-mediated cell adhesion is altered in the otocyst, but not in early Pax2\textsuperscript{+} precursors

β-Catenin is not only a key mediator of the canonical Wnt signaling pathway, but also one of the main components of cadherin-mediated adhesion complexes (Bienz, 2005; Harris and Peifer, 2005; Nelson and Nusse, 2004). To determine whether the loss of otocyst tissue in β-catenin CKO embryos was due to a failure in cadherin-mediated cell adhesion, we analyzed E-cadherin adhesion complexes in CKO embryos. Despite the fact that Pax2-Cre mice delete β-catenin from E8.0, we still observed low levels of membrane-localized β-catenin protein in otic-level ectoderm at E8.75 (Fig. 4A). This probably reflects the fact that β-catenin preferentially associates with adherens junctions when present in limiting amounts (Hinck et al., 1994) and suggests that at least 12 hours is required to clear all β-catenin protein from membrane junction complexes. We did not observe significant differences in the levels of either E-cadherin, γ-catenin or α-catenin at E8.75 (Fig. 4A). These results suggest that the loss of otic markers and the concomitant expansion of epidermal markers seen in CKO embryos is unlikely to account for a result of deleting β-catenin.

When we examined β-catenin CKO embryos at E9.0, we noticed that the small otic cup remaining at this time consisted of a mixture of epithelial cells strongly expressing β-catenin protein and some expressing no β-catenin protein at all (Fig. 4A). We have previously noted that a small number of Pax2\textsuperscript{+} cells fail to undergo recombination in Pax2-Cre transgenic mice (Ohyama and Groves, 2004b), and believe this is responsible for the residual number of β-catenin\textsuperscript{+} otic cells in E9.0 CKO embryos. The small number of remaining otocyst cells in which β-catenin protein was absent contained significantly lower levels of E-cadherin and α-catenin compared with controls, but normal levels of γ-catenin (Fig. 4A, lower panels). By E10.5, the residual otocyst in CKO mice consists of a series of small cysts fused to one another. The cysts tended to consist entirely of either β-catenin\textsuperscript{+} or β-catenin\textsuperscript{−} cells (Fig. 4B).

The β-catenin\textsuperscript{−} cysts contained both E-cadherin and α-catenin localized to the cell membrane. As γ-catenin has been reported to localize to the cell membrane. As γ-catenin has been reported to
localize to adherens junctions in the absence of β-catenin (Haegel et al., 1995), we believe that it is responsible for maintaining epithelial integrity in cells lacking β-catenin. We also observed a small number of Neurod1+ and TuJ1+ β-catenin– neurons delaminating from the small remaining otocysts of CKO embryos (Fig. 4C), suggesting that both otocyst formation and neurogenesis could proceed in the very small numbers of β-catenin– cells that gave rise to the CKO otocyst. We also observed a reduction in the size of the geniculate and petrosal placodes after E9.0 (see Fig. S2 in the supplementary material).

Conditional activation of β-catenin in Pax2+ precursor cells causes an expansion of the otic placode at the expense of epidermis

To confirm that loss of canonical Wnt signaling causes a fate change from otic placode to epidermis, we performed a complementary experiment by conditionally activating a stabilized form of β-catenin in Pax2+ precursors. We made use of a conditional β-catenin allele which selectively deletes exon 3 in the presence of Cre recombinase (Harada et al., 1999). This eliminates all serine and threonine residues whose phosphorylation by the APC/axin/GSK3 complex normally targets β-catenin for degradation. As a result, the mutant form of β-catenin becomes stabilized and acts in a dominant active fashion (Harada et al., 1999). We crossed these mice with Pax2-Cre transgenic mice to constitutively activate the canonical Wnt pathway in all Pax2+ precursor cells.

The otic placode of conditionally activated (cAct) β-catenin mutant embryos formed at the level of rhombomeres 4 and 5, and patterning of the posterior hindbrain was normal based on Krox20, Fgf3, Eph4 and Hoxb expression (see Fig. S1B in the supplementary material). By E8.75, the otic placode expanded laterally and ventrally into the cranial ectoderm, as assayed by Dlx5, Pax8 and Pax2 expression (Fig. 5A). Significantly, expansion of the otic placode in cAct embryos was accompanied by a concomitant loss of Foxi2+ epidermal cells (Fig. 5A). Immunostaining of cAct embryos at E8.75 showed high levels of β-catenin in the invaginating placodal epithelium. By E9.5, the greatly expanded region of thickened placodal epithelium extended over much of the lateral surface of the embryo, extending ventrally to the level of the ventral pharynx and occasionally invaginating in ectopic locations (Fig. 5B). This epithelium was verified to be otic in character by its expression of Dlx5, Gbx2 and Msx1 (Fig. 6A). The huge expansion of placodal epithelium appeared not to be due to an increase in cell proliferation, as we could detect no significant increase in pH3.
staining in the epithelium between E8.75 and E9.5 (Fig. 5B,C). Together, these results suggest that activation of the canonical Wnt signaling pathway in \( \text{Pax}^2 \) precursors forces them to differentiate into otic placode cells and prevents them from differentiating into epidermis.

**Wnt signaling promotes dorsal otocyst cell fates**

A number of studies suggest that signaling activity from the dorsal neural tube may regulate dorsal cell fates in the otocyst (Bok et al., 2005; Riccomagno et al., 2005; Wu et al., 1998). To test whether this activity was due to Wnt signaling, we analyzed the expression of a battery of regional markers of the otocyst in control and cAct embryos. Two markers of the dorsal otocyst, \( \text{Dlx}^5 \) and \( \text{Gbx}^2 \), and a marker of the future endolymphatic duct, \( \text{Msx}^1 \), were expressed throughout the thickened placodal epithelium in E9.5 cAct embryos (Fig. 6A). \( \text{Dlx}^5 \) and \( \text{Msx}^1 \) were expressed at lower levels in the most deeply invaginated part of the placode, which tended to lay closest to the ventral midline (Fig. 6A). Expression of other dorsal otocyst markers such as \( \text{Hmx}^3 \) did not appear to be expanded in cAct embryos (Fig. 6B). By E9.5, \( \text{Pax}^2 \) and \( \text{Pax}^8 \) are normally restricted to ventromedial and dorsomedial regions of the otocyst, respectively. We saw virtually no \( \text{Pax}^2 \) expression in cAct embryos and \( \text{Pax}^8 \) expression was expressed in an expanded region of the invaginating placode (Fig. 6B). Markers of neurogenesis in the ventral otocyst such as lunatic fringe, \( \text{Neurod}^1 \) (Fig. 6C) and \( \text{Ngn}^1 \) (not shown) were absent in cAct embryos, and the posteroverentral marker \( \text{Tbx}^1 \) was also entirely absent in mutant embryos. Taken together, these results suggest that in addition to directing \( \text{Pax}^2 \) precursor cells to an otic placode fate, Wnt signaling also promotes dorsal cell fates within the otic epithelium.

**DISCUSSION**

Development of a particular region of the embryo frequently occurs by the specification of a broad cellular domain, followed by refinement into different tissue types. In the present study, we provide evidence that Wnt signaling acts instructively on \( \text{Pax}^2 \) precursors to direct them to an otic placode fate, and in the absence of Wnt signaling, \( \text{Pax}^2 \) precursors differentiate as epidermis. We also show that in addition to mediating the placode-epidermis fate decision, Wnt signaling also specifies a dorsal identity in otic placode cells early in their development.

**Wnt signals as candidates to pattern \( \text{Pax}^2 \) precursor cells**

We monitored activation of the canonical Wnt signaling pathway in \( \text{Pax}^2 \) precursor cells using a TCF/Leff-lacZ transgenic reporter (Mohamed et al., 2004). We observed activation of the reporter in a subset of \( \text{Pax}^2 \) cells in cranial ectoderm from E8.25 onwards, with cells closest to the neural tube displaying high levels of reporter activity and more lateral ectoderm displaying little or no reporter activity.
activity. This pattern of activation suggested that the source of Wnt signaling emanated from the dorsal midline – most likely the neural tube – and declined in more lateral and ventral ectoderm. Significantly, expression of the TCF/Lef reporter at E8.25 occurs after the onset of Pax2 expression in the presumptive placodal ectoderm (Fig. 1A,B), suggesting that Wnt signaling is unlikely to be involved in the initial induction of the Pax2 cell field. We have only been able to reproducibly detect nuclear β-catenin expression at later stages in the inner ear (T.O., O.A.M., M.M.T., D.D. and A.K.G., unpublished), suggesting that very low levels of nuclear β-catenin drive TCF/Lef reporter expression at the otic placode stage. This may explain why reporter activity can be observed in the presumptive placode in our reporter mice which carry six TCF/Lef-binding sites, but not in mice with only three sites (DasGupta and Fuchs, 1999; Dorsky et al., 2002).

What is the source and identity of the Wnt signals observed in our study? Wnt8 homologues are expressed from a very early stage in rhombomere 4 in mouse and chick (Bouillet et al., 1996; Hume and Dodd, 1993; Kil et al., 2005; Niederreither et al., 2000), and in rhombomeres 5 and 6 in zebrafish (Lekven et al., 2001). We show that Wnt8a is expressed in mouse rhombomere 4 between E8.0 and E8.5 (Fig. 1A,B), after which time it disappears, to be replaced by Wnt1 and Wnt7a in the dorsal hindbrain (Parr et al., 1993). We note that the most posterior regions of the otic placode and otocyst – regions that lie farthest from rhombomere 4 – expressed the lowest levels of β-galactosidase reporter (Fig. 1). However, in the absence of gene expression data for other Wnt family members and loss-of-function data for Wnt8a in mice, the case for a Wnt8a signaling source in rhombomere 4 must still be considered circumstantial.

**Wnt signaling mediates a cell fate decision between otic placode and epidermis**

Fate-mapping experiments suggest that the earliest events in inner ear development involve the induction of a broad field of Pax2+ cells, some of which will maintain Pax2 expression and form the otic placode, and others which will differentiate as epidermis. In the present study, we provide evidence from loss- and gain-of-function mutations of β-catenin that activation of the canonical Wnt signaling pathway in the initial Pax2+ field causes cells to differentiate into otic placode tissue, while Pax2+ cells that are not exposed to Wnt signaling differentiate as epidermis. In E8.5 β-catenin CKO embryos, expression of the early otic markers Pax8 and Dlx5 is greatly reduced, while Pax2 – which by E8.5 is localized to the otic placode – is also reduced compared with controls. We have previously shown that Foxi2 labels a region of epidermal cells adjacent to the otic placode (Ohyma and Groves, 2004). In β-catenin CKO embryos, Foxi2 expression expands to occupy the territory that would normally form the otic placode. Conversely, forced activation of β-catenin in the initial Pax2+ field causes a great expansion of thickened placodal epithelium, and expansion of Pax8, Dlx5 and other ear markers, such as Gbx2 and Max1, as well as Pax2 itself. This expansion of otic placode territory is accompanied by a corresponding loss of epidermal Foxi2 expression. Our results thus support the idea that Wnt signaling is mediating a cell fate decision in Pax2+ cells between otic placode and epidermis. Most studies of otic placode induction – including those from our own laboratory – have tended to view Pax2 as an exclusive marker of the otic placode. Our present results now reinforce the notion from fate mapping and gene expression studies (Ohyma and Groves, 2004b; Streit, 2002) that induction of Pax2 in cranial ectoderm and formation of the otic

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**Fig. 5. Conditional activation of β-catenin in Pax2+ cells expands the otic placode at the expense of epidermis.** (A) Whole-mount in situ hybridization of otic and epidermal markers in β-catenin cAct embryos. Expression of the early otic markers Pax2, Pax8 and Dlx5 are upregulated in ectodermal lateral to the otic cup (arrows) at E8.75, while the epidermal marker Foxi2 is downregulated at E9.5. The broken line indicates the outline of the Foxi2+ placodal domain. Markers of the mid-hindbrain boundary (asterisk) are also expanded in cAct embryos. (B) Expansion of the placodal domain without an increase in cell division in cAct embryos. The ectoderm lateral to the otic cup is thickened from E8.75 in cAct embryos and β-catenin protein (red) is expressed strongly in the placodal epithelium. By E9.5, the placodal epithelium fails to close in cAct embryos, forming a grossly enlarged placode extending ventrally to the level of the pharynx, with accumulated β-catenin and ectopic invaginating regions (asterisk). (C) The average number of pH3-positive cells is not significantly increased in cAct placodal epithelium. Numbers are normalized to the length of the apical surface of the placode (see Materials and methods). Scale bars: 100 μm.
placode are actually experimentally separable events, and that Pax2 should more properly be thought of as initially marking a ‘pre-otic’ pool of both placodal and epidermal progenitor cells.

β-Catenin also functions as an important component of adherens junctions, and so the phenotypes we observe by activating or deleting β-catenin might be explained by changes in cell adhesion. However, although we observe some changes in cell adhesion at late stages of otocyst formation in CKO embryos, these occur well after placodal and epidermal defects are already apparent at E8.5. Similarly, the changes in cell survival we observe in β-catenin conditional deletion mutants also occur after changes in the expression of placodal and epidermal markers seen at E8.5, and are almost exclusively restricted to Foxi2* epidermis. Thus, we feel that the most parsimonious explanation for the changes in the size of the otic placode at E8.5 in both our gain- and loss-of-function results is a Wnt signaling-mediated change in cell fate. In the CKO embryos, clearance of β-catenin protein from adherens junctions by E9.0 leads to secondary morphological defects in the remnants of the otic placode. As otic vesicle invagination is driven in part by contacts with the neural tube (Brown et al., 1998; Gerchman et al., 1995; Moro-Balbas et al., 2000; Visconti and Hilfer, 2002), it is likely that physical constraints prevent the greatly expanded placode of cAct embryos from invaginating to form an enlarged otic vesicle.

### Wnt and Fgf signaling in otic placode induction

Ladher and colleagues proposed a scheme for induction of the chick inner ear whereby Fgf19 and Wnt8c act synergistically to induce Pax2 and other markers in unspecified ectoderm (Ladher et al., 2000). By contrast, Riley and colleagues have questioned the role of Wnt signaling in zebrafish otic placode formation in a study where overexpression of the secreted Wnt antagonist dickkopf 1 (Dkk1) failed to block otic placode induction (Phillips et al., 2004). It is possible, however, that such Dkk1 experiments do not represent a complete loss of Wnt function. In the present study we have attempted to overcome these problems, and problems of lethality associated with a null mutation of β-catenin, by conditionally inactivating β-catenin only in Pax2-expressing cells.

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**Fig. 6. Activation of β-catenin promotes dorsal otocyst cell fates.**

(A) Whole-mount in situ hybridization of dorsal otocyst markers in cAct embryos at E9.5. Dlx5, Gbx2 and Msx1 are upregulated in the placodal epithelium, although Dlx5 and Msx1 are downregulated in the most deeply invaginated part of the placode, closest to the ventral midline (asterisks). Borders of the Dlx5 and Gbx2 expressions in control embryos are indicated with bars. Msx1 expression is just starting to appear in the control endolymphatic bud at E9.5 (arrowhead). (B) Pax2 expression in cAct is downregulated at E9.5, while Pax8 is expanded. A dorsal marker, Hmx3, is not expanded. (C) Expression of neurogenic and non-neurogenic markers. Neurod1 and Lfng (anteroventral markers) and Tbx1 (posteroventral marker) are not expressed in cAct embryos. Broken lines represent the malformed otocyst and open rim. Anterior (A) and posterior (P) orientation is indicated with arrows.
We reconcile our results with those of Ladher and Riley by proposing that Wnt signaling is required to stabilize the otic placode cell state by enhancing, sensitizing or perpetuating the response of ectoderm to Fgf signaling, either at some step in the signaling pathway, or else by synergizing with Fgf-induced transcription in the nucleus. Indeed, recent observations suggest that potential for crosstalk between the Wnt and Fgf pathways exist, particularly at the level of regulation of GSK3 (Dailey et al., 2005; Hashimoto et al., 2002; Holinthen et al., 2002; Israsena et al., 2004; Mansukhani et al., 2005). A simple model summarizing our results is shown in Fig. 7A. An initial field of Pax2⁺ cells – induced by Fgf signaling – receives a source of Wnt signaling from the midline. Above a certain threshold of Wnt signaling, Pax2⁺ cells differentiate into the otic placode and its derivatives. Below this threshold, cells either differentiate into epidermis or, in the presence of additional inducing signals, into epibranchial placodes (Begbie et al., 2002; Begbie et al., 1999; Begbie and Graham, 2001). In β-catenin CKO embryos, the absence of Wnt signaling causes loss of early otic markers such as Pax8 and Dlx5, and an expansion of epidermal markers such as Foxi2. Conversely, activation of the Wnt signaling pathway in β-catenin cAct embryos causes an expansion of early otic markers, and concomitant loss of epidermal markers. In support of our model, morpholino knockdown of Wnt8 mRNA in zebrafish, or inhibition of Wnt signaling with Dkk1 results in greatly reduced otocysts (Phillips et al., 2004), whereas exposure of Xenopus embryos to lithium chloride [which activates canonical Wnt signaling by inhibiting GSK-3β (Klein and Melton, 1996; Stambolic et al., 1996)] leads to multiple expanded and interconnected otic vesicles (Gutknecht and Fritzsch, 1990).

The role of Wnt signaling in specifying dorsoventral polarity of the inner ear

The work of Fekete, Wu and others has clearly shown that the developing ear becomes patterned in all three embryonic axes shortly after its formation (reviewed by Brigande et al., 2000; Cantos et al., 2000; Fekete, 1996; Fekete and Wu, 2002). The signals that specify the dorsoventral, mediolateral and anteroposterior axes are beginning to be understood – for example, signals from the ventral midline such as sonic hedgehog (Shh) are required to specify ventral fates in the otocyst (Riccomagno et al., 2002). More recently, Riccomagno and colleagues have shown Wnt1 and/or Wnt3a signaling from the dorsal neural tube acts to specify dorsal otocyst fates, and that a balance of opposing Wnt and Shh signals is required for correct dorsoventral otocyst polarity (Riccomagno et al., 2005).

Our results confirm aspects of these previously published studies. We find that activation of the canonical Wnt signaling pathway in cAct embryos causes a large expansion of dorsal otocyst markers at the expense of ventral markers (Fig. 6). We also show that signals from the ventral midline such as Shh are capable of counteracting Wnt signaling in at least some cases: for example, while expression of Dlx5 and Msx1 is seen throughout the placode of cAct embryos, these genes are expressed at their lowest levels in regions of the placode closest to the ventral midline (Fig. 6A, left and right panels). By contrast, Gbx2 expression seemed unaffected by proximity to the midline (Fig. 6A, center panel). We have confirmed that Shh was still expressed in cAct embryos, and as such is a good candidate to downregulate Msx1 and Dlx5 in ventral cAct otic tissue (see Fig. S3 in the supplementary material). Our results also confirm the observation of Riccomagno and colleagues that some dorsally expressed genes such as Hmx3 appear to be refractory to changes in Wnt signaling (Fig. 6B), suggesting that some aspects of dorsal identity in the otocyst are regulated by Wnt-independent signals.

In Fig. 7B, we describe a revised model of otic placode induction, in which Fgf signaling first establishes a field of ‘pre-otic’ Pax2⁺ cells. We suggest that subsequent Wnt signaling not only instructs Pax2⁺ precursors to an otic placode fate, but also specifies dorsal otic identity in these cells. As the placode invaginates, the most ventral regions of

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**Fig. 7. Proposed model for the role of Wnt signaling in mediating the otic placode-epidermis fate decision and dorsal otocyst cell fates.** (A) Summary of the β-catenin loss-of-function (CKO) and gain-of-function (cAct) phenotypes. Control: Wnt-TCF/Lef is activated. (magenta) in Pax2⁺ surface ectoderm by Wnt (blue) from hindbrain (HB) to delineate the size of the otic placode (dark blue). Pax2⁺ cells that do not receive Wnt signals differentiate as epidermis (gray). In CKO, in the absence of Wnt signaling, epidermis (gray) is expanded at the expense of the otic placode. In cAct, stabilized β-catenin activates Wnt-responsive genes in a wide region of the surface ectoderm, thus expanding the otic placode at the expense of epidermis. Ovals in the top panels represent the otic placode. A, anterior; P, posterior. Lower panels represent a transverse section at the level shown in the top panels as a broken line. The strength of the Wnt-mediated response is shown as a graph. (B) A model of the induction and polarization of the otic placode. E8.0: Pax2 in the surface ectoderm (SE) is induced by Fgf signaling from underlying tissues and/or the hindbrain. E8.5: Wnt signaling from rhombomere (r) 4 directs the medial-most region of the otic placode closest to the ventral midline (Fig. 6A, center panel). We have confirmed that Shh is still expressed in cAct embryos, and as such is a good candidate to downregulate Msx1 and Dlx5 in ventral cAct otic tissue (see Fig. S3 in the supplementary material). Our results also confirm the observation of Riccomagno and colleagues that some dorsally expressed genes such as Hmx3 appear to be refractory to changes in Wnt signaling (Fig. 6B), suggesting that some aspects of dorsal identity in the otocyst are regulated by Wnt-independent signals.
otic epithelium is brought close to the ventral midline, exposing it to ventralizing signals such as Shh. Cells that do not receive Shh signals – even those at the most lateral edge of the otic placode – continue to express dorsal fates, and upon closure of the otic pit, these most lateral cells will contribute to the dorsal otocyst. It is interesting to speculate in this regard that Wnt signaling may be required continuously for the correct development of the dorsal structures. For example, Fekete and colleagues have shown that ectopic activation of β-catenin in the ventral otocyst can ultimately give rise to vestibular hair cells within the cochlea (Stevens et al., 2003).

The processes that specify the size of a mature organ coordinate inductive interactions with cell division and cell death (e.g. Conlon and Raff, 1999; Day and Lawrence, 2000; Jacobson et al., 1997; Johnston and Gallant, 2002; Kenyon et al., 2003). In the present study, we show that Wnt signaling subdivides a field of Pax2-expressing cells into otic placode and epidermal derivatives, and that by modulating Wnt signaling, we are able to increase the size of one derivative at the expense of the other. It is possible that the strength and range of Wnt signaling acts to specify the particular size of the otic placode with respect to adjacent ectoderm in different species.

We thank Rebecca Ferreira and her staff for animal maintenance; Juan Llamas, Welly Makmura and Sheri Juntilla for colony management; and Juemei Wang for excellent technical support. We are grateful to Doris Wu, Greg Dressler, Martin Bienz, M. and Juan Llamas, for animal maintenance, and Raff, 1999; Day and Lawrence, 2000; Jacobson et al., 1997; Johnston and Gallant, 2002; Kenyon et al., 2003). In the present study, we show that Wnt signaling subdivides a field of Pax2-expressing cells into otic placode and epidermal derivatives, and that by modulating Wnt signaling, we are able to increase the size of one derivative at the expense of the other. It is possible that the strength and range of Wnt signaling acts to specify the particular size of the otic placode with respect to adjacent ectoderm in different species.

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Wnt signals in otic placode development


