The development of semicircular canals in the inner ear: role of FGFs in sensory cristae

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Summary

In the vertebrate inner ear, the ability to detect angular head movements lies in the three semicircular canals and their sensory tissues, the cristae. The molecular mechanisms underlying the formation of the three canals are largely unknown. Malformations of this vestibular apparatus found in zebrafish and mice usually involve both canals and cristae. Although there are examples of mutants with only defective canals, few mutants have normal canals without some prior sensory tissue specification, suggesting that the sensory tissues, cristae, might induce the formation of their non-sensory components, the semicircular canals. We fate-mapped the vertical canal pouch in chicken that gives rise to the anterior and posterior canals, using a fluorescent, lipophilic dye (DiI), and identified a canal genesis zone adjacent to each prospective crista that corresponds to the *Bone morphogenetic protein 2* (*Bmp2*)-positive domain in the canal pouch. Using retroviruses or beads to increase Fibroblast Growth Factors (FGFs) for gain-of-function and beads soaked with the FGF inhibitor SU5402 for loss-of-function experiments, we show that FGFs in the crista promote canal development by upregulating *Bmp2*. We postulate that FGFs in the cristae induce a canal genesis zone by inducing/upregulating *Bmp2* expression. Ectopic FGF treatments convert some of the cells in the canal pouch from the prospective common crus to a canal-like fate. Thus, we provide the first molecular evidence whereby sensory organs direct the development of the associated non-sensory components, the semicircular canals, in vertebrate inner ears.

Supplemental data available online

Key words: FGF2, FGF3, FGF10, Sensory organ, Semicircular canals, Common crus, BMP2, BMP7

Introduction

The vertebrate inner ear is a highly intricate sensory organ that relays two vital sensory inputs to the brain, hearing and balance. Within the membranous labyrinth of the inner ear, a relatively small population of cells are directly involved in sensory transduction. The rest of the labyrinth consists of non-sensory components that are equally important, and which are responsible, albeit indirectly, for proper mechanotransduction. For example, in the vestibular system, the apparatus responsible for sensing angular acceleration consists of three non-sensory components: the anterior, posterior and lateral semicircular canals. Each canal is connected at one end to an ampulla that houses the sensory tissue – the crista ampullaris – and at the other end to a non-sensory structure known as the common crus (Fig. 1). Truncations and size reductions of the semicircular canals have been shown to result in vestibular deficits in both mice and zebrafish (Deol, 1983; Ponnio et al., 2002; Whitfield et al., 1996).

In birds and mammals, the anterior and posterior canals develop from a common vertical outpouch in the dorsal otocyst starting at around E3.5 in chicken, whereas the lateral canal develops from a horizontal outpouch in the middle otocyst. In the vertical outpouch, the opposing epithelia approach each other forming two fusion plates that then fuse and resorb, leaving behind the two tube-shaped canals (anterior and posterior) connected in the middle by the common crus (Fig. 1) (Bissonnette and Fekete, 1996). Although there are mouse and zebrafish mutants with defects only in the semicircular canals, there are no well-characterized mutants in which the semicircular canals develop normally in the absence of sensory tissue development (Chang et al., 2004; Anagnostopoulos, 2002; Whitfield et al., 1996). These observations led us to propose that non-sensory development may require prior specification by sensory tissues (Cantos et al., 2000).

Analyses of mouse inner ear mutants have identified a number of genes that are important for the proper formation of the semicircular canals and their cristae, such as *Dlx5*, *Hmx2*, *Hmx3* and *Fgf10* (for a review, see Chang et al., 2004). The role of FGFs in canal development is demonstrated by the loss of all three semicircular canals and the posterior crista in *Fgf10* knockout mice, and an occasional loss of the posterior canal in one of the reported *Fgf3* knockout mouse lines (Pauley et al., 2003; Mansour et al., 1993). Identification of the molecular pathways underlying these phenotypes is complicated by the multiple expression domains of *Fgf3* and *Fgf10*: both genes are expressed in tissues surrounding the otic placode, as well as in the neurogenic and sensory regions of the otocyst proper (Pirvola et al., 2000; Wright, 2003).

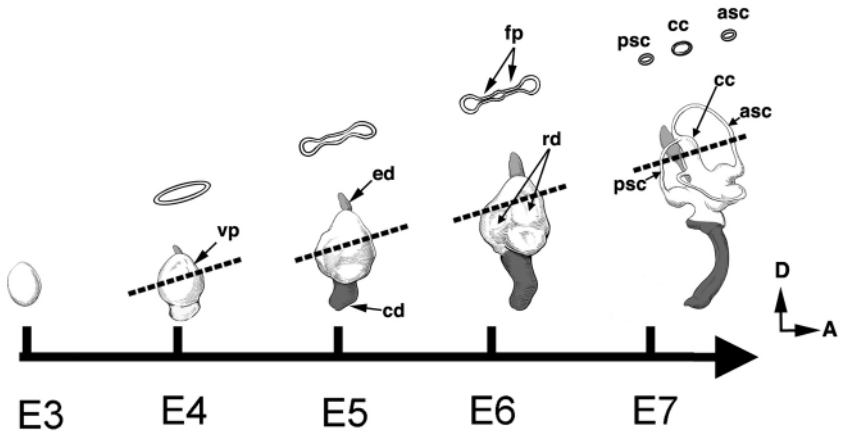

Fig. 1. A schematic diagram of the chicken inner ear from E3 to E7. Dotted lines represent the plane of a cross section through the vertical canals, shown above each stage. asc, anterior semicircular canal; cc, common crus; cd, cochlear duct; ed, endolymphatic duct; fp, fusion plate; psc, posterior semicircular canal; rd, resorption domain; vp, vertical canal pouch; A, anterior; D, dorsal.

A requirement for Bone Morphogenetic Proteins (BMPs) in canal and crista development is suggested by manipulating the activities of the proteins in chicken embryos. Inner ears treated with exogenous Noggin, an antagonist to BMPs, displayed semicircular canal truncations as well as defective sensory organs (Chang et al., 1999; Gerlach et al., 2000). However, at least three BMPs are expressed in the chicken otocyst, *Bmp2*, *Bmp4*, and *Bmp7* (Chang et al., 1999; Oh et al., 1996; Wu and Oh, 1996). It is not clear which BMP(s), or combination of these proteins, is directly required for the formation of these structures.

Here, we fate map the vertical canal pouch in chicken using DiI. We identify a canal genesis region immediately adjacent to the sensory tissues, which contributes to a majority of the cells in the canals. By delivering exogenous FGFs using beads soaked with FGF2 or FGF10 proteins, or recombinant avian retroviruses encoding *Fgf3* or *Fgf10*, we demonstrate that FGFs in the presumptive cristae promote canal development, most likely by inducing *Bmp2* in the canal genesis zone. Ectopic FGF treatments convert some of the cells in the dorsal region of the canal pouch to a canal-like fate.

Materials and methods

Chicken embryos

Fertilized chicken eggs (SPAFAS) were incubated at 38°C, and embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos for paint-fill analysis were fixed in Bodian's fixative and processed as described by Bissonnette and Fekete (Bissonnette and Fekete, 1996).

Fate mapping

Glass micropipettes (5 μm in diameter) were prepared using a Sutter Micropipetter Puller P87 and backfilled with a 0.05% solution of Celltracker CM-DiI (C-7000, Molecular Probes) in 0.3 M sucrose. To visualize the lumenal side of the otic epithelia, otocysts were first injected with 0.05% Fast Green in PBS. Then, a small opening was made in an E4 or E5 otic canal pouch at a location away from the injection site using a tungsten needle. A micropipette filled with DiI solution was inserted tangentially through the opening into the otocyst cavity with the aid of a micromanipulator. DiI was then pressure-injected to the designated area using Pneumatic Picopump PV820 (World Precision Instrument) under a fluorescent microscope (Leica MZFLIII). Only embryos without dye leakage into the otic lumen were kept. To further ensure there was no additional labeling due to possible leakage from the pipette, the lumen was flushed repeatedly with 0.05% Fast Green solution immediately after labeling. Each successfully labeled specimen was photographed immediately after injection, and then again at E7, after harvest and partial dissection.

In situ hybridization

Whole-mount and in situ hybridization experiments were carried out as described (Wu and Oh, 1996). In situ hybridization results presented for each stage are representative of at least three experiments. Riboprobes for chicken *Bmp2*, *Bmp4*, *Bmp7* (Chang et al., 2002), *SOHo-1* (Kiernan, 1997), *Fibroblast growth factor receptor 1-3* (*Fgfr*) (Walshe and Mason, 2000), *Fgf10* (Ohuchi et al., 1997) and *Fgf3* (Mahmood et al., 1995) were also prepared according to procedures described in the cited references.

Retroviral infection

An avian retrovirus encoding mouse *Fgf3* was generated by subcloning the coding region of mouse *Fgf3* (provided by Dr Ivor Mason, King's College, London) into the *Cla*I site of an RCAS(A) vector (Petropoulos and Hughes, 1991). As a negative control, mouse *Fgf3* was subcloned in the reverse orientation (RCAS-*Fgf3*-RO). The RCASBP(A)-*Fgf10* construct containing a 700 bp fragment of the rat *Fgf10* cDNA was obtained from Dr Sumihare Noji (University of Tokushima). Retroviruses were prepared according to procedures described in Morgan and Fekete (Morgan and Fekete, 1996), and viral stocks with titers of approximately 1×10^8 infectious units per ml were used. Viruses were injected into either the lumen of otocysts or the surrounding mesenchyme as described in the Results section. The monoclonal anti-gag antibody 3C2 was used to determine the extent of viral infection (Chang et al., 1999).

Bead implantation

Affi-Gel Blue Beads (Bio-Rad) pre-soaked with mouse Noggin-Fc recombinant fusion protein (R&D Systems) (Chang et al., 2002), or human recombinant FGF2 (Invitrogen) or FGF10 (R&D Systems) protein, were prepared as described (Chang et al., 1999). Briefly, for our standard treatment, 30 beads were incubated with 1 μl of PBS containing 1 μg of Noggin, FGF2 or FGF10 plus heparin (10 μg/μl), for one hour at room temperature and then stored on ice until implantation. For a standard implantation, a single bead was implanted into an otocyst. The total number of beads used in the soaking stage for both Noggin and FGFs were empirically determined, such that a single bead is sufficient to elicit a canal phenotype after implantation into an E5 otocyst (see Results). To reduce the amount of protein being delivered in rescue experiments with Noggin in ovo, the total number of beads used during the soaking stage was increased by 4-fold. To increase the amount of protein being delivered, multiple beads prepared by the standard method were implanted. Beads pre-

soaked with bovine serum albumin (BSA), or BSA plus heparin (BSA-heparin) were used as controls and did not result in inner ear abnormalities.

For delivery of SU5402 (Sugen), positively charged AG1 beads (BioRad, AG1-X8) were used. Briefly, 30 AG beads were incubated for 20 minutes at room temperature in 1 μl of 2.5, 5 or 10 mM SU5402 dissolved in DMSO for the delivery of low, medium and high dosages, respectively. After incubation, SU5402-soaked beads were washed three times with sterile PBS, and were stored on ice until implantation. Beads prepared with DMSO alone were used as controls.

FGF- or SU5402-soaked beads were implanted directly into the lumen of the otocyst at the stages indicated, whereas Noggin-soaked beads were implanted into the mesenchyme adjacent to the dorsal region of the vertical canal pouch.

Results

Fate mapping of the canal pouch

We labeled the canal pouch on E4 with DiI at different locations and observed the fate of labeled cells on E7. The labeling positions are illustrated in Fig. 2A, with positions 3 and 9 o'clock located dorsal to the presumptive anterior and posterior cristae, respectively (based on *Bmp4* expression), and the 12 o'clock position at the dorsal tip of the canal pouch. Our results show that cells labeled between 10 and 2 o'clock develop solely into the common crus (Fig. 2B-G,K; *n*=11/11), with the exception that injections at 12 o'clock sometimes show additional sporadically labeled cells in the endolymphatic duct and sac. Injections at 3 and 9 o'clock label primarily the semicircular canal (Fig. 2H-J,K; *n*=17/18), and to a lesser extent the common crus (Fig. 2I,J; *n*=13/18). Only one out of 18 specimens injected at the 3 and 9 o'clock positions show DiI labeling in the crista, indicating that we have targeted successfully regions dorsal to the sensory tissues. Most of the specimens show a region of canal close to the ampulla that is devoid of DiI labeling (Fig. 2J, arrowheads), suggesting the possibility that new cells are continually being generated at the 3 and 9 o'clock positions. Similar labeling patterns are observed when the same locations are labeled at E5 (Fig. 2L; *n*=5). Taken together, these results suggest that there is a canal genesis zone adjacent to the prospective sensory region that contributes to the majority of the canal outgrowth.

Bmp2 is expressed in the canal genesis zone

Previously, we have shown that *Bmp2* expression is associated with the outer rim but not the center of the canal pouch at E5, and that its expression remained associated with the outer rim

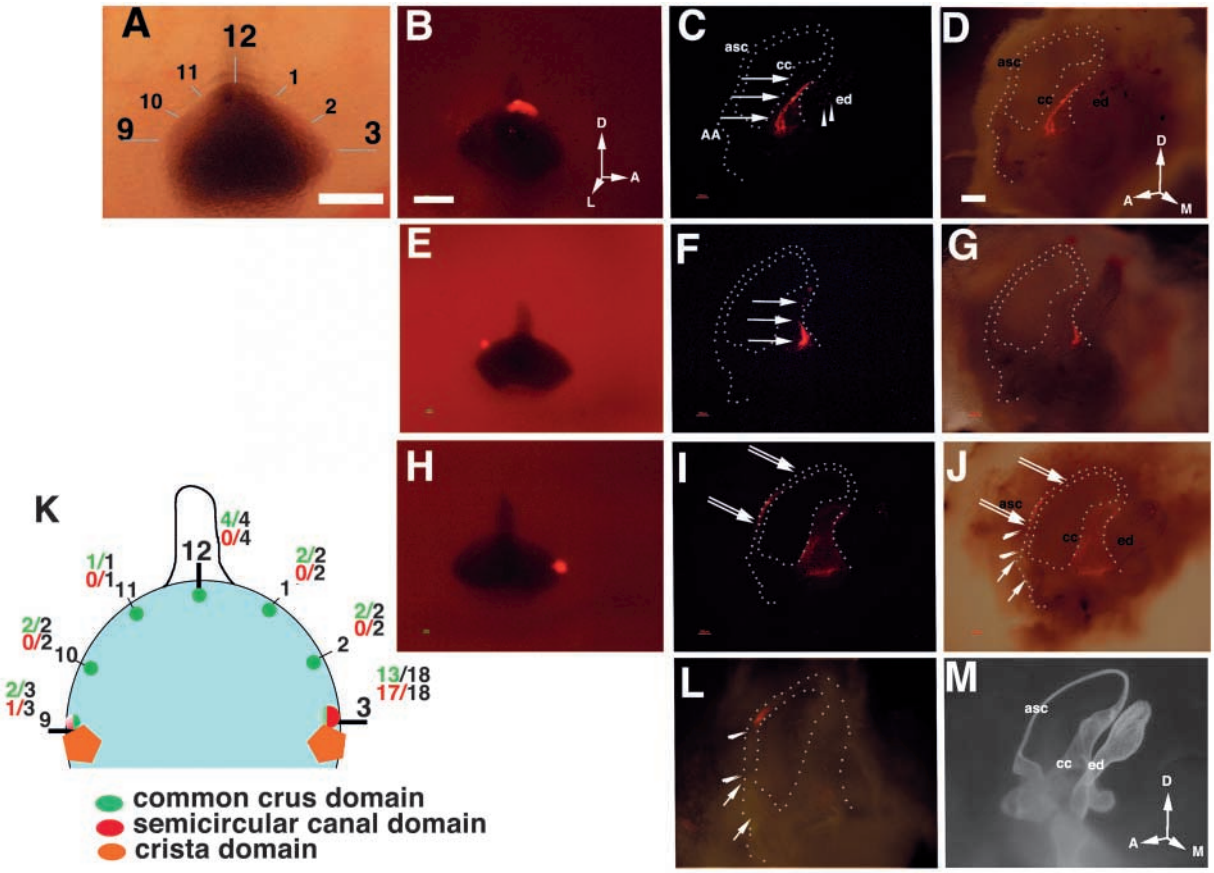

Fig. 2. Fate mapping the canal pouch. (A) Otic epithelia were labeled with DiI at the positions shown. Examples are shown at the time of injection on E4 (B,E,H) and again at E7 (C,D,F,G,I,J,L,M). Partially dissected E7 ears were imaged under fluorescence (C,F,I,L), combined fluorescence and bright field microscopy (D,G,J) or as a paint-fill for orientation (M). (K) Summary of fate mapping data. The number of specimens with DiI-labeled cells in the canals and common crus at E7 are shown in red and green, respectively. The common crus (long arrows), anterior canal (double arrows) and anterior ampulla (short arrows) are indicated. A region between the anterior ampulla and canal (flanked by arrowheads) is consistently unlabeled. See text for further details. Orientations in B applies to A, E and H. Orientations in D applies to C,F,G,I,J and L. M, medial; L, lateral; AA, anterior ampulla. Scale bars: in A, 200 μm; in B, 200 μm for E,H; in D, 200 μm for C,F,G,I,J,L.

of the canals after formation (Chang et al., 2002). We now show, at the earliest stage of detection for *Bmp2* transcripts (E3.5), there are two distinctive wedges of expression along the anterior and posterior limits of the vertical canal pouch. The ventral margin of each wedge (Fig. 3A, arrowheads) abuts the presumptive cristae domains as revealed by *Bmp4*-hybridization signals (Fig. 3B,C). These *Bmp2*-positive regions correspond to the canal genesis region as indicated by the fate mapping study.

The prospective common crus domain is *Bmp2* and *Bmp7* negative

Bmp2 transcripts are not detectable in the dorsal-central region of the canal pouch that fate maps to the common crus (Fig. 3A, arrows). This was confirmed by in situ hybridization using cryostat sections. The association of *Bmp2* expression with the canals but not the common crus persists well after the canals are formed at E7; at least up to E12 (Fig. 3D).

In contrast to *Bmp2*, *Bmp7* expression is not restricted to the prospective canal rim, and instead becomes elevated in the central region of the canal pouch by E6 (Chang et al., 2002). However, similar to *Bmp2*, *Bmp7* is not expressed in the dorsal region of the canal pouch at E3.5 (Fig. 5C, double arrows). By E7, after the canals and common crus are formed, *Bmp7* is not expressed in the common crus but in the inner rim of the canals (Chang et al., 2002).

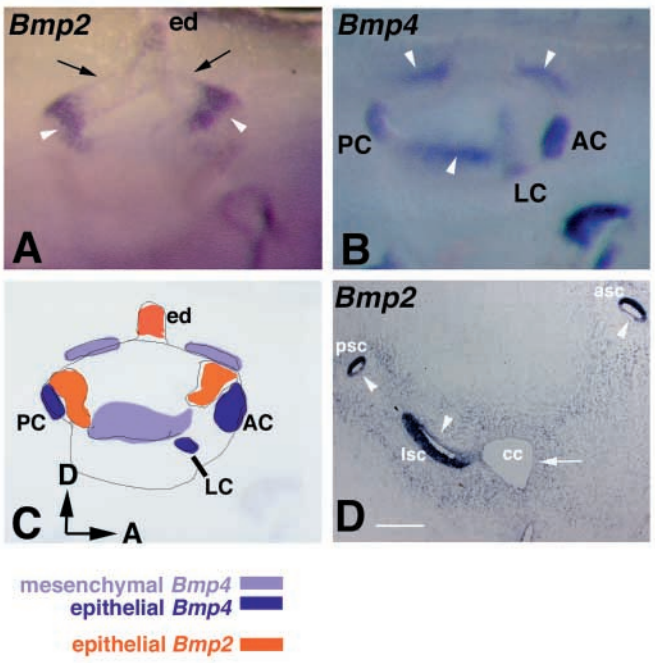

Fig. 3. Expression patterns of *Bmp2* and *Bmp4* in E3.5 canal pouches. (A) Arrows indicate the *Bmp2*-negative domain in the dorsal region of the canal pouch that will develop into the common crus. The ventral margins of the two *Bmp2* expression domains (arrowheads in A) abut the *Bmp4* sensory domains shown for another specimen in B. Arrowheads in B indicate *Bmp4* expression in the mesenchyme. (C) Schematic showing the *Bmp2* and *Bmp4* expression domains superimposed. (D) Horizontal section through the lateral canal at E9. The *Bmp2* hybridization signal is strong in the thick, outer rim, but weak in the thin, inner rim of the semicircular canals (arrowheads) and absent in the common crus (arrow). Orientations in C apply to A and B. Abbreviations are the same as for Fig. 1. AC, anterior crista; LC, lateral crista; lsc, lateral semicircular canal; PC, posterior crista. Scale bar in D: 100 μm.

Phenotypes elicited by RCAS-*Fgf3*, RCAS-*Fgf10*, FGF2- and FGF10-soaked beads

Both *Fgf3* and *Fgf10* transcripts are associated with the neurogenic and sensory regions of the inner ear, similar to what has been reported in mice (Pirvola et al., 2000) (see Fig. S1 at http://dev.biologists.org/supplemental/). In addition, two of the FGF receptors, *Fgfr1* and *Fgfr2*, are weakly expressed in the canal pouch and highly expressed in the endolymphatic duct and surrounding otic mesenchyme. To examine the functions of FGF3 and FGF10 during otocyst development, we ectopically expressed FGFs in the developing chicken inner ear by infection with recombinant avian retrovirus encoding *Fgf3* (RCAS-*Fgf3*) or *Fgf10 (*RCAS-*Fgf10)*, and implantation of beads soaked with FGF2- or FGF10-heparin.

Injection of RCAS-*Fgf3* into the lumen of the otocyst at E2.5-3 (Stages 18-20) results in a failure of canal resorption at E7 when compared with controls (Fig. 4A,B; arrowhead). By E9, the infected inner ears show multiple epithelial protrusions from the non-resorbed canal pouches (Fig. 4D, arrows), although the overall size and relative position of the canal pouches appear normal (Fig. 4C,D; arrowheads; *n*=15/15). RCAS alone or RCAS-*Fgf3*-RO did not lead to inner ear defects (*n*=15). RCAS-*Fgf10* yielded a similar phenotype to RCAS-*Fgf3*, with undulated epithelial outgrowths (Fig. 4F; *n*=10/16).

The phenotypes induced by both RCAS-*Fgf3* and RCAS-*Fgf10* are complicated and appear to involve several developmental processes in the canal pouch, including resorption and common crus formation. In order to pinpoint the developmental process(es) and stage(s) that are most sensitive to FGF treatment, and to explore the downstream targets of FGFs, we tested the effects of adding FGFs at different stages of canal development. As FGF3 is not commercially available and FGF2 has been shown to activate multiple FGF receptors (Ornitz et al., 1996), the effects of the FGF2 protein on canal development were tested. Even though weak FGF2 immunostaining was reported in the otic placode and otocysts (Vendrell et al., 2000), we did not detect any *Fgf2* transcripts in chicken otocysts using in situ hybridization. Nevertheless, implanting a bead soaked with FGF2 into the lumen of the inner ear at E4-E5 under standard conditions (see Materials and methods) interferes with common crus formation by E9 (Fig. 4G, arrows; Table 1, *n*=40/43). The extent of the common crus loss is variable, ranging from a complete absence (Fig. 4G, arrows) to a partial loss of its most dorsal extent (Fig. 6B, arrows). In the absence of the common crus, the anterior and posterior canals are continuous with each other, whereas the lateral canal is often truncated at the location where it normally joins the common crus (Fig. 4G, arrowhead). Implantation at an earlier age (E3) using a reduced dose of FGF2 causes a similar common crus phenotype (*n*=38/40). A standard dose of FGF2 at E3, however, causes additional defects in structures that include the endolymphatic duct and cochlear duct (*n*=36/40) that have not been examined in detail. Previous studies have shown that implantation of FGF2 beads anterior to the otic placode increased the size of the cochleovestibular ganglion (Adamska et al., 2001). We did not examine whether our treatments here, conducted at a later stage of otic

development, also affect ganglionic development. In the present study, implantation beyond E5.5 results in normal inner ears with an intact common crus (*n*=22/22), suggesting that only the early stages of common crus specification are sensitive to excess FGF.

FGF2-bead treatment prevents canal pouch resorption when assayed on E7, which is prior to the loss of common crus phenotype first seen at E9 (*n*=14/15). This lack of resorption is similar to that seen on E7 following infection with RCAS-*Fgf3* (Fig. 4B). These results suggest that both FGF treatments affect similar developmental processes, even though the phenotypes subsequently diverged by E9.

To verify that the two gain-of-function approaches are disrupting the same developmental processes, we conducted two additional experiments in which the effective dosages of FGFs were altered. We increased the dosage of exogenous FGF2 by implanting more FGF2-soaked beads into the lumen of the otocyst at E5, and, as a result, resorption was delayed at least up until E9, although epithelial protrusions similar to RCAS-infected inner ears were not evident (Table 1). By contrast, in an attempt to reduce viral spread, a small dose of RCAS-*Fgf3* was injected into the mesenchyme dorsal to the otocyst at E4, 1-1.5 days later than the lumenal injections. A small percentage of these infected inner ears show a milder phenotype on E9, characterized by slightly enlarged semicircular canals (Fig. 4E, asterisk) and a thin common crus (Fig. 4E, arrow; *n*=4/35 from four separate experiments), thus resembling the bead-implanted inner ears. The phenotypes in the rest of the specimens are similar to the one illustrated in Fig. 4D.

Furthermore, to verify that these phenotypes are elicited by perturbing the FGF pathway, we simultaneously implanted FGF2 beads and beads soaked with SU5402, an inhibitor of FGF receptors (Mohammadi et al., 1997). As expected, SU5402 is able to prevent the loss of the common crus phenotype caused by exogenous FGF2 (Fig. 4H,I; arrows; *n*=5/6).

Fgf10 is expressed endogenously in the developing inner ear prior to canal pouch formation. FGF10-soaked beads have no effect on common crus formation. However, FGF10-heparin beads elicit the loss of the common crus similar to the common crus phenotype induced by FGF2 bead implantation (Fig. 4J, arrows; Table 1, *n*=5/8). Taken together, these results suggest that a transient presence (bead implantation) or a modest increase (focal mesenchymal RCAS infection) of FGFs during canal pouch development delays the normal resorption process and alters the formation of the common crus. By contrast, prolonged FGF treatment (lumenal RCAS infection) completely blocks resorption and converts the entire canal pouch into a canal duct-like fate.

FGFs induce *Bmp2* and *Bmp7* in the common crus domain

We next sought to explore downstream effects of the FGFs. Inner ears implanted with FGF2 beads at E3 to E5 show an induction of *Bmp2* and *Bmp7* in the dorsal otocyst within 24 hours, particularly in regions corresponding to the prospective resorption domains and common crus (Fig. 5A-D, double arrows). Although the dorsal epithelium of the canal pouch is normally thin (Fig. 5E, arrowheads), FGF2 bead implantation causes an induction of *Bmp2* expression and an increase in the thickness of the epithelium that resembles canal-type epithelium (Fig. 5F, arrowheads). In a more ventral region of the pouch, where *Bmp2* expression is normally restricted to the outer rim (Fig. 5G, arrows), treatment with FGF2 expands the

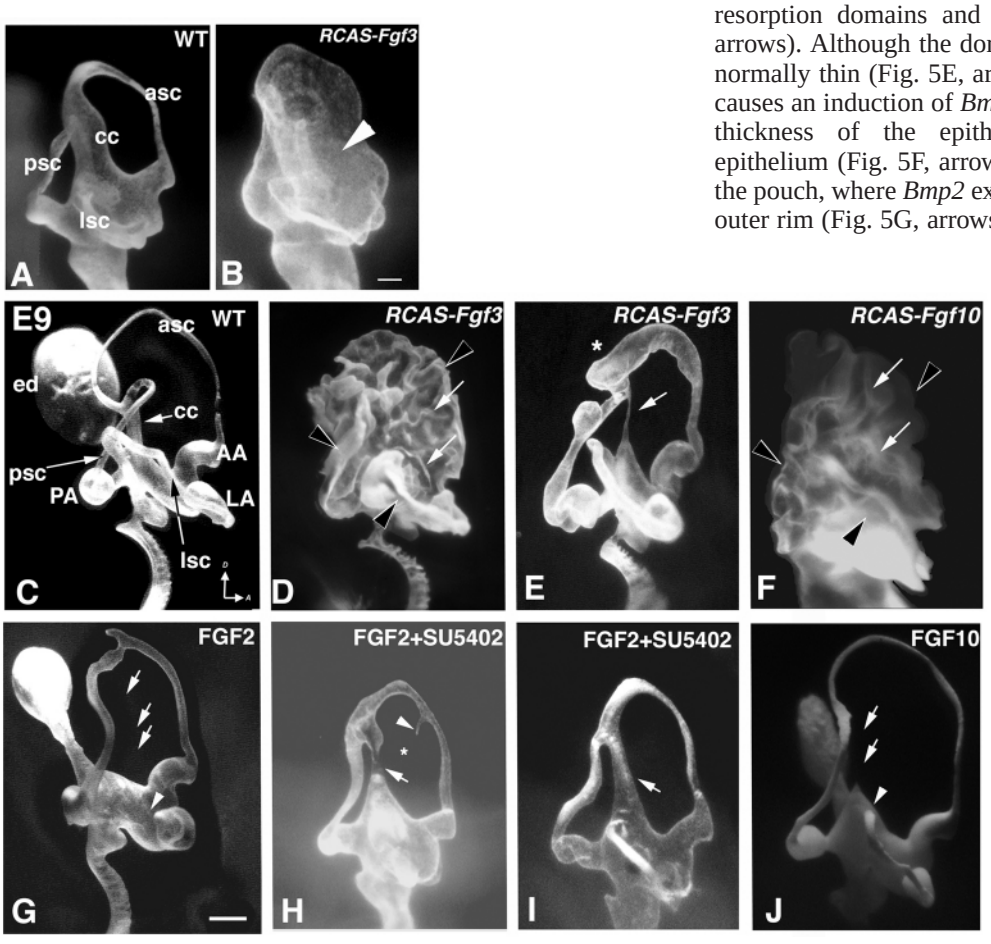

Fig. 4. Ectopic FGF treatments. Paint-filled inner ears were harvested on E7 (A,B) or E9 (C-J). Embryos were injected with viruses (A-F) or implanted with beads (G-J), as indicated in each panel. Viruses were delivered to the otocyst lumen on E2.5-3, except for the ear shown in E, which received injections into the periotic mesenchyme on E4. Beads were implanted on E4 (G,J) or E5 (H,I). White arrows denote defective regions and arrowheads are described in detail in the text. Black arrowheads in D and F indicate normal locations of the canal pouches. For illustration of the common crus phenotype, the endolymphatic duct was removed from the inner ears shown in E, H and I. Abbreviations are as in Fig. 1. AA, anterior ampulla; LA, lateral ampulla; PA, posterior ampulla. Orientations in C apply to all panels. Scale bars: in B, 100 μm for A,B; in G, 200 μm for C-J.

Table 1. Quantitation of FGF-induced phenotypes in the vertical canal pouch

Treatments	Age of operation	Age of harvest	Absence of common crus*,¶	Canal pouch non-resorption*
FGF2 bead	E3	E9	38/40	0/40
FGF2 bead	E5	E7	0/15	14/15
FGF2 bead	E4-E5	E9	40/43	0/43
FGF2 bead	E5.5	E9	0/22	0/22
Multiple FGF2 beads†	E5	E9	0/5	2/5
FGF10 bead	E5	E9	5/8	0/8
RCAS-Fgf3 (lumen)	E2.5-3	E7	0/10	10/10
RCAS-Fgf3 (lumen)	E2.5-3	E9	0/15	15/15
RCAS-Fgf10 (lumen)	E2.5	E9	0/16	10/16
RCAS-Fgf10 (dorsal mesenchyme)	E4	E9	4/35‡	31/35§

*Number of specimens with the indicated phenotype/total number of specimens scored.
†The common crus is present but narrower than in wild type.
‡Partial loss of the common crus.
§Partial non-resorption of the canal pouch.
¶Numbers represent those specimens that underwent excessive resorption resulting in the loss of the common crus.

Bmp2 expression domain towards the center of the pouch (presumptive resorption and common crus regions; arrowheads, Fig. 5G,H). An increase in the thickness of the otic epithelium is also observed (Fig. 5H, arrowheads). Likewise, implantation with FGF10-heparin beads induces *Bmp2* expression in the canal pouch (Fig. 5K,L; *n*=10/11) and increases the thickness of the epithelium. BSA-soaked beads or BSA-heparin beads do not change *Bmp2* expression (*n*=6).

We verified that these thickened epithelia still retain their canal pouch properties by probing for *Soho1*, a gene normally expressed throughout the entire canal pouch (Fig. 5M). Despite the change in cellular morphology, *Soho1* expression persists

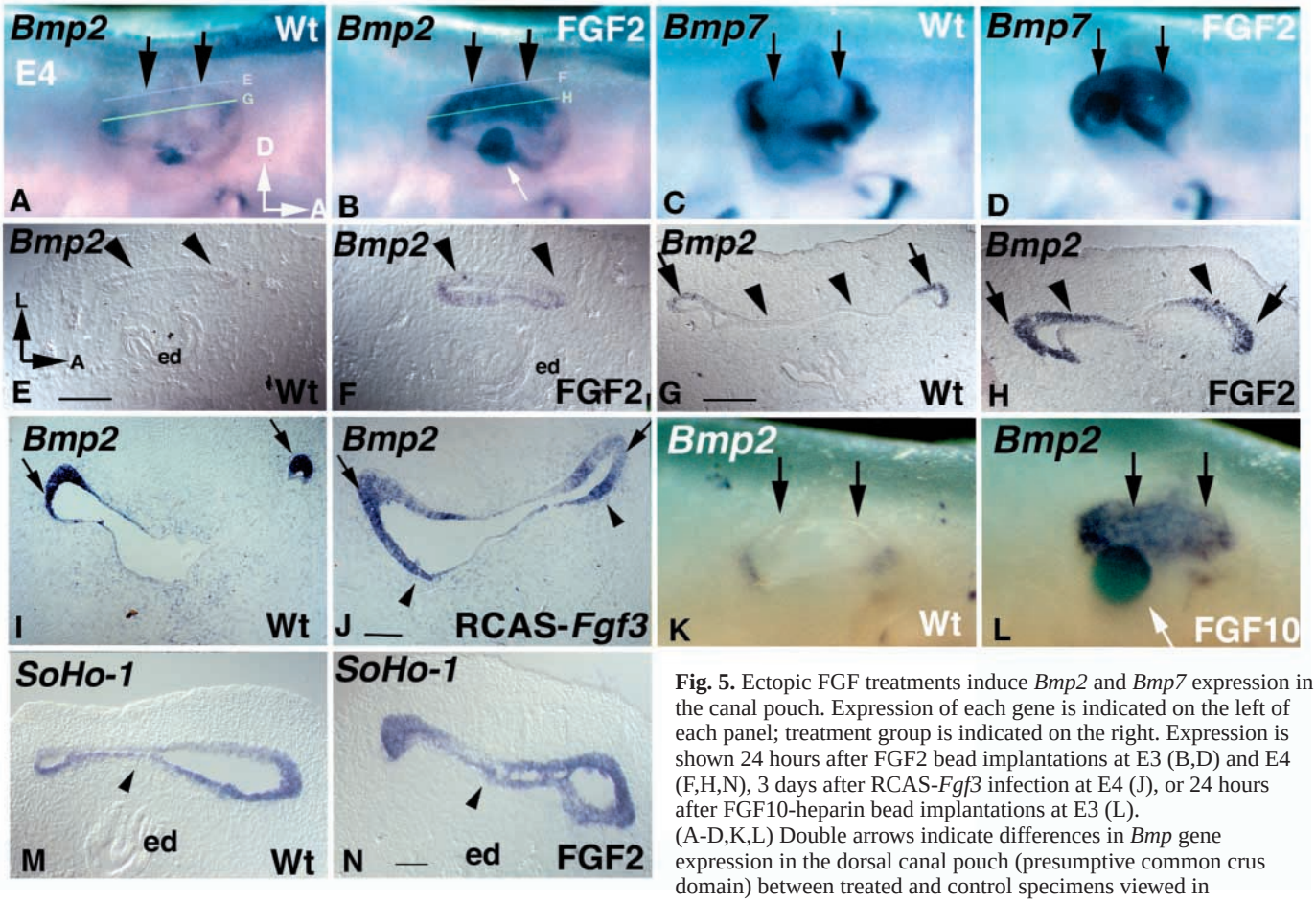

Fig. 5. Ectopic FGF treatments induce *Bmp2* and *Bmp7* expression in the canal pouch. Expression of each gene is indicated on the left of each panel; treatment group is indicated on the right. Expression is shown 24 hours after FGF2 bead implantations at E3 (B,D) and E4 (F,H,N), 3 days after RCAS-*Fgf3* infection at E4 (J), or 24 hours after FGF10-heparin bead implantations at E3 (L). (A-D,K,L) Double arrows indicate differences in *Bmp* gene expression in the dorsal canal pouch (presumptive common crus domain) between treated and control specimens viewed in wholemounts. (E-H) The approximate level of each section is indicated in either A or B. (E-J) Arrows indicate *Bmp2* expression normally evident in the rim of the prospective canal pouch; arrowheads indicate differences between experiments and controls in *Bmp2* expression and epithelial thickness in the central part of the canal pouch. (M,N) *Soho1* expression persists in the central region of the canal pouch (arrowhead) in both control and treated ears. Orientations in A applies to B-D,K,L; E applies to E-J,M,N. White arrows in B and L indicate the implanted beads. Scale bars: in E, 100 μm for E,F; in G, 100 μm for G,H; in J, 50 μm for I,J; in N, 50 μm for M,N.

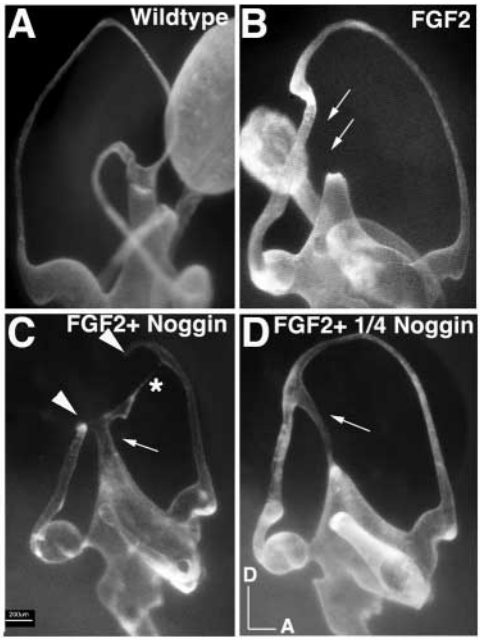

Fig. 6. Noggin rescues the loss of common crus induced by FGFs. Paint-filled inner ears at E9 from (A) controls or (B-D) embryos implanted at E5 with beads as indicated. Arrows point to the region of the common crus. Arrowheads indicate truncation of canals. Asterisk denotes an ectopic epithelial stump. The endolymphatic ducts in C and D were removed prior to photography. Orientations in D apply to A-C. Scale bar in C: 200 μm for A,B,D.

in the FGF2-treated ears, suggesting that a conversion to a sensory fate has not occurred (Fig. 5N; *n*=5).

Noggin rescues the loss of common crus induced by FGF2

To determine whether the induction of *Bmp2/7* in the prospective common crus region is a cause or a consequence of the loss of this structure, we investigated whether the phenotype could be rescued with a BMP inhibitor. We implanted beads soaked with Noggin into the mesenchyme surrounding the dorsal region of the common crus, concurrent with implanting FGF2-soaked beads to the lumen of inner ears. Noggin rescues the loss of common crus (Fig. 6C, arrow) but also results in a partial loss of the semicircular canals (Fig. 6C, arrowheads; *n*=4/4). Presumably, Noggin blocks endogenous BMP activities in the canal pouch (i.e. canal rim formation), in addition to blocking exogenous BMPs induced by FGF treatments (i.e. rescue of the common crus). However, by using a weaker dose of Noggin, the two functions are separable: canal formation is normal while the common crus phenotype is still rescued (Fig. 6D; *n*=5/9). We conclude that BMP induction by FGF2 is indeed causal to the absence of the common crus.

Ectopic FGF treatments cause some canal pouch cells to change fate

As the prospective common crus is normally *Bmp2* and *Bmp7* negative, we used fate-mapping studies to determine whether the loss of the common crus with FGF2 treatment is possibly due to a change in cell fates. We implanted chicken otocysts with BSA- or FGF2-soaked beads at E4 and fate mapped the dorsal rim of canal pouch by injecting DiI into the 12 o'clock position at E5. By E9, DiI-labeled cells are observed only in the common crus of the BSA-treated specimens (Fig. 7A-C, arrows; *n*=6/6), whereas DiI-labeled cells are incorporated in the canals of specimens treated with FGF2 (Fig. 7D-F, double arrows; *n*=5/6). Similar results are observed when the implantation of FGF2 beads is concurrent with DiI labeling at E5 (Fig. 7G-I, double arrows; *n*=3/3). Under both treatment conditions, some DiI-labeled cells are associated with the mesenchyme outside of the labyrinth (Fig. 7E,F,H,I; arrowheads), a result not observed with BSA implants (Fig. 7B,C). These results suggest FGF treatments cause some cells in the dorsal rim of canal pouch that normally give rise to the common crus to become incorporated into the canals instead.

FGFs induce *Bmp2* expression before canal pouch formation

Next, we investigated whether ectopic FGF treatments altered *Bmp2* expression prior to canal pouch formation. We implanted FGF2-soaked beads into the otocyst at E2.5, before canal pouch formation, and harvested specimens 18 or 24 hours later for *Bmp2* gene expression analysis. In addition, RCAS-*Fgf3* was injected into the mesenchyme surrounding the otic cup at E2, 12 hours earlier than the bead implantation experiments, to allow sufficient time for viral integration and transcription, and then inner ears were harvested at E3 to E3.5. Our results show that ectopic *Bmp2* is increased both by FGF2 beads (Fig. 8A,B; arrowhead; *n*=12/15) and by RCAS-*Fgf3* (Fig. 8C,D; arrowhead; *n*=9/14), at an age before the endogenous expression of *Bmp2* is obvious (Fig. 8A,C; arrows). No significant changes in *Bmp2* expression are observed in control experiments using RCAS-*Fgf3*-RO (*n*=16), or BSA beads (*n*=6). These results indicate that FGFs are capable of inducing/upregulating *Bmp2* expression at the otocyst stage before canal pouch formation.

Precocious induction of *Bmp7* by FGFs cannot be evaluated because the otic epithelium is already *Bmp7*-positive by E2.5, prior to the initiation of FGF expression (Oh et al., 1996). Therefore, FGFs in the sensory domain are unlikely to be required for the induction of *Bmp7* expression in the inner ear.

Endogenous FGF activities are required for *Bmp2* expression and formation of the semicircular canals and ampullae

We used a loss-of-function approach to address whether endogenous FGFs are required to initiate or maintain *Bmp2* expression in the canal pouch. Otocysts were treated with varying dosages of the FGF inhibitor SU5402 (see Materials and methods) at E2.5 to E3, i.e. before the initiation of canal pouch formation, and were assayed after 24 hours for *Bmp2* expression. No change in *Bmp2* expression is observed with low doses of SU5402. At a medium dose, however, the *Bmp2* expression domains in the canal pouch are reduced (Fig. 8E,F, *n*=5/6), with the posterior wedge (arrowheads) more affected than the anterior wedge (arrows). Similar results are obtained when SU5402-soaked beads are implanted at E5 (*n*=17/22, data not shown). Control experiments using DMSO-soaked beads show no reduction of *Bmp2* expression (*n*=7). These results indicate that *Bmp2* expression in the canal pouch requires FGFs.

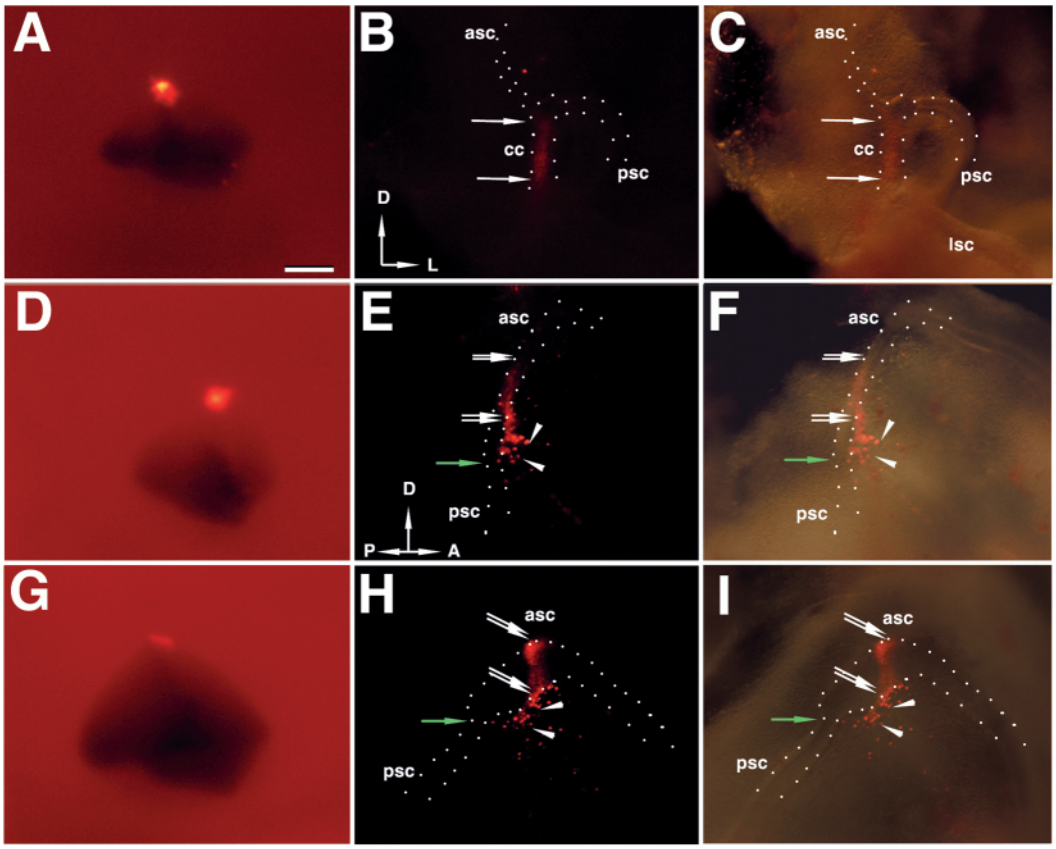

Fig. 7. Fate mapping of the FGF2-treated canal pouch. Otocysts were treated with either BSA (A-C) or FGF2 (D-F) at E4, and DiI was delivered to the 12 o'clock position of the canal pouch at E5; inner ears were harvested at E9. (G-I) FGF2 bead implantation and DiI injection were carried out at the same time, at E5. A, D and G show the location of DiI injection. The same three specimens shown in A, D and G are shown on E9 as fluorescent (B,E,H) or combined fluorescent and bright field images (C,F,I). DiI-labeled cells are associated with the common crus only in BSA-treated specimens (B,C; arrows), whereas some DiI-labeled cells in FGF2-treated specimens are associated with the canals (E,F,H,I; double arrows). Arrowheads indicate cells outside the membranous labyrinth and green arrows indicate the junction of the anterior and the posterior canals. A, anterior; D, dorsal; L, lateral; P, posterior. Scale bar in A: 200 μm for A-I.

To determine the effect of reduced *Bmp2* expression on canal formation, SU5402-treated inner ears were paint-filled at E6 and E9. Consistent with the changes observed in *Bmp2* expression, a medium dose of SU5402 affects the posterior canal pouch (Fig. 8G, asterisk). Also, the posterior canal (Fig. 8I, asterisk) is more severely affected than the anterior canal (n=20/31). In addition, the posterior ampulla is absent. However, with high doses of SU5402, both the vertical and horizontal canal pouches are affected (Fig. 8H), and all three canals fail to form, although their associated ampullae are sometimes present (arrowhead, Fig. 8J). By contrast, the common crus is intact in all affected specimens analyzed (Fig. 8H,J, arrows; n=20). These results lend further support to the proposal that FGFs promote canal development but are not required for specification of the common crus.

Discussion

Canal formation

The vertical and horizontal canal pouches that develop into the three semicircular canals are primarily derived from the lateral wall of the otocyst. In the chicken, fate mapping of the rim of the otic cup indicates that during otic cup closure, a medial-lateral lineage boundary is established such that the medial region of the otocyst develops into the endolymphatic duct and the lateral region forms the primordial canal pouches (Brigande et al., 2000). The first physical sign of the endolymphatic duct and canal pouch primordium as separate entities occurs around E3.5 (stage 21). However, what drives the continual growth of either of these structures is not known. Here, we show that a canal genesis zone located adjacent to the prospective sensory region is a key component in the continual growth of the canal pouch (Fig. 9, blue stars). Cells in this genesis zone give rise to canal epithelium as well as to the common crus. The dorsal region of the canal pouch (Fig. 9, light blue color) gives rise to the common crus. Both regions most likely contribute to cells in the resorption domains.

Bmp2-positive domain and the canal genesis zone

Fate mapping places the canal genesis zone in close proximity to the *Bmp2*-positive domain. However, it is not clear whether both domains completely overlap because a detailed comparison of the two domains cannot be performed. It is possible that the two domains do not overlap, and that cells in the genesis zone are *Bmp2* negative and only those that acquire canal fate become *Bmp2* positive. If indeed the two domains

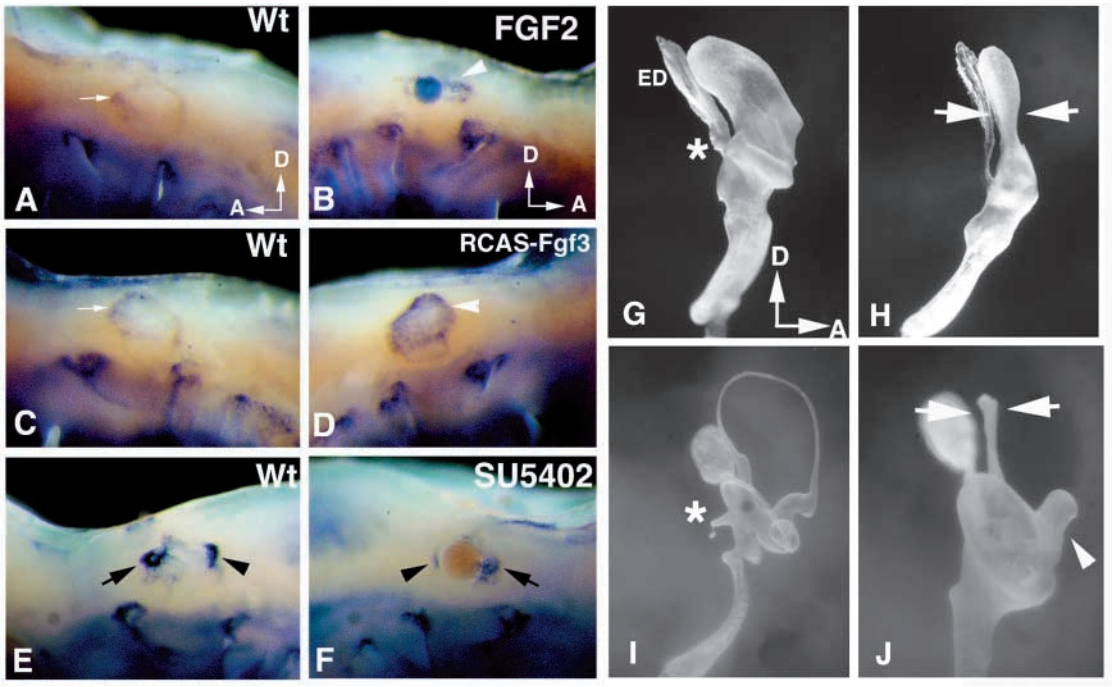

Fig. 8. Effects of FGF and SU5402 treatments prior to canal pouch formation. (A-F) *Bmp2* expression in FGF- (B,D) or SU5402-treated (F) otocysts. A, C and E show left untreated ears of the same embryos that are shown in B, D and F, respectively. Otocysts implanted with an FGF2-soaked bead at E2.5 (B), infected with RCAS-*Fgf3* at E2 (D), or implanted with SU5402-soaked beads at E3 (F) were analyzed 24 hours later. At E3-E3.5, *Bmp2* expression in control inner ears is barely detectable (arrows, A,C), but it is upregulated with FGF2 and *RCAS-Fgf3* treatments (white arrowhead in B and D). By E4, the two wedge-shaped *Bmp2* expression domains are evident in the control ear (E), and this expression is downregulated in the SU5402-treated ear (F). The reduction of *Bmp2* expression is more pronounced in the posterior domain (arrowhead) than the anterior domain (arrow). (G-J) Paint-filled inner ears treated with medium (G,I) and high (H,J) doses of SU5402 at E2.5 and harvested at E6 (G,H) or E9 (I,J). (G,I) A medium dose of SU5402 causes the loss of the posterior canal pouch at E6 (asterisk, G) and prevents formation of the posterior canal and ampulla at E9 (asterisk, I). (H,J) A high dose of SU5402 causes a severely deformed canal pouch at E6 (arrows, H) and absence of all three canals and ampullae, but the common crus is intact (arrows, J). Arrowhead in J indicates the presence of the anterior ampulla. Orientations in A apply to C and E; orientations in B apply to D and F; orientations in G apply to H-J.

overlap completely, then the result of labeled cells found in both the canals and common crus after injection to the genesis zone would suggest that some cells migrate out from the *Bmp2*-positive domain to form the common crus. Alternatively, these results can also be explained if the canal genesis zone/*Bmp2* domain is relatively small compared with the labeled area from a single DiI injection. In this case, the chance of simultaneously labeling cells giving rise to both canal and common crus is high.

FGFs in sensory tissues promote canal development

Using gain-of-function (FGF2, FGF3 and FGF10) as well as loss-of-function (SU5402) approaches in the developing chicken inner ear, we demonstrated the requirement of FGFs for canal development. The endogenous sources of FGF3 and FGF10 are postulated to arise from the neurosensory primordial, and mediate canal development by inducing *Bmp2* in the adjacent canal pouch (Fig. 9). The restricted expression of *Fgf3* and *Fgf10* in the pro-sensory domains, and the ubiquitous expression of FGF receptors in the otic epithelium, support this hypothesis.

The significance of BMPs in canal development is supported by our previous ectopic Noggin treatment studies, even though these studies did not address which BMP(s) were directly involved (Chang et al., 1999). The association of *Bmp2* in the prospective canal regions, its upregulation by FGF2, FGF3 and FGF10, and its downregulation by an FGF inhibitor, all implicate *Bmp2*. We suggest that endogenous *Bmp2* activity in the canal pouch is regulated by FGFs associated with the neurosensory primordia. The requirement of *Bmp2* in canal development, and its possible interactions with other genes known to be important for canal development, could be addressed by using *Bmp2* conditional-knockout mice, as this FGF-*Bmp2* pathway is likely to be conserved in mice (see below). These experiments are currently underway.

Even though FGFs could also mediate their effects through *Bmp7*, the timing of *Bmp7* expression tends not to support this; the onset of *Bmp7* expression precedes that of FGFs in the inner ear, suggesting that *Bmp7* induction is not dependent on FGF signaling. Also, the downregulation of *Bmp7* expression in the prospective canal region at E6 (Chang et al., 2002), when the canal pouch is still undergoing rapid growth, suggests that *Bmp7* may not play a role in maintaining canal development. Finally, unlike *Bmp2*, no obvious downregulation of *Bmp7* expression was observed in inner ears treated with SU5402 (data not shown).

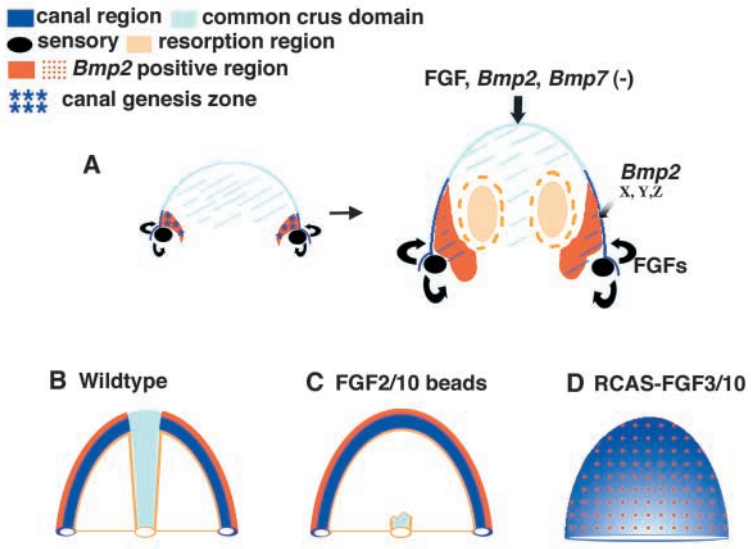

Fig. 9. Model demonstrating how FGFs originating from sensory primordia regulate semicircular canal and common crus formation in adjacent epithelium. (A) Progression of canal pouch development from E3.5 to E5.5. FGF3 and FGF10 emanating from sensory regions (black ovals) promote canal outgrowth by inducing a canal genesis zone (blue stars), possibly by activating *Bmp2* expression (orange) and, potentially, other factors (X, Y and Z). Either through physical distance from the sources of FGFs or through other uncharacterized mechanisms, a FGF-negative, prospective common crus domain is established (light blue). This prospective common crus domain is *Bmp2* and *Bmp7* negative in the dorsal region. Two resorption domains (light gold ovals surrounded by gold dashes) are established on both sides of the common crus region in which the epithelia eventually disappear leaving behind two canals and the common crus (B). (C) A transient, exogenous dose of FGFs applied before E5 expands the BMP territory and prevents the normal resorption process. As the level of exogenous FGF diminishes over time, the resorption process resumes, and includes the rest of the epithelia in the common crus domain that failed to be properly specified. (D) Sustained FGF overexpression by RCAS blocks the resorption process so that the pouches remain open and a canal-rim fate is adopted.

Ectopic FGF treatments affect common crus formation

Ectopic FGF treatments also affected formation of the common crus. The common crus normally forms as a result of resorption of epithelial cells in the fusion plate, a process that involves programmed cell death in the chicken (Fekete et al., 1997). It is not clear whether the formation of the common crus is dictated solely by regulated resorption of the fusion plate. Alternatively, the prospective common crus region could be molecularly distinct from the surrounding fusion plate and could play an active role in regulating the resorption process. This scenario is supported by the differential *Bmp2* expression in the canal rim but not in the common crus primordia. Other known canal pouch markers, such as *Hmx2*, *Hmx3*, *Soho1*, *Netrin1* and *Nor1*, do not distinguish between the two primordial structures (Fedorov et al., 1998; Kiernan et al., 1997; Ponnio et al., 2002; Salminen et al., 2000).

The undulation of the otic epithelium, and the absence of resorption in the canal pouch treated with a normal dose of RCAS-*Fgf*, might be explained by over-proliferation or a lack of programmed cell death (Fig. 4D, Table 1). However, the thinning or lack of the common crus resulting from reduced levels of the *RCAS-Fgf* or FGF2 bead implantation supports an excess of programmed cell death rather than over-proliferation (Fig. 4E,G; Table 1). Therefore, FGF-induced phenotypes cannot be explained easily by either process. Instead, our fate-mapping data indicate that a cell fate change might be involved. Ectopic FGF2 treatments cause some of the cells in the dorsal rim of the canal pouch, which normally develop into the common crus, to form part of the canals (Figs 2, 7). We hypothesize that cell fate conversion might also derail the normal resorption process. Presumably, as the amount of exogenous FGF in the bead-implanted specimens diminished over time, the resorption process, although resumed, was misregulated and included the common crus domain. Thus, the common crus was absent in FGF2- and FGF10-treated ears (Fig. 9C). This could also explain the extensive DiI labeling in the mesenchyme of FGF-treated specimens at E9 (Fig. 7). However, with sustained FGF expression by viral infection, the resorption was not initiated and the entire canal pouch epithelium adopted a canal-like fate (Fig. 9D). While fate change is one plausible explanation at this point, other as yet unknown mechanisms normally responsible for this epithelial remodeling process might be affected by FGF treatments. Regardless of the mechanisms involved, FGFs can no longer elicit a phenotype beyond E5.5. Furthermore, our results suggest that the prospective common crus plays an active role in regulating the resorption process during normal canal genesis.

It is not clear how FGF concentration is modulated in the dorsal canal pouch in vivo. So far, expression of *Fgfr*, or the known FGF antagonist *Sprouty* (data not shown) (Hacohen et al., 1998; Minowada et al., 1999), has not revealed any regional differences in expression patterns that could account for the low FGF activity in the dorsal region of the canal pouch. Physical distance from the sources of FGFs could be one plausible explanation.

Although low levels or absence of FGF activity is required to specify or maintain a common crus fate, FGF is unlikely to be the only factor required for this fate. Regulated levels of BMPs are important (see below), and insensitivity to retinoic acid might also be involved because the common crus is particularly resilient to retinoic acid treatments (Choo et al., 1998). In addition, blocking FGF activity with SU5402 is insufficient to recruit the surrounding canal pouch epithelium to form an ectopic common crus (Fig. 3G,H).

Even though Noggin was able to rescue the loss of the common crus by blocking BMP activities induced by ectopic FGF treatments, the normal development of the common crus most likely requires regulated levels of BMP activities rather than a complete absence of BMPs. This is evident by the absence of a common crus in some of the specimens treated with high levels of Noggin (Chang et al., 1999).

Evolutionarily conserved role of FGFs in mediating canal development

In mice, both *Fgf3* and *Fgf10* are expressed in the neurogenic and sensory regions of the inner ear (Pirvola et al., 2000). Possible functional redundancy of *Fgf3* and *Fgf10* in the

sensory regions cannot be addressed easily in mice because double knockouts of *Fgf3* and *Fgf10* have no inner ear. Presumably, this absence of otic vesicle formation is due to the lack of earlier FGF3 and FGF10 functions in the hindbrain and mesoderm, respectively (Alvarez et al., 2003; Wright and Mansour, 2003). However, canal phenotypes reported in the knockout of either *Fgf3* or *Fgf10* support the role of FGFs that is proposed here (Mansour et al., 1993; Pauley et al., 2003). In addition, the posterior canal and ampulla are the most affected in *Fgf10* knockout mice, similar to the SU5402-treated specimens in the chicken. Furthermore, *Bmp2* also has a similar spatial and temporal expression pattern in the canal pouch of mice as in chicken (W.C. and D.K.W., unpublished). These results suggest that the role of FGFs in specifying non-sensory development in the inner ear is most likely evolutionarily conserved across birds and mammals.

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