FGF/FGFR-2(IIIb) Signaling Is Essential for Inner Ear Morphogenesis

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Interactions between FGF10 and the IIIb isoform of FGFR-2 appear to be crucial for the induction and growth of several organs, particularly those that involve budding morphogenesis. We determined their expression patterns in the inner ear and analyzed the inner ear phenotype of mice specifically deleted for the IIIb isoform of FGFR-2. FGF10 and FGFR-2(IIIb) mRNAs showed distinct, largely nonoverlapping expression patterns in the undifferentiated otic epithelium. Subsequently, FGF10 mRNA became confined to the presumptive cochlear and vestibular sensory epithelia and to the neuronal precursors and neurons. FGFR-2(IIIb) mRNA was expressed in the nonsensory epithelium of the otocyst that gives rise to structures such as the endolymphatic and semicircular ducts. These data suggest that in contrast to mesenchymal–epithelial-based FGF10 signaling demonstrated for other organs, the inner ear seems to depend on paracrine signals that operate within the epithelium. Expression of FGF10 mRNA partly overlapped with FGF3 mRNA in the sensory regions, suggesting that they may form parallel signaling pathways within the otic epithelium. In addition, hindbrain-derived FGF3 might regulate otocyst morphogenesis through FGFR-2(IIIb). Targeted deletion of FGFR-2(IIIb) resulted in severe dysgenesis of the cochleovestibular membraneous labyrinth, caused by a failure in morphogenesis at the otocyst stage. In addition to the nonsensory epithelium, sensory patches and the cochleovestibular ganglion remained at a rudimentary stage. Our findings provide genetic evidence that signaling by FGFR-2(IIIb) is critical for the morphological development of the inner ear.

Key words: FGFR-2; FGF10; FGF3; gene expression; gene disruption; inner ear development; cochleovestibular neurons

Fibroblast growth factors (FGFs 1–19) comprise a family of signaling molecules that regulate cellular proliferation, differentiation, and migration by binding to and activating members of a family of tyrosine kinase receptors (FGFRs 1–4) (for review, see Wilkie et al., 1995; Ornitz, 2000). A number of studies have implicated FGF signaling as being fundamental for pattern formation during the genesis of several tissues, suggesting that it has been repeatedly adapted for use during organogenesis. FGFs are found throughout metazoan evolution. For example, *Drosophila* homologs of vertebrate FGFs and FGFRs, *branchless* and *breathless*, respectively, regulate epithelial branching in the tracheal system (Klambt et al., 1992; Murphy et al., 1995; Sutherland et al., 1996). In mammals, FGF–FGFR interactions appear to regulate budding morphogenesis of the limb and the branching morphogenesis in lung formation. Recent studies have provided evidence that mesenchyme-derived FGF10 is the key factor that initiates and/or maintains outgrowth of FGFR-2-expressing epithelial appendages (Ohuchi et al., 1997). Compelling evidence for this suggestion has come from FGF10 null mutations and from different approaches to delete the *FGFR-2* gene (Peters et al., 1994; Jackson et al., 1997; Celli et al., 1998; Min et al., 1998; Xu et al., 1998; Arman et al., 1999; Sekine et al., 1999; DeMoerlooze et al., 2000). Furthermore, recent data imply that reciprocal signaling loops between different FGFs function during morphogenesis, as exemplified in the limb bud (Ohuchi et al., 1997; for review, see Hogan, 1999).

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In comparison to the limb bud, the inner ear is derived from a simple ectodermal thickening, the otic placode. Although relatively little is known about the mechanisms that generate the different cell types, even less is known about how the three-dimensional morphology of the cochleovestibular labyrinth is generated (Fekete, 1996, 1999). Genetic evidence and expression data have suggested that FGF3 influences early development of the mammalian inner ear, specifically by regulating formation of the endolymphatic duct (Mansour et al., 1993; McKay et al., 1996). However, the inner ear phenotype of *FGF3* null mutants shows reduced penetrance and variable expressivity (Mansour et al., 1993). Therefore, a prerequisite for understanding FGF3 function in the inner ear is to characterize its receptor(s) and the possible FGF compensatory signals that could account for the partially penetrant phenotype. Of the four FGF receptors, FGFR-3 is expressed in the cochlear sensory epithelium during late embryogenesis and postnatal life (Peters et al., 1993; Pirvola et al., 1995), and a *FGFR-3* null mutation leads to deafness attributable to disturbances in differentiation of the cochlear sensory epithelium (Colvin et al., 1996). Expression data have suggested that cochlear neuron-derived FGF1 (Pirvola et al., 1995) and inner hair cell-derived FGF8 (Pirvola et al., 1998) may serve as ligands for FGFR-3 in the late embryonic and postnatal cochlea. FGF9 mRNA has been localized to the otic vesicle and to the later developing nonsensory epithelium and ganglion of the cochlea (Colvin et al., 1999). In addition, exogenous FGF2 has been shown to stimulate neuronal migration and differentiation in explants of the early chick inner ear (Hossain et al., 1996; Brumwell et al., 2000).

The extracellular domain of FGFRs are composed of three Ig-like domains, designated as Ig-loops-I, -II and -III. Ligand binding involves the second and third Ig-loop, although specificity is determined predominantly by Ig-loop-III. The proximal part of this loop is encoded by alternatively spliced exons for FGFRs 1–3, thereby generating two distinct receptor isoforms, designated as IIIb and IIIc isoforms, which have different ligand-binding specificities. For example, FGFR-2(IIIb) is activated by FGF1, FGF3, FGF7, and FGF10, whereas FGFR-2(IIIc) is activated by FGF1,

FGF2, FGF4, FGF6, and FGF9 (Ornitz et al., 1996). Furthermore, the IIIb and IIIc isoforms of FGFR-2 have mainly exclusive expression patterns (Orr-Urtreger et al., 1993). A null mutation and a hypomorphic mutation of the complete *FGFR-2* gene show early lethality or die in midgestation, respectively (Arman et al., 1998; Xu et al., 1998). More recently, mice lacking the IIIb but not the IIIc isoform of FGFR-2 have been generated by using a Cre-mediated excision approach. These mice are viable until birth and show disturbances in the development of multiple organs, including the inner ear (DeMoerlooze et al., 2000).

MATERIALS AND METHODS

Transgenic embryos

We analyzed the inner ear phenotype of *FGFR-2(IIIb)* null mutant mice that were generated by using Cre recombination to excise exon IIIb from the genome of targeted embryonic stem (ES) cells as described previously (DeMoerlooze et al., 2000). Genotyping of embryos was performed by PCR as described in that article.

Histological analysis

Embryos. Embryonic day 8 (E8), E9, E10, E11, E13, E14, E16, and E18 mice were collected for histological studies. Between E9 and E16, the histology of two or three mutant embryos and five control littermates was analyzed at each time point. At E8, five control embryos were studied. At E18, eight inner ears of mutant mice and six inner ears of control littermates were analyzed. The day of appearance of the vaginal plug was taken as day 0 of embryogenesis. Whole embryos and dissected E18 inner ears were fixed in 4% paraformaldehyde (PFA) in PBS, pH 7.4, overnight at 4°C, dehydrated, embedded in paraffin, and sectioned at 5 μm in the transverse (whole embryos) and midmodiolar (dissected inner ears) plane. Sections were placed on triethoxysilane-coated slides, dried overnight at 37°C, and stored at 4°C. For conventional histology, sections were stained with hematoxylin and eosin.

Postnatal inner ears. Dissected inner ears of postnatal day 2 (PN2), PN7, PN14, and adult mice were fixed by perilymphatic perfusion with 4% PFA and immersed in the same fixative overnight at 4°C. Specimens were decalcified in 0.5 M EDTA, pH 8.0, at 4°C for 2–7 d, then dehydrated, embedded in paraffin, and sectioned as described above.

In situ *hybridization and probes*

The antisense and sense cRNA probes were labeled with ^{35}S-UTP by *in vitro* transcription. *In situ* hybridization was performed according to Wilkinson and Green (1990) with the modifications described earlier (Pirvola et al., 1992, 1994). Sections were counterstained with hematoxylin. The plasmids used to make the antisense and control-sense cRNA probes have been described previously: FGF10 (Bellusci et al., 1997), FGFR-2(IIIb) (Orr-Urtreger et al., 1993), FGFR-2(IIIc) (Kettunen et al., 1998), FGF3 (Wilkinson et al., 1989), neurotrophin-3 (NT-3; Pirvola et al., 1992), brain-derived neurotrophic factor (BDNF; Pirvola et al., 1992), light-chain neurofilament (NF) (NF68; Pirvola et al., 1994), and Pax2 (Dressler et al., 1990). At each stage of development and during adulthood, these expressions were verified in at least four inner ears of control mice. Similarly, at least four inner ears of *FGFR-2(IIIb)* null mutants were used for *in situ* analysis at each stage of development. Sense probes did not produce any specific signal above the background level (data not shown). Bright- and dark-field illuminations were digitized by using an Olympus Provis microscope (Tokyo, Japan) and a Photometrics SenSys CCD video camera (Tucson, AZ). Figures were processed by using Image-Pro Plus 3.0 (Silver Spring, MD), Adobe Photoshop 4.0 (Adobe Systems, San Jose, CA), and Micrografx Designer 6.0 (Micrografx, Richardson, TX).

Histochemistry and detection of apoptotic cells

Immunostaining of paraffin sections with a mouse monoclonal anti-neurofilament antibody was performed as described previously (Pirvola et al., 1994). The avidin–biotin–peroxidase method and 3,3′diaminobenzidine were used for detection. To detect apoptotic cells, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay was performed on paraffin sections according to manufacturer's instructions (Fluorescein *In Situ* Cell Death Detection Kit, Boehringer Mannheim, Mannheim, Germany). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. Whole-mount staining of *NT-3* null mutant embryos for β-galactosidase activity was performed as described (Farinas et al., 1994). After staining, the embryos were prepared for paraffin sectioning as above.

RESULTS

Localization of FGFR2, FGF10, and FGF3 mRNAs in the developing cochlea

Initial stages of development

In E8 mouse embryos, the otic placode is identifiable as a thickening of the head ectoderm (Fig. 1*A*), which by E9 becomes invaginated to form a vesicle that can be seen detaching from the surface ectoderm. The E9 otic epithelium appeared homogenous in thickness, without bulges or outpocketings, indicating its undifferentiated state (Fig. 1*C*). Between E9 and E10, neuronal precursors started to delaminate from the ventral wall of the otic vesicle and migrate to form the cochleovestibular ganglion. In addition, the thin epithelium of the dorsal wall, the presumptive nonsensory epithelium, became distinguishable from the thicker epithelium of the ventral half of the vesicle (Fig. 1*E*,*G*). The outpocketing of the endolymphatic duct emerged from the dorsomedial wall between E10 and E11.

In the present description, "presumptive sensory epithelium" will refer to epithelial cell types that form the greater epithelial ridge of the cochlea and the sensory epithelia of vestibular organs (hair cells and supporting cells) (Fekete et al., 1998; Morsli et al., 1998). NT-3 and BDNF are used as known molecular markers for the presumptive sensory epithelium (Pirvola et al., 1992, 1994). "Neuronal compartment" will refer to migrating neuronal precursors and to the sensory neurons of the cochleovestibular ganglion.

Weak expression of FGFR-2(IIIb) mRNA was seen in the otic placode of E8 embryos (Fig. 1*A*,*B*). This expression became regionalized to the dorsal portion of the vesicle by E9 (Fig. 1*C*,*D*). At E10, the FGFR-2(IIIb) signal was found in the dorsal and medial walls but not in the early sensory patches of the vesicle (Fig. 1*E–H*). The IIIc isoform-specific probe revealed low level expression of FGFR-2(IIIc) in the mesenchyme surrounding the early inner ear (data not shown).

Similar to FGFR-2(IIIb), expression of its ligand FGF10 mRNA was detected in the otic placode of E8 mice (Fig. 1*I*), but at higher levels than FGFR-2(IIIb) mRNA. At E9, FGF10 mRNA was prominently expressed throughout the otic vesicle but was excluded from the dorsal region expressing FGFR-2(IIIb) mRNA (Fig. 1*J*). Between E9 and E10, FGF10 transcripts became concentrated to a broad region in the ventral half and to a small patch in the posterodorsal wall [devoid of FGFR-2(IIIb) expression] (Fig. 1*K*,*L*). By using adjacent sections, a limited degree of overlap between the FGF10 and FGFR-2(IIIb) signals was seen in the medial region of the vesicle. FGF10 mRNA was most intensely expressed in the delaminating and migrating neuronal precursors and in the early cochleovestibular ganglion, but it was not detected in the surrounding mesenchyme (Fig. 1*K*,*L*).

FGF3 mRNA, encoding another ligand for FGFR-2(IIIb), was not detected in the otic placode (Fig. 1*M*) but became apparent in the ventrolateral wall of the vesicle, where it colocalized with FGF10 mRNA, as revealed from adjacent sections (Fig. 1*J*,*N*). In addition, FGF3 mRNA was expressed at high levels in a large region of the hindbrain adjacent to the otic placode and early otic vesicle (Fig. 1*M*,*N*), as described previously (Wilkinson et al., 1989; McKay et al., 1996).

Together, these data provide good circumstantial evidence for a role of FGF10/FGFR-2(IIIb) signaling within the otic epithelium before and at the initiation of morphological differentiation. In addition, they provide support of a role for FGF3/FGFR-2(IIIb) in patterning of the early inner ear.

Differentiating otocyst

At E11, when morphogenetic changes of the otocyst become apparent, FGFR-2 and FGF10 mRNAs show distinct expression patterns (Fig. 2). FGFR-2(IIIb) mRNA was expressed in the thin-layered nonsensory epithelium of the dorsal half of the otocyst, in the dorsomedial outpocketing of the endolymphatic duct and in the medial wall (Fig. 2*A–D*). A weak signal of the IIIb isoform was also detected in the surrounding mesenchyme (Fig. 2*A–D*), but IIIc was the main isoform found in that compartment, although its expression level was low. The IIIc signal was also detected in the otic epithelium, but at very low levels and in a diffuse pattern (data not shown).

At E11, FGF10 mRNA was expressed in the otocyst in a pattern complementary to FGFR-2 mRNA. It was seen in the thickened patches of the presumptive sensory epithelia (Fig. 2*E*,*F*), where it

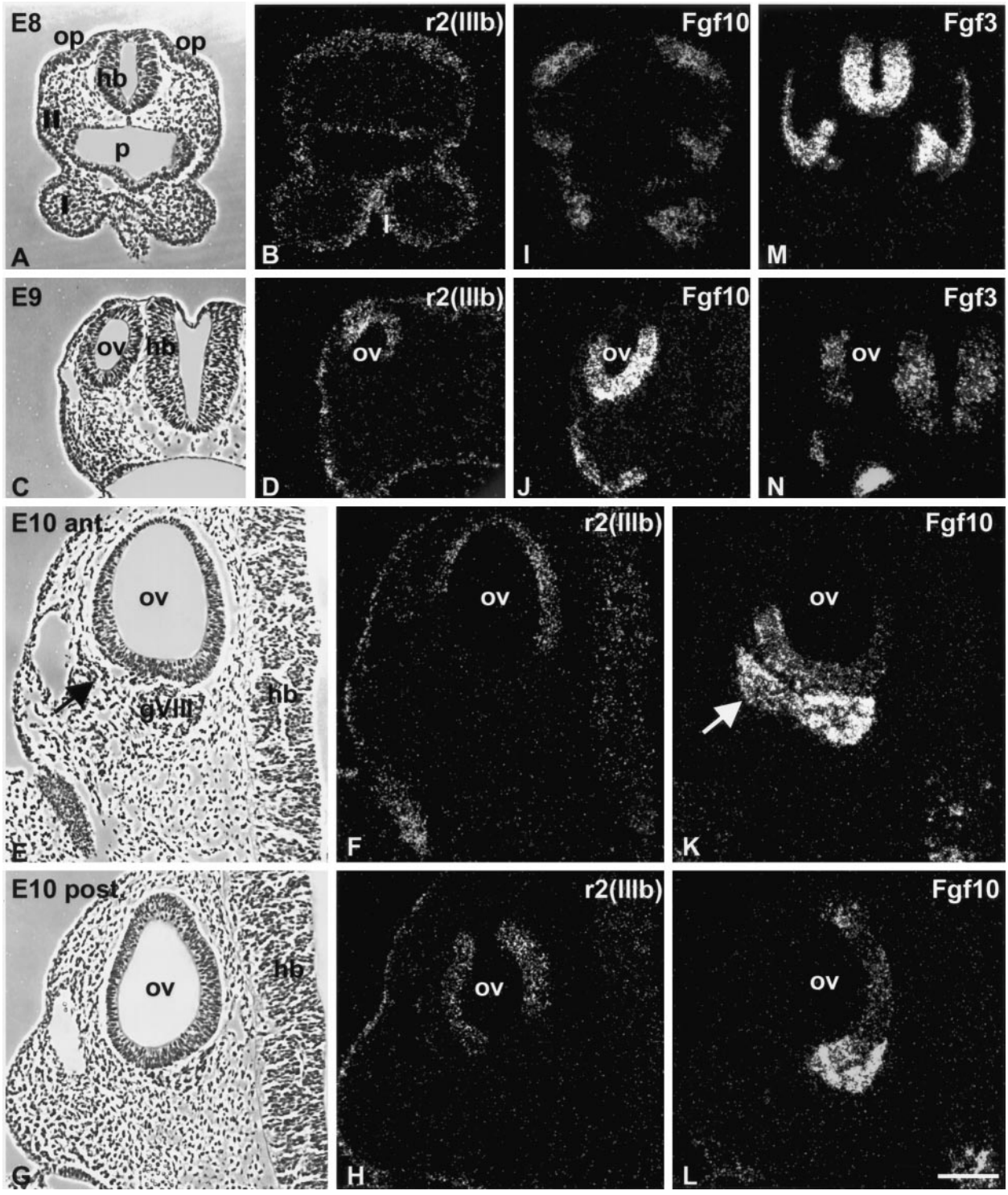

Figure 1. Expression of FGFR-2(IIIb), FGF10, and FGF3 mRNAs at the earliest stages of inner ear formation. *In situ* hybridizations on transverse sections photographed under phase-contrast and dark-field optics (*A*, *B*, *I*, *M*; *C*, *D*, *J*, *N*; *E*, *F*, *K*; *G*, *H*, *L*, adjacent sections). *A*, *B*, FGFR-2(IIIb) mRNA is expressed in the otic placode of E8 mouse embryos. *C*, *D*, At E9, FGFR-2(IIIb) mRNA is found in the dorsal portion of the otic vesicle that is detaching from the surface ectoderm. Anterior (*E*, *F*) and posterior (*G*, *H*) sections through the E10 vesicle show the FGFR-2(IIIb) signal in the dorsal and medial walls, except for the posterodorsal domain. *I*, FGF10 mRNA is expressed in the otic placode. *J*, At E9, strong FGF10 signal is found in the otic epithelium, except for its dorsal portion. At E10, anterior (*K*) and posterior (*L*) sections show the FGF10 signal in ventral and medial walls, in the migrating neuronal precursors, and in the cochleovestibular ganglion. *M*, At E8, FGF3 mRNA is prominently expressed in the hindbrain but not in the otic placode. *N*, At E9, FGF3 signal is seen in the lateral wall of the vesicle and in the region of hindbrain flanking the inner ear. *ov*, Otic vesicle; *gVIII*, cochleovestibular ganglion; *hb*, hindbrain; *I*, first branchial arch; *II*, origin of the second branchial arch; *p*, pharynx. The *arrows* in *E* and *K* mark the migrating neuronal precursors. Scale bar: *A–L*, 120 μm.

overlapped with NT-3 (Fig. 2*G*) and BDNF mRNAs (data not shown), which are markers for the presumptive sensory regions (Pirvola et al., 1992, 1994). From the ventrolaterally situated patch that continued ventromedially in an anterior-to-posterior plane, FGF10 mRNA extended into the pathway of migrating neuronal precursors and into the cochleovestibular ganglion (Fig. 2*E*). FGF10 mRNA was also found dorsally in a small patch of presumptive vestibular sensory epithelium (Fig. 2*F*). However, it was

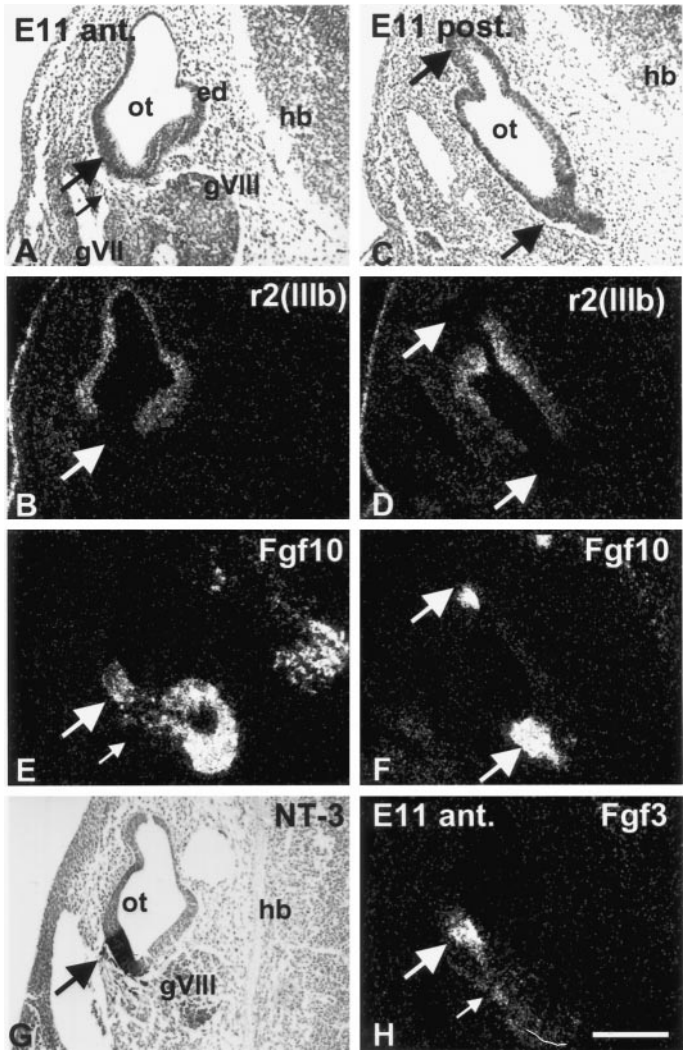

Figure 2. FGFR-2(IIIb), FGF10, FGF3, and NT-3 signals in the otocyst of E11 mouse. *In situ* hybridizations (*A*, *B*, *E*, *H*; *C*, *D*, *F*, adjacent sections) on transverse sections photographed under bright- and dark-field optics, and β−galactosidase-staining (*G*) photographed under bright-field illumination. As seen in anterior (*A*, *B*) and posterior (*C*, *D*) views, FGFR-2(IIIb) mRNA is expressed in large areas of the otic epithelium, except for the sensory patches (*large arrows*). The dorsal sensory patch is the presumptive posterior crista. Note an abundance of transcripts in the outpocketing of the endolymphatic duct. A low level of FGFR-2(IIIb) signal is seen in the surrounding mesenchyme. As seen in anterior (*E*) and posterior (*F*) views, FGF10 mRNA is expressed in the presumptive sensory regions (*large arrows*), in migrating neuronal precursors (*small arrow*), and in the cochleovestibular but not facial ganglion. At E11, FGF10 mRNA is also found in the hindbrain. *G*, As revealed in a *NT-3* null mutant embryo, NT-3 signal is found in the ventrolateral sensory region (*large arrow*). *H*, FGF3 mRNA is detected in the ventrolateral sensory domain (*large arrow*) and in the migrating neuronal precursors (*small arrow*). *ot*, Otocyst; *ed*, endolymphatic duct; *gVIII*, cochleovestibular ganglion; *gVII*, facial (geniculate) ganglion; *hb*, hindbrain. Scale bar: *A–H*, 160 μm.

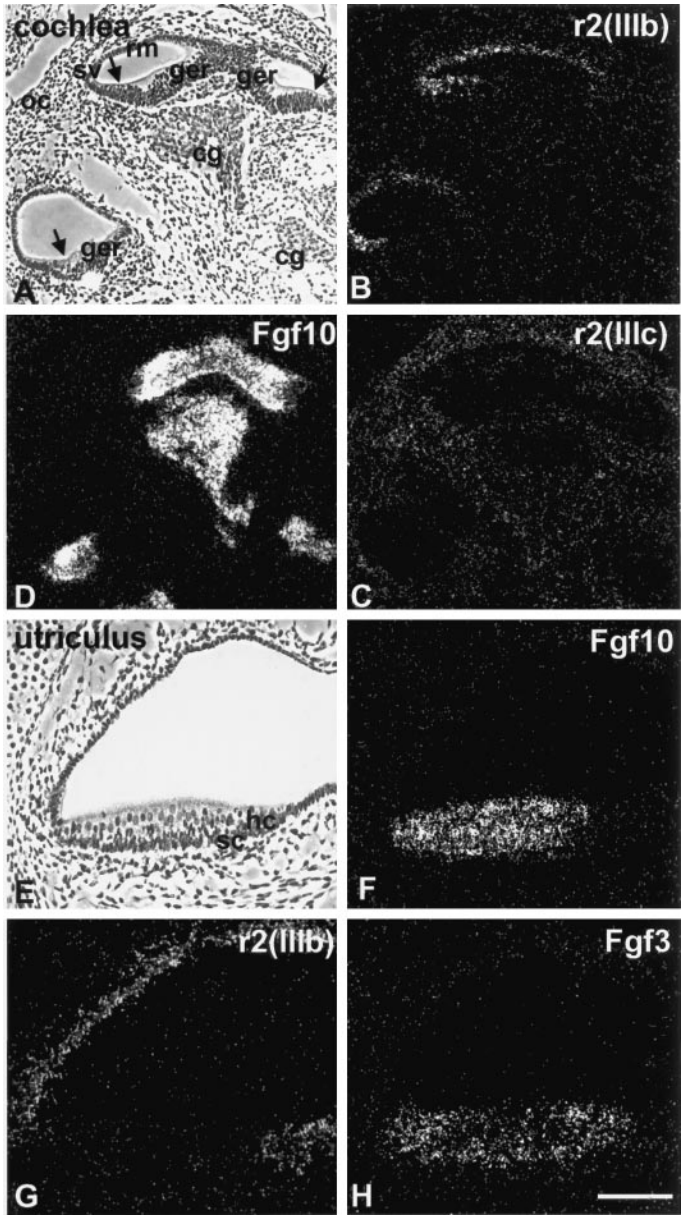

Figure 3. Expression of FGFR-2(IIIb), FGFR-2(IIIc), FGF10, and FGF3 mRNAs in the cochlea and utriculus at E16. *In situ* hybridizations (*A–D*; *E–H*, adjacent sections) on midmodiolar sections photographed under phase-contrast and dark-field optics. *A*, *B*, The IIIb isoform of FGFR-2 is found in the lateral wall of the cochlear duct and at very low levels in the surrounding mesenchyme. *C*, The IIIc isoform is expressed at low levels in the periotic mesenchyme and otic capsule. *D*, FGF10 mRNA is expressed in the greater epithelial ridge of the cochlear duct and in the sensory neurons of the cochlear ganglion. *Arrows* mark the differentiating hair cells. FGF10 (*E*, *F*) and FGF3 (*H*) mRNAs are expressed in the hair cells and supporting cells of utricular sensory epithelium. *H*, FGFR-2(IIIb) mRNA is found in the nonsensory epithelium of utriculus. *hc*, Hair cells; *sc*, supporting cells; *ger*, greater epithelial ridge; *cg*, cochlear ganglion; *rm*, Reissner's membrane; *sv*, stria vascularis; *oc*, otic capsule. Scale bar: *A–D*, 120 μm; *E–H*, 60 μm.

not expressed in the mesenchyme surrounding the otocyst or in the nearby VIIth (Figs. 2*E*, 6*A*,*B*) and IX–Xth (see Fig. 6*A*,*B*) cranial ganglia.

At E11, FGF3 mRNA was expressed in the ventrolaterally located thickened patch of the otic epithelium and weakly in the pathway of neuronal precursors (Fig. 2*H*), as described previously (Wilkinson et al., 1989; McKay et al., 1996). By analyzing adjacent sections, the expression pattern of FGF3 in the early inner ear was more restricted than that of FGF10 mRNA (Fig. 2*E*,*H*). The prominent FGF3 expression seen in the hindbrain of younger embryos had downregulated to low levels by E11 (Fig. 2*H*).

These findings show that FGF10 and FGF3 mRNAs are confined to the presumptive sensory and neuronal regions of the otocyst, whereas the mRNA for their receptor, FGFR-2(IIIb), is expressed in the nonsensory domains. The largely nonoverlapping expression patterns suggest the presence of a paracrine signaling mechanism.

Late gestational inner ear

Expression of FGFR-2, FGF10, and FGF3 mRNAs continued in the later developing cochleovestibular labyrinth, as analyzed at E16 and E18 (Fig. 3). In the cochlear duct, FGFR-2(IIIb) transcripts were located laterally to the differentiating hair cells and to the lateral and ventral walls (Figs. 3*A*,*B*) that form the stria vascularis

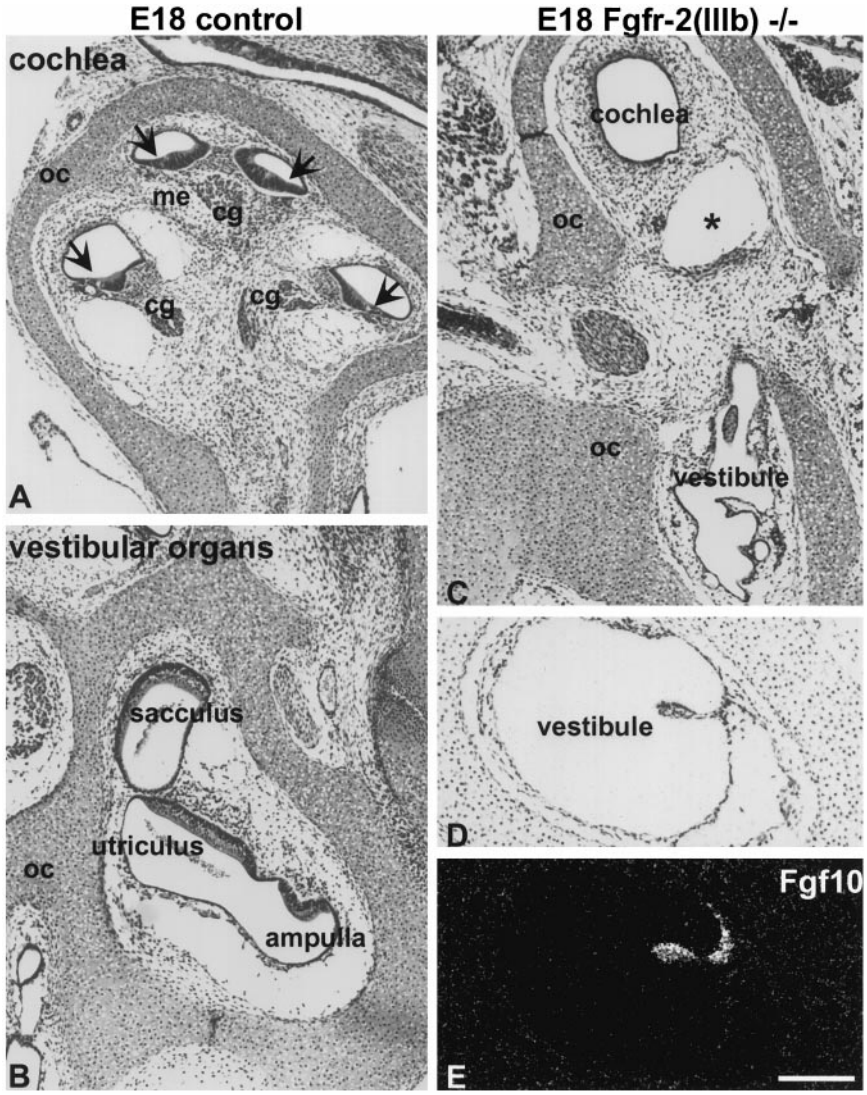

Figure 4. Targeted deletion of the IIIb isoform of FGFR-2 results in dysgenesis of the cochleovestibular membraneous labyrinth as revealed at E18. *A*, A midmodiolar section through the cochlea of a control mouse shows the two and one-half turns of the cochlear duct in which the differentiating sensory epithelium (*arrows*) and the innervating cochlear ganglion are seen. *B*, A section through the vestibular labyrinth at the level of the sensory organs of sacculus, utriculus, and ampullae of a control embryo. *C*, Both the cochlea and the vestibular labyrinth are disrupted in *FGFR-2(IIIb)* null mutants, showing no cellular compartments of the normal morphology. Note that the otic capsule is developed in the mutant mice, although its size is reduced as compared with controls. The *asterisk* marks the combined scala vestibuli/tympani. *D*, *E*, *In situ* hybridization shows restricted expression of FGF10 mRNA, marking the inner ear sensory cells, in the vestibule of one mutant embryo. However, there are no signs of overt sensory organ formation that allows identification. *oc*, Otic capsule; *me*, mesenchyme; *cg*, cochlear ganglion. Scale bar: *A–C*, 250 μm; *D*, *E*, 120 μm.

and Reissner's membrane (Sher, 1971). The IIIb isoform was also expressed at very low levels in the mesenchyme. Weak and diffuse expression of the IIIc isoform was detected in the otic capsule and periotic mesenchyme (Fig. 3*C*). In addition to the cochlear duct, FGFR-2(IIIb) signal was found in the endolymphatic and semicircular ducts and in the nonsensory epithelium of vestibular organs (Fig. 3*G*).

During late gestational stages, FGF10 mRNA was prominently expressed in the greater epithelial ridge of the cochlear duct (Fig. 3*D*), where it colocalized with NT-3 mRNA (data not shown). FGF10 mRNA was also strongly expressed in the cochlear (Fig. 3*D*) and vestibular sensory neurons. In addition, FGF10 mRNA was found in the sensory epithelium of all vestibular organs. The hybridization signal was seen in the differentiating hair cells and supporting cells (Figs. 3*E*,*F*). FGF3 transcripts colocalized with FGF10 mRNA in the vestibular sensory epithelia (Fig. 3*F*,*H*), but FGF10 expression was more intense. FGF3 mRNA showed restricted expression in the organ of Corti of late embryonic inner ears (data not shown), as described previously (Wilkinson et al., 1989).

Postnatal inner ear

Expression of FGFR-2 and FGF10 mRNAs persisted in the cochlea of postnatal mice (data not shown), although at lower levels than during embryogenesis. In the mature cochlea, FGFR-2(IIIb) mRNA was expressed in the inner and outer sulcus, spiral limbus, and stria vascularis. The postnatal reduction of the greater epithelial ridge was accompanied by a reduced expression of FGF10 mRNA to a small cell population located medially to the inner hair cells. This expression, as well as the expression in the vestibular hair cells and supporting cells, disappeared during the second postnatal week, but the inner ear neurons continued to express FGF10 mRNA in the adult.

Inner ear phenotype of *FGFR-2(IIIb)* null mutants

The above expression data suggested that FGF/FGFR-2 signaling could play an important role in inner ear formation. Next, we began to analyze the inner ear phenotype of mice carrying a null mutation of the IIIb isoform of FGFR-2 (DeMoerlooze et al., 2000). We were interested in the isoform-specific null mutation, because only the IIIb isoform is able to bind FGF10 and FGF3 (Mathieu et al., 1995; Ornitz et al., 1996). We were particularly interested in the receptor rather than the ligand null mutations, because of a potential functional redundancy within the FGF family in the developing inner ear (Mansour et al., 1993). Nevertheless, our unpublished results indicate an inner ear phenotype also in *FGF10* null mutants.

FGFR-2 IIIb null mutant mice die at birth, because of lung agenesis (DeMoerlooze et al., 2000). Analysis at E18 showed that the cochleovestibular membraneous labyrinth was severely disrupted in the mutant mice (Fig. 4*A–E*); it was replaced by cystic cavities or chambers that were lined by simple epithelium. Of the eight inner ears analyzed, seven specimens did not show any evidence of sensory cell development, as verified by the lack of expression of NT-3 and FGF10 mRNAs. In one ear, the rudimen-

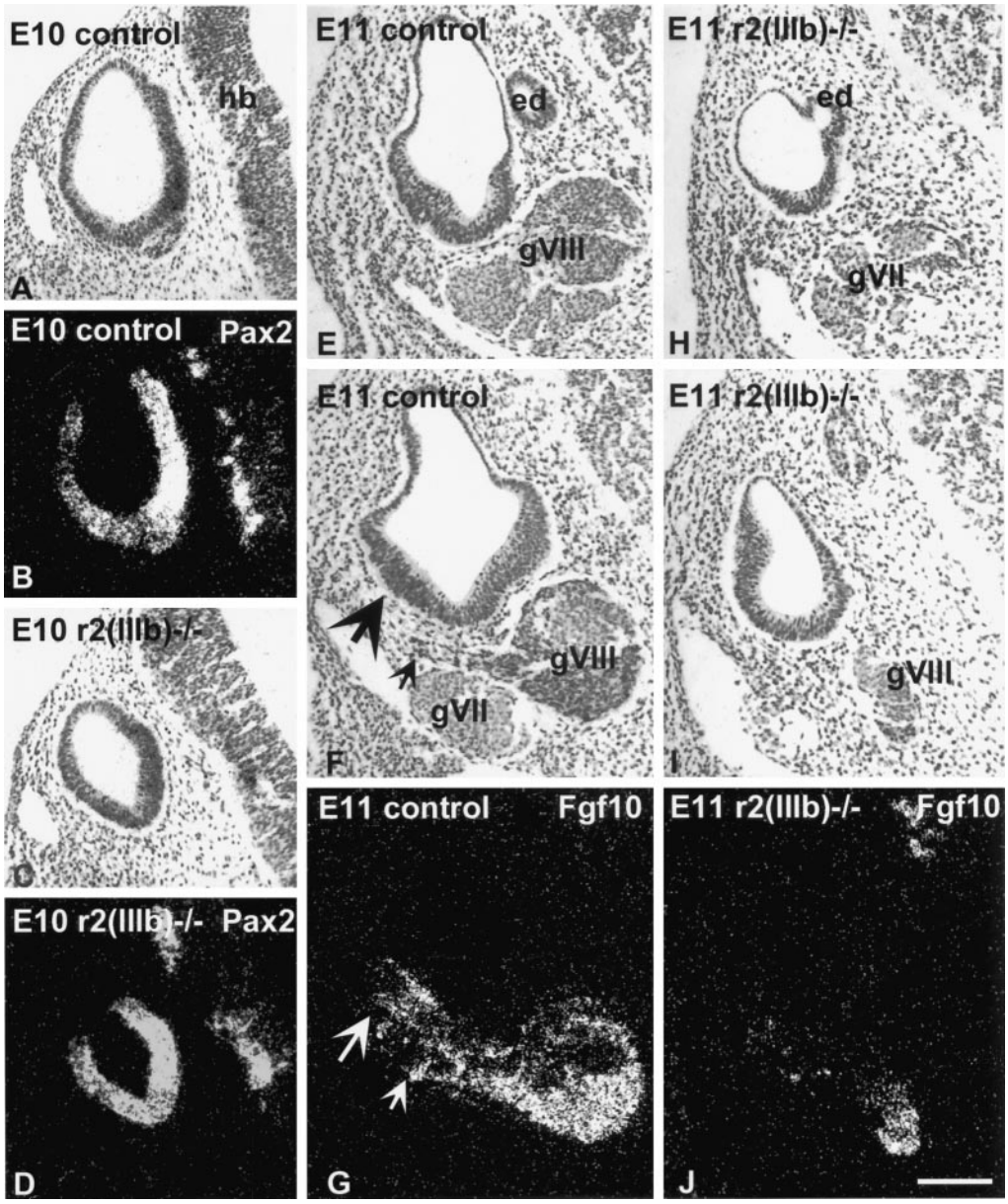

Figure 5. Targeted deletion of the IIIb isoform of FGFR-2 results in a blockade of early inner ear development as revealed in transverse sections of E10 and E11 mice. Sections were hybridized with Pax2 (*A–D*) and FGF10 (*F*, *G*, *I*, *J*) riboprobes and photographed under bright- and dark-field illumination. *A*, *B*, At E10, Pax2 mRNA is prominently expressed in the otic epithelium of a control embryo, except for its dorsalmost region. *C*, *D*, In the smaller otic vesicle of an E10 *FGFR-2(IIIb)* null mutant, Pax2 shows a similar expression pattern as seen in controls. *E*, A section from the anterior part of the otic vesicle of an E11 control mouse shows the outpocketing of the endolymphatic duct and the early cochleovestibular ganglion. *F*, *G*, A more posterior section of the same specimen shows FGF10 expression in the ventrolaterally located sensory patch (*large arrow*), in migrating neuronal precursors (*small arrow*), and in the cochleovestibular but not the facial ganglion. Also the initial outgrowth of semicircular ducts from the dorsal half of the vesicle is seen. *H*, The small otic vesicle of an E11 mutant embryo shows initiation of endolymphatic duct outgrowth. *I*, *J*, A more posterior section of the same specimen shows that the migratory path of neuronal precursors and the cochleovestibular ganglion are poorly developed, as also evidenced by insignificant expression of FGF10 mRNA (*E*, *F*). *ed*, Endolymphatic duct; *hb*, hindbrain; *gVII*, facial (geniculate) ganglion; *gVIII*, cochleovestibular ganglion. Scale bar: *A–J*, 130 μm.

tary epithelium of both the cochlea and vestibule showed restricted expression of FGF10 mRNA, but no sign of sensory organ formation was seen (Fig. 4*D*,*E*). In mutant embryos, the otic capsule was reduced in size as compared with control littermates (Fig. 4*A–C*). The capsule, despite being deformed, appeared normal, also with respect to the perilymphatic system orifices (oval and round window). As anticipated, the IIIc rather than IIIb isoform of FGFR-2 predominates in the inner ear mesenchyme. In heterozygous mice, the inner ear membraneous labyrinth did not have any obvious abnormalities (data not shown).

The inner ear dysgenesis of *FGFR-2(IIIb)* mutant mice at birth suggested that development is inhibited at an early stage, when major morphogenetic events take place (Sher, 1971; Fritzsch et al., 1998). At E10 and E11, otic vesicles form in mutant mice, but they were significantly smaller when compared with control littermates (Fig. 5). At E10, FGF10 (present study) and Pax2 mRNAs (Nornes et al., 1990; present study) (Fig. 5*A*,*B*) were widely expressed in the otic vesicle, except for the dorsal region. A similar pattern of gene expressions was found in the otic epithelium of the mutants (Fig. 5*C*,*D*), indicating that the vesicle is at least partially subdivided into domains similar to those seen in the normal embryos. Moreover, at E10, initial delamination and migration of neuronal precursors from the ventral wall of the otic vesicle was seen in both mutant and control embryos (data not shown).

Although the otocysts of E11 mutant mice were rudimentary in size, early outgrowth of the endolymphatic and cochlear ducts could be found, similar to control littermates (Fig. 5*E*,*H*). However, there was variability in the penetrance and expressivity of the

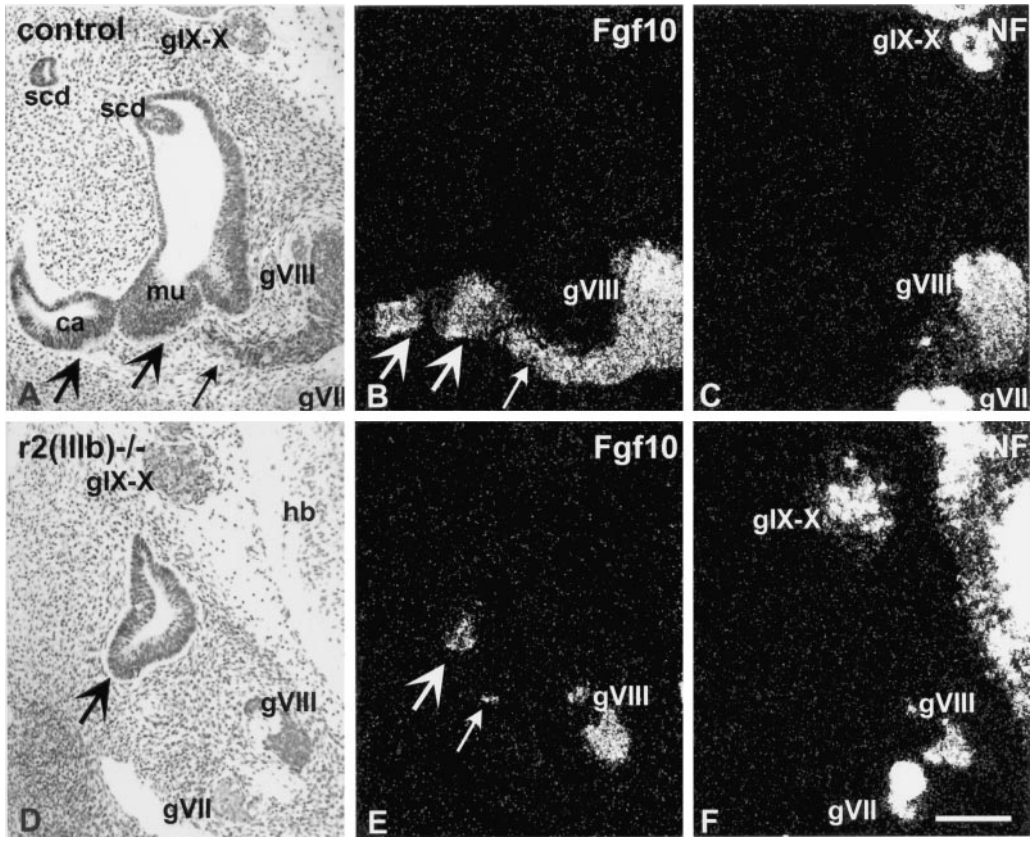

Figure 6. Targeted deletion of the IIIb isoform of FGFR-2 results in a blockade of otocyst morphogenesis as revealed in transverse sections of E13 mice. Adjacent sections were hybridized with FGF10 (*A*, *B*, *D*, *E*) and NF (*C*, *F*) riboprobes and photographed under bright- and dark-field illumination. *A*, *B*, In the otocyst of a control embryo, semicircular ducts and the FGF10 mRNA-expressing vestibular sensory epithelia (*large arrows*) are formed. FGF10-expressing neuronal precursors migrate (*small arrow*) to form the cochleovestibular ganglion. Inner ear neurons express FGF10 (*A*, *B*) and NF (*C*) mRNAs. Note that the facial ganglion shows NF but not FGF10 mRNA expression. *D*, *E*, The otocyst, the migratory path of neuronal precursors, and the cochleovestibular ganglion of *FGFR-2(IIIb)* mutant mice are rudimentary, as also revealed by insignificant expression of FGF10 mRNA. *F*, NF mRNA is used as a marker to show that the facial and glossopharyngeal–vagal ganglia are unaffected in the mutant mice, in contrast to the cochleovestibular ganglion. *scd*, Semicircular duct; *ca*, crista ampullaris; *mu*, macula utriculi; *hb*, hindbrain; *gVII*, facial (geniculate) ganglion; *gVIII*, cochleovestibular ganglion; *gIX-gX*, glossopharyngeal–vagal ganglia. Scale bar: *A–F*, 150 μm.

endolymphatic outpocketing. Approximately 50% of otocysts of null embryos between E11 and E14 ($n = 16$ otocysts) did not show any signs of initiation of endolymphatic duct formation. In control mice, NT-3, BDNF and FGF10 (Fig. 5*F*,*G*) mRNAs were expressed in the thickened patches of the presumptive sensory epithelia. In the otocysts of mutant mice, there was only a slight thickening of the ventrally located epithelium, and it was associated with very restricted expression of the markers for the presumptive sensory epithelia (Fig. 5*I*,*J*). The vestibular sensory regions located in the dorsal part of the otocyst were even more rudimentary and did not show expression of neurotrophin or FGF10 mRNAs. Moreover, although initial delamination and migration of neuronal precursors could be seen in E10 mutant embryos, this process and the formation of the cochleovestibular ganglion were severely retarded in E11 mutants, as indicated by reduced FGF10 (Fig. 5*F*,*G*,*I*,*J*) and NF mRNAs levels used as markers for the inner ear neuronal compartment.

At E13 and E14, the otocysts of *FGFR-2(IIIb)* mutant embryos do not show the prominent changes in size and shape that were seen in control littermates. There was no fusion of canal plates or elongation of the semicircular or endolymphatic ducts (Fig. 6). Control embryos also formed vestibular sensory epithelia and showed distinct expression of neurotrophin and FGF10 mRNAs (Fig. 6*A*,*B*), whereas the otocysts of mutant mice showed no evidence of vestibular organ formation (Fig. 6*D*,*E*). Weak expression of FGF10 (Fig. 6*D*,*E*) and NT-3 mRNAs could be seen only in the ventral portion of the rudimentary otocyst.

At E13 and E14, the migratory pathway of neuronal precursors and the cochleovestibular ganglion were rudimentary in the *FGFR-2(IIIb)* mutant mice, as verified by using FGF10 (Fig. 6*A*,*B*,*D*,*E*) and NF riboprobes (Fig. 6*C*,*F*). At E13, TUNEL staining revealed a large number of cochleovestibular neurons dying through apoptosis in mutant embryos, but not in controls (Fig. 7*A–D*). On the contrary, the ventromedial region of otocysts of control embryos at E13 showed a high apoptotic profile, in accordance with earlier data (Fekete et al., 1997), but this pattern was not seen in the rudimentary otocysts of mutant mice (Fig. 7*A–D*). NF immunostaining revealed that neurons of the rudimentary cochleovestibular ganglion emanated fibers toward the periphery, but in contrast to control specimens, these neurites did not penetrate the otic epithelium, where their peripheral targets, the hair cells, are located (Fig. 7*E*,*F*). The nearby VIIth and IX–Xth cranial ganglia appeared normal in the mutant mice (Fig. 6*A–F*). The otocyst morphogenesis of heterozygous embryos appeared to proceed similarly as in wild-type littermates. Taken together, these results show that initial inner ear development occurs in *FGFR-2(IIIb)* mutant mice, but subsequent morphogenesis of the otocyst fails.

DISCUSSION

FGF10/FGFR-2(IIIb) signaling regulates inner ear development

The present study examines the mRNA expression patterns of FGF10 and its cognate receptor, FGFR-2(IIIb), in the inner ear. The results suggest that a largely paracrine signaling mechanism operates within the early otic epithelium and later in the cochleovestibular labyrinth. Mice deficient for *FGFR-2(IIIb)* develop otic vesicles from ectodermal placodes (DeMoerlooze et al., 2000;

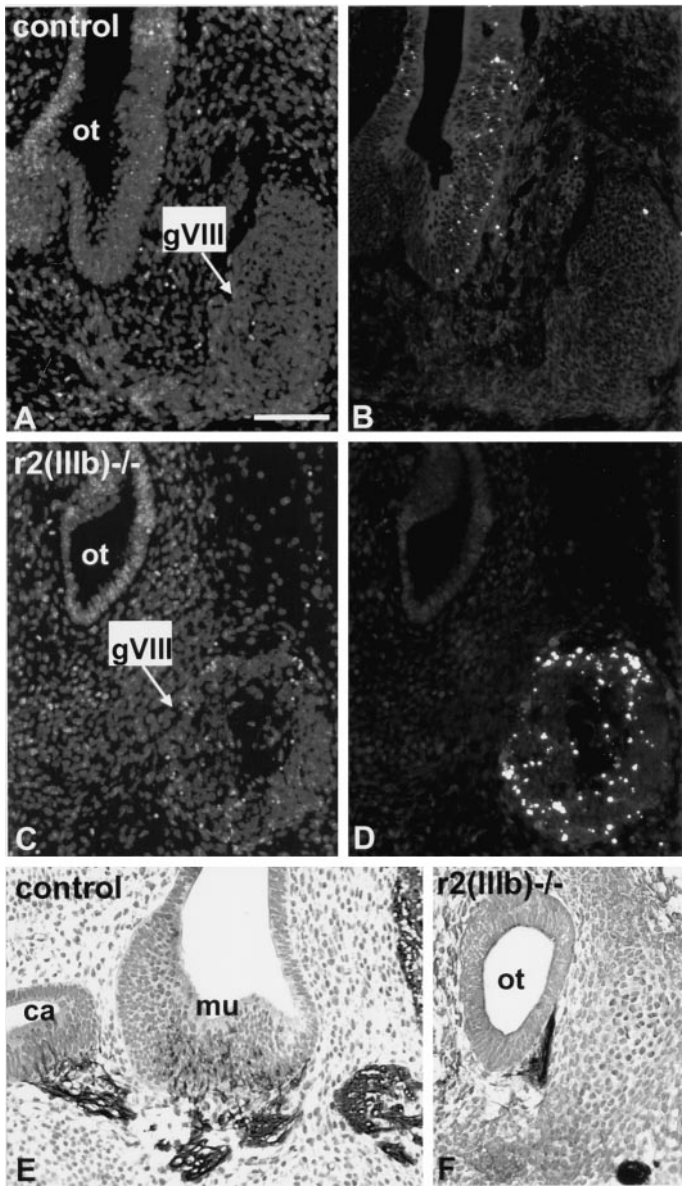

Figure 7. Targeted deletion of the IIIb isoform of FGFR-2 results in failures in the development of cochleovestibular neurons. Sections of E13 control and *FGFR-2(IIIb)* null mutant mice were TUNEL-stained (*B*, *D*) and counterstained with DAPI (*A*, *C*). Other sections were stained by neurofilament antibodies (*E*, *F*). *A*, *B*, Control embryos show high numbers of apoptotic profiles in the ventromedial wall of the otocyst, but not in the cochleovestibular ganglion. *C*, *D*, In contrast to controls, many apoptotic neurons are seen in the cochleovestibular ganglion of a mutant mouse, and the ganglion shows signs of degeneration. Apoptotic cells are not found in the ventromedial wall of the otocyst of the mutant embryo. *E*, At E13, nerve fibers innervate the differentiating vestibular sensory epithelia of control embryos. *F*, In mutant mice, a low number of nerve fibers have grown toward the otocyst, but they do not penetrate the epithelium. Differentiation of the vestibular sensory epithelia is not evident in the mutant embryo. *ot*, Otocyst; *gVIII*, cochleovestibular ganglion; *mu*, macula utriculi; *ca*, crista ampullaris. Scale bar: *A–D*, 60 μm; *E*, *F*, 80 μm.

present study), indicating that FGFR-2(IIIb) signaling is not critical for the earliest stages of inner ear formation. This finding is in accord with earlier data on mice with a homozygous hypomorphic allele (Xu et al., 1998). The latter null mutants, however, die at the early otic vesicle stage, at the time when its morphogenesis is started. With an isoform-specific targeted deletion, we now provide evidence that FGFR-2(IIIb) is essential for morphogenesis of the otic vesicle. Our observations of an inner ear rudiment at birth are in agreement with the findings of another study that used a loss-of-function approach using a secreted dominant-negative form of FGFR-2(IIIb) (Celli et al., 1998).

Dynamic expression patterns of FGF10 and FGFR-2(IIIb) mRNAs

Initially, FGF10 mRNA was widely expressed in the undifferentiated otic epithelium but became subsequently restricted to the presumptive cochlear and vestibular sensory patches (a broad ventral region and a small posterodorsal patch). In addition, strong expression of FGF10 mRNA was found in the otic epithelium-derived neuronal precursors and in the neurons of the cochleovestibular ganglion. It has been shown that neuronal precursors detach and migrate from the ventral portion of the otic vesicle to form the cochleovestibular ganglion (Altman and Bayer, 1982; Carney and Silver, 1983). The expression pattern of FGF10 mRNA and its colocalization with neurotrophin mRNAs in the ventral patch suggest that the inner ear neurons and part of the sensory epithelium have a common origin in this epithelial domain. Within the cranial ganglia, expression of FGF10 mRNA was found uniquely in the cochlear and vestibular ganglia and not in the nearby VIIth and IX–Xth ganglia. Whether FGF10 expression relates to the unique colocalization of neurotrophin receptors, *trkB* and *trkC*, in the inner ear sensory neurons (Pirvola et al., 1994; Fritzsch et al., 1999) remains to be tested in *FGF10* null mutant mice.

A previous study has shown expression of FGFR-2(IIIb) mRNA in the epithelium and FGFR-2(IIIc) mRNA in the mesenchyme of the midgestational inner ear (Orr-Urtreger et al., 1993). Here we show that this differential expression pattern of the two isoforms is maintained throughout inner ear development. In addition, we define the specific epithelial domains for the expression of FGFR-2(IIIb) mRNA. It was expressed in the undifferentiated otic epithelium, specifically in the dorsal portion of the early otic vesicle, a region suggested to give rise to the nonsensory epithelium (Li et al., 1978). Accordingly, this mRNA was subsequently found in the nonsensory epithelium of the cochlea and vestibular organs and in the nonsensory outpocketings such as the endolymphatic duct and semicircular canals. Importantly, *FGFR-2(IIIb)* null mice are defective for these nonsensory structures, showing an early blockade of their formation. Incorporating these results together with the findings of largely nonoverlapping expression patterns for FGFR-2(IIIb) and FGF10 mRNAs, we suggest that this signaling regulates specification of the nonsensory epithelium of the inner ear. Because FGFs are thought to act as short-range diffusible morphogens (Hogan, 1999), FGF10/FGFR2(IIIb) signaling might happen along the boundaries of the expression domains (or within their limited overlap), and these boundary regions would then define the origin of the nonsensory outpocketings. In some mutant mice, initial development of the endolymphatic duct was observed, but this outgrowth did not proceed beyond the earliest stages. Therefore, in addition to the initiation of morphogenesis, FGF10, secreted from the differentiating sensory and neurogenic regions, might regulate further development of the FGFR-2(IIIb)-expressing nonsensory epithelium.

Altogether, on the basis of the budding morphogenesis theme, it appears that correlations can be made between the developing inner ear and organs such as the limb bud and lung. In all cases, the outgrowth of appendages seems to be regulated by FGF10/FGFR-2(IIIb) signaling (Bellusci et al., 1997; Xu et al., 1998; DeMoerlooze et al., 2000; Kettunen et al., 2000). However, in contrast to some other organs in which this signaling appears to involve a mesenchymal to epithelial interaction (for review, see Hogan, 1999), in the inner ear it appears to operate primarily within the epithelium.

The present histological and molecular analyses show that the sensory epithelia and the cochleovestibular ganglion, as well as the nonsensory structures, are rudimentary in *FGFR-2(IIIb)* null mutant mice. Failure to develop inner ear neurons appears to result from disturbances at the level of neuronal precursors. However, in contrast to the inner ear nonsensory epithelium, the sensory and neuronal regions were largely devoid of FGFR-2(IIIb) expression. Therefore, the failure of sensory structures to develop might be caused by disturbances at the initial stages of specification of different epithelial domains, the null mutation inhibiting the estab-

lishment of boundaries between dorsal and ventral cell types. However, partial axis fixation was observed in the vesicles of *FGFR-2(IIIb)* mutants, based on regionalization of FGF10 and Pax2 expressions and initial delamination of neuronal precursors from the ventral wall. Alternatively, reciprocal interactions between the sensory/neuronal and nonsensory domains or even more intricate signaling loops between the epithelium and the surrounding mesenchyme might occur and could explain the loss of the sensory components found in *FGFR-2(IIIb)* null mice. It has become increasingly evident that FGF signaling operates on the basis of reciprocal paracrine interactions (for review, see Hogan, 1999). In accordance with our suggestion, grafting experiments have pointed to communication between different domains of the otic epithelium (Swanson et al., 1990). However, although the present study suggests the candidate molecules acting from the sensory compartments to the nonsensory epithelium, the converse remains obscure.

FGFR-2(IIIb) null mutants fail to form the cochleovestibular ganglion at the level of neuronal precursors. However, a small neuronal population was formed, and these neurons extended fibers to the periphery but did not innervate the otic epithelium. High numbers of apoptotic neurons were seen within the early cochleovestibular ganglion of mutant mice. This distinct apoptosis is likely to result from a failure of the sensory patches to differentiate and to promote survival of the innervating neurons by producing neurotrophic factors (Pirvola et al., 1994; Fritzsch et al., 1999).

Because of the severe effects of the targeted deletion of *FGFR-2(IIIb)* on early inner ear development, these mutant mice did not shed light on the possible role of FGF10/FGFR-2(IIIb) signaling during later developmental events. In late gestational inner ears, FGF10 mRNA continued to be expressed in the sensory compartments and FGFR-2(IIIb) mRNA in the nonsensory epithelial structures. Hence, it is possible that FGF10, secreted from the greater epithelial ridge, may impact on the development of the FGFR-2(IIIb)-expressing Reissner's membrane and stria vascularis. However, on the basis of our unpublished results, FGF10 might signal through a receptor other than FGFR-2(IIIb) within the late embryonic cochlear duct, specifically within the sensory compartment. This signaling would be in line with the short-range mode of action of FGFs in general (Hogan, 1999). Because expression of FGF10 mRNA was maintained in the vestibular hair cells and supporting cells during the first 2 postnatal weeks, FGF10 might act on the FGFR-2(IIIb)-expressing nonsensory epithelial structures of the vestibular labyrinth even after birth.

FGF3 and FGF10 may regulate inner ear development through FGFR-2(IIIb)

Both FGF3 and FGF10 bind and activate the IIIb isoform of FGFR-2 (Mathieu et al., 1995; Ornitz et al., 1996). Although FGF3 mRNA is localized to the otic epithelium, more attention has been paid to the putative role of hindbrain-derived FGF3 on the early inner ear, based on the expression of FGF3 mRNA in the rhombomeres adjacent to the otic vesicle (Wilkinson et al., 1989; McKay et al., 1996). Hindbrain-derived FGF3 has been suggested to regulate patterning of the inner ear, particularly endolymphatic duct formation, based on the phenotype of *FGF3* null mutants (Mansour et al., 1993). This suggestion is attractive, especially because we now show that FGFR-2(IIIb) mRNA is expressed in the dorsomedial wall of the vesicle that is flanking the hindbrain and later in the outpocketing of the endolymphatic duct. However, not excluding the suggestion of a hindbrain-derived FGF3 signal, FGF3/FGFR-2(IIIb) signaling within the otic epithelium is also possible. By analysis throughout development, we found that FGF3 mRNA is expressed in the ventrolateral domain of the otic vesicle at the same stage when it is seen in the hindbrain, raising the possibility that FGF3 derived from both sources concomitantly acts on the otic epithelium. Similar to FGF10, FGF3 expression continued in the otocyst and later in the sensory epithelia of the cochlea and vestibular organs. FGF3 and FGF10 mRNAs showed partly overlapping expression patterns, but FGF10 mRNA was more intensely and more widely expressed. Hence, local production of both FGF3 and FGF10 could act on the FGFR-2(IIIb)-expressing nonsensory epithelium. It remains to be shown whether FGF3 signals derived from the otic wall and hindbrain have the same site(s) of action within the broad FGFR-2(IIIb) expression domain, taking into account that FGFs in general act as short-range signals.

In addition to the possibility that hindbrain-derived FGF3 might have an impact on the FGFR-2(IIIb)-expressing otic epithelium, the present expression data suggest a potential redundancy for the otocyst-derived FGF3 and FGF10 signals. Support for this comes from the variable inner ear phenotype reported for *FGF3* null mutant mice (Mansour et al., 1993), which share similarities with the *FGFR-2(IIIb)* mutant mice reported here. For example, in both *FGF3* and *FGFR-2(IIIb)* mutants, formation of the nonsensory epithelial outpocketings and the cochleovestibular ganglion were disrupted. In *FGFR-2(IIIb)* null mice and in the severely affected *FGF3* null mice, slight initial differentiation of these structures could be found. Also in line with the *FGFR-2(IIIb)* mutants, the otic capsule did not show major defects in *FGF3* mutants, consistent with the predominance of IIIc isoform of FGFR-2 in the mesenchymal compartments of the inner ear (Orr-Urtreger et al., 1993; present study). However, although the phenotype of inner ears of *FGF10* null mutants (Min et al., 1998; Sekine et al., 1999) has not been reported, our unpublished results suggest a milder phenotype in *FGF10* than in *FGF3* null mutants. These preliminary results argue for an important role of hindbrain-derived FGF3 in otocyst morphogenesis. In conclusion, the present study provides evidence that FGF3 and FGF10 function as paracrine regulators of gross morphological patterning of the inner ear by activating the IIIb isoform of FGFR-2. Our results indicate that disruption of this ligand–receptor interaction leads to dysgenesis of the inner ear membraneous labyrinth.

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