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Fgf9 signaling regulates inner ear morphogenesis through epithelial–mesenchymal interactions

Ulla Pirvola[a,*], Xiuqin Zhang[b], Johanna Mantela[a], David M. Ornitz[b], Jukka Ylikoski[c]

[a]*Institute of Biotechnology, University of Helsinki, 00014 Helsinki, Finland*
[b]*Department of Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, MO 63110, USA*
[c]*Department of Otolaryngology, University of Helsinki, 00014 Helsinki, Finland*

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Abstract

The mammalian inner ear comprises the cochleovestibular labyrinth, derived from the ectodermal otic placode, and the encasing bony labyrinth of the temporal bone. Epithelial–mesenchymal interactions are thought to control inner ear development, but the modes and the molecules involved are largely unresolved. We show here that, during the precartilage and cartilage stages, *Fgf9* is expressed in specific nonsensory domains of the otic epithelium and its receptors, *Fgfr1(IIIc)* and *Fgfr2(IIIc)*, widely in the surrounding mesenchyme. To address the role of Fgf9 signaling, we analyzed the inner ears of mice homozygous for *Fgf9* null alleles. *Fgf9* inactivation leads to a hypoplastic vestibular component of the otic capsule and to the absence of the epithelial semicircular ducts. Reduced proliferation of the prechondrogenic mesenchyme was found to underlie capsular hypoplasticity. Semicircular duct development is blocked at the initial stages, since fusion plates do not form. Our results show that the mesenchyme directs fusion plate formation and they give direct evidence for the existence of reciprocal epithelial–mesenchymal interactions in the developing inner ear. In addition to the vestibule, in the cochlea, *Fgf9* mutation caused defects in the interactions between the Reissner's membrane and the mesenchymal cells, leading to a malformed scala vestibuli. Together, these data show that Fgf9 signaling is required for inner ear morphogenesis.

Keywords: Inner ear; Otic capsule; Semicircular duct; Reissner's membrane; Epithelial—Mesenchymal interactions; Proliferation; Fgf; Fgfr

Introduction

The mammalian inner ear contains the cochleovestibular membranous labyrinth and the encasing labyrinth of the temporal bone. The cochlear duct and the vestibular organs together with the semicircular ducts and the endolymphatic duct house the membranous labyrinth. Its chambers and ducts are filled with endolymph, an inner-ear-specific fluid. The walls of the membranous labyrinth are made of sensory and nonsensory epithelium. The hair cells of the cochlear and vestibular sensory epithelia are the key cellular components in hearing and balance function, respectively, and they are innervated by the sensory neurons of the cochlear and vestibular ganglia. The epithelial and neuronal compartments of the inner ear are derived from an ectodermal thickening, the otic placode. The placode invaginates and closes to a vesicle, called the otocyst, which develops through a series of morphogenetic events into the mature inner ear. The mesenchyme surrounding the otocyst starts to condense during early otocyst stages and the outer domain of the condensing mesenchyme differentiates into the cartilaginous otic capsule, which forms the temporal bone.

Fibroblast growth factor (Fgf)–Fgf receptor (Fgfr) signaling functions at multiple stages of development of the mouse inner ear, as shown by *Fgf* and *Fgfr* loss-of-function mutations. *Fgfs* and *Fgfrs* have been shown to be

* Corresponding author. Institute of Biotechnology, University of Helsinki, P.O. Box 56, 00014 Helsinki, Finland. Fax: +358 9 19159366.
E-mail address: ulla.pirvola@helsinki.fi (U. Pirvola).

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critical for otic placode induction (Alvarez et al., 2003; Wright and Mansour, 2003), otocyst morphogenesis (Mansour et al., 1993; Pauley et al., 2003; Pirvola et al., 2000), formation of the pool of precursor cells giving rise to the auditory sensory epithelium (Pirvola et al., 2002), and differentiation of supporting cells within this sensory epithelium (Colvin et al., 1996). By a double gene inactivation approach, *Fgf3* and *Fgf10* have been shown to function redundantly in the formation of the otic placode of the mouse (Alvarez et al., 2003; Wright and Mansour, 2003). These two Fgfs appear to function at later stages of inner ear development too, as evidenced by their expression in the otocyst epithelium (Pirvola et al., 2000; Wilkinson et al., 1989). *Fgf3* and *Fgf10* single null mutant mice show dysmorphic inner ears (Mansour et al., 1993; Pauley et al., 2003). Furthermore, *Fgfr2(IIIb)*, encoding the main receptor for Fgf3 and Fgf10 (Igarashi et al., 1998; Lu et al., 1999; Ornitz et al., 1996), is expressed in the otic epithelium (Orr-Urtreger et al., 1993; Pirvola et al., 2000) and its inactivation leads to a severe inner ear phenotype, a developmental arrest at the otocyst stage (De Moerlooze et al., 2000; Pirvola et al., 2000). *Fgfr2(IIIb)* is distinctly expressed in the nonsensory regions and *Fgf10* in the sensory regions of the otocyst; these results, together with the knockout phenotypes, suggest that Fgf10/Fgfr2(IIIb) signaling is essential for inner ear morphogenesis. In addition to *Fgf10* and *Fgf3*, *Fgf9* has been shown to be expressed in the otic epithelium (Colvin et al., 1999) and its preferred receptors, *Fgfr1(IIIc)* and *Fgfr2(IIIc)* (Ornitz et al., 1996; Santos-Ocampo et al., 1996), are mainly expressed in the otic mesenchyme (Orr-Urtreger et al., 1993; Pirvola et al., 2000, 2002).

Reciprocal signaling between the epithelium and mesenchyme is a fundamental process for the morphogenesis of diverse organs. In organs such as the limb bud, lung, and tooth, Fgf10 and/or Fgf7 are essential mesenchymal signals regulating epithelial morphogenesis, whereas epithelial Fgf9, Fgf8, Fgf4, and/or Fgf17 control proliferation, patterning, survival, and differentiation of the mesenchyme (Colvin et al., 2001a; Hogan, 1999; Jernvall and Thesleff, 2000; Min et al., 1998; Sekine et al., 1999; Sun et al., 2002). Compared to most other developing organs studied, it is quite unique that, during the otocyst stages, *Fgf10* is expressed in the otic epithelium rather than in the surrounding mesenchyme (Pirvola et al., 2000). Although Fgf10 signaling appears not to regulate inner ear morphogenesis through epithelial–mesenchymal interactions, several lines of evidence suggest that interactions between these two compartments are essential, the otocyst providing a chondrogenic stimulus for the mesenchyme (Frentz and Van de Water, 1991; McPhee and Van de Water, 1985) and the mesenchyme regulating global morphogenesis of the otocyst (Swanson et al., 1990). However, the molecular basis of epithelial–mesenchymal interactions in the inner ear is poorly understood.

In the present study, we describe the spatiotemporal expressions of *Fgf9*, *Fgfr1(IIIc)*, and *Fgfr2(IIIc)* in the embryonic inner ear and study the in vivo functions of Fgf9 signaling. We demonstrate that Fgf9 is an otic epithelial signal stimulating growth and remodeling of the otic mesenchyme where its receptors are expressed. *Fgf9* null mice show distinct defects in the vestibular part of the inner ear where decreased mesenchymal proliferation leads to a hypoplastic otic capsule and this, in turn, seems to inhibit the formation of the epithelial fusion plates, which normally initiate semicircular duct development. These results provide for the first time genetic evidence of an otic epithelium-derived growth factor regulating expansion of the surrounding mesenchyme and of the existence of reciprocal epithelial–mesenchymal interactions directing gross development of the inner ear.

Materials and methods

Mutant mice

Generation of mice homozygous for a targeted disruption of *Fgf9* has been previously described (Colvin et al., 2001a,b). Genotypes were determined by PCR as described in these papers. *Fgf9* mutant mouse line used was on the C57BL/6 background. At E10.5, E12.0, E12.5, E14.5, E15.5, E16.5, and E18.5, both ears of 5–10 mutant embryos were analyzed per developmental stage. In addition, eight ears of mutants were prepared for paint-filled whole mounts at E18.5.

Tissue preparation and histology

Whole heads of E16.5 and younger embryos and dissected inner ears of E18.5 embryos were fixed overnight in 4% paraformaldehyde (PFA) and embedded in paraffin (Shandon). Five 5-μm-thick sections were stained with hematoxylin (Shandon). For immunohistochemistry, a rabbit polyclonal antibody directed against myosin VI (Hasson et al., 1997) was used. Detection was done with the Vectastain Elite ABC kit and the diaminobenzidine (DAB) substrate (Vector Laboratories). Methyl green (Sigma) was used for counterstaining and, following dehydration, sections were mounted in Permount (Fisher). For cartilage staining, Scott's Alcian Blue (Sigma) method (Scott and Dorling, 1965) was used. Paraffin sections were immersed in the Alcian Blue staining buffer overnight, thereafter counterstained with nuclear fast red (Sigma), dehydrated, and mounted in Permount.

Proliferation and apoptosis assays

For proliferation studies, pregnant females received an intraperitoneal injection of bromodeoxyuridine (BrdU)-labeling reagent (Amersham-Pharmacia) 1 h before sacri-

fice. Deparaffinized sections were denatured with 2 N HCl for 15 min, incubated with a monoclonal BrdU-antibody (Neomarkers), and the reaction was detected with the Vectastain MOM ABC kit and DAB. To detect phospho-histone H3-positive cells, deparaffinized sections were microwaved for 10 min in 10 mM citrate buffer (pH 6.0), incubated with a polyclonal antibody (Cell Signaling Technology), followed by detection with the Vectastain Elite ABC kit and DAB. Sections were counterstained with methyl green. To detect apoptotic cells, the fluorescein in situ cell death detection kit was used according to manufacturer's (Roche) instructions.

In situ hybridization

In situ hybridization was performed with ^{35}S-labeled riboprobes on PFA-fixed paraffin sections according to the protocol by Wilkinson and Green (1991). Expression of *Fgf9*, *Fgf10*, *Fgfr1(IIIc)*, *Fgfr2(IIIb)*, and *Fgfr2(IIIc)* was studied on each embryonic day between E9.5 and E18.5. At least four normal inner ears per embryonic day and per probe were analyzed. In addition, expression of *Sox9*, *Dlx5*, *Hmx2*, *Netrin1*, and *Fgf10* was compared in the inner ears of control and *Fgf9* mutants between E10.5 and E18.5. At each stage, at least four inner ears of both mutants and controls were analyzed per probe.

Paint-filled whole mounts

Dissected inner ears of E18.5 control and mutant embryos were fixed overnight in 4% PFA, washed in phosphate-buffered saline, and postfixed for 2 days in Bodian's fixative at +4°C. Specimens were dehydrated in ethanol and cleared in methyl salicylate (Sigma). White paint was diluted in methyl salicylate to a concentration of about 1% and microinjected into the cochleovestibular membranous labyrinth as previously described (Martin and Swanson, 1993; Morsli et al., 1998).

Results

Expression of Fgf9 and its receptors in the embryonic inner ear

Epithelial–mesenchymal interactions have been suggested to control inner ear development, but only little is known about the molecules and cellular mechanisms behind these interactions. The importance of Fgf signaling in epithelial–mesenchymal interactions is well established

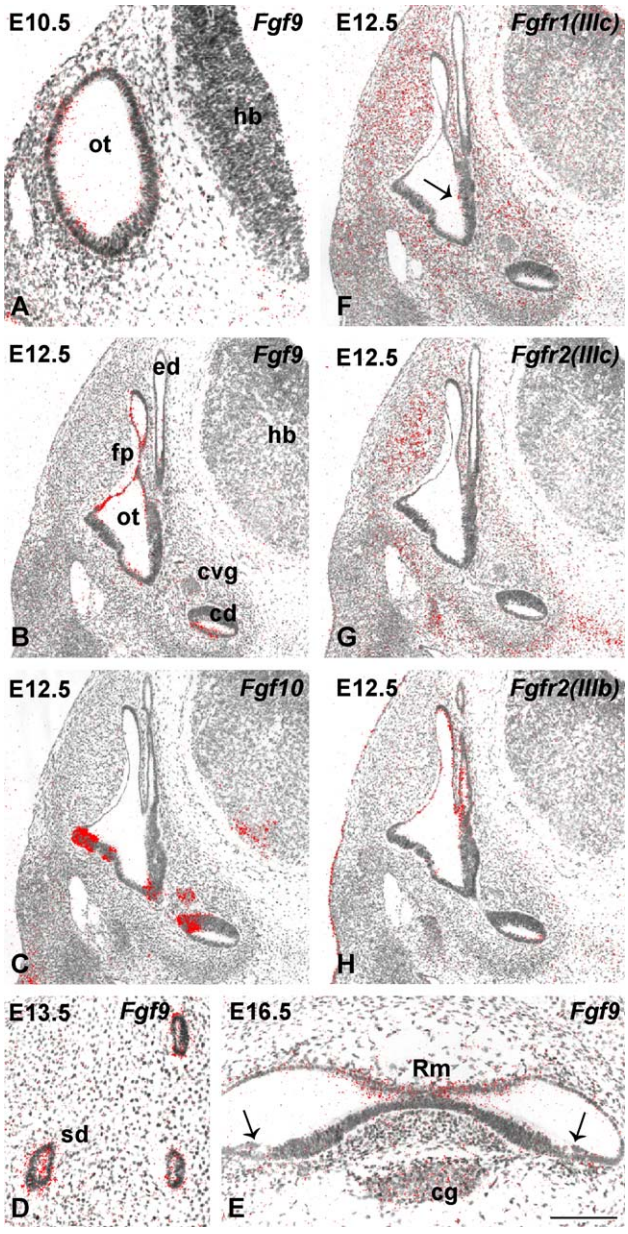

Fig. 1. Expression of *Fgf9*, *Fgf10* and their receptors in the embryonic mouse inner ear as revealed by radioactive in situ hybridization. (A) At E10.5, *Fgf9* is expressed in the otic epithelium, most distinctly in its dorsolateral and ventrolateral domains. (B) At E12.5, *Fgf9* is expressed in the thin-walled nonsensory epithelium of the otocyst except the endolymphatic duct. The cochleovestibular ganglion shows weak expression. (C) As shown in an adjacent section, *Fgf10* is expressed in the otic epithelium in a pattern nonoverlapping to *Fgf9* distribution. *Fgf10* is expressed in the thickened regions of the otic epithelium, the presumptive sensory epithelia. Similar to *Fgf9*, it is expressed in the cochleovestibular ganglion, but the levels of expression are much higher. (D) At E13.5, *Fgf9* is expressed in the growing semicircular ducts. (E) At E16.5, *Fgf9* is expressed in the Reissner's membrane of the cochlear duct and in the neurons of the cochlear ganglion. Arrows mark the negative auditory sensory epithelium, comprising the hair cells. (F,G) At E12.5, *Fgfr1(IIIc)* (F) and *Fgfr2(IIIc)* (G) are expressed in the otic mesenchyme, most prominently around the dorsal part of the otocyst. In addition, weak *Fgfr1(IIIc)* expression is seen in the ventromedial region of the otocyst epithelium (arrow). (H) At E12.5, *Fgfr2(IIIb)* expression is partly overlapping with the distribution of *Fgf9* transcripts (B) in the nonsensory otic epithelium. However, the endolymphatic duct is positive only for *Fgfr2(IIIb)*. Abbreviations: ot, otocyst; hb, hindbrain; ed, endolymphatic duct; cd, cochlear duct; cvg, cochleovestibular ganglion; fp, fusion plate; sd, semicircular duct; Rm, Reissner's membrane; cg, cochlear ganglion. Scale bar in E, 80 μm (A, D, E); 120 μm (B, C, and F–H).

during organogenesis. We hypothesized that this might be the case in the developing inner ear as well. Consistent with an earlier study (Colvin et al., 1999), we found *Fgf9* expression in the embryonic otic epithelium (Fig. 1). The expression was very weak in the otocyst at E9.5 (data not shown), but became stronger and showed initial regionalization by the next embryonic day (Fig. 1A). Between E11.5 and E12.5, *Fgf9* was expressed in the thin-layered nonsensory epithelium of the otocyst with the exception of the endolymphatic duct (Fig. 1B). This expression pattern was nonoverlapping with *Fgf10* expression, which is confined to the sensory and neurogenic patches of the otocyst (Fig. 1C) as previously shown (Pirvola et al., 2000). Specifically, at E12.0, *Fgf9* was prominently expressed in the outpocketings representing the primordia of the semicircular ducts (data not shown) and, at E12.5, in the forming fusion plates, which initiate semicircular duct development (Fig. 1B). At E13.5 and E14.5, *Fgf9* was expressed in the growing semicircular ducts (Fig. 1D), but this expression was downregulated by E16.5. In the extending cochlear duct of E12.0 to E14.5 embryos, *Fgf9* was expressed in the ventral wall (Fig. 1B), which comprises the nonsensory epithelium giving rise to the Reissner's membrane (Sher, 1971). Consistently, cochlear ducts of E16.5 and E18.5 mice showed *Fgf9* expression in the developing Reissner's membrane (Fig. 1E). In addition to the otic epithelium, low numbers of *Fgf9* transcripts were detected in the neurons of the embryonic inner ear ganglia (Fig. 1E).

Fgf9 preferentially activates the IIIc isoform of Fgfrs (Ornitz et al., 1996; Santos-Ocampo et al., 1996). *Fgfr1(IIIc)* and *Fgfr2(IIIc)* were widely expressed in the otic mesenchyme. As shown at E12.5, these receptors were expressed in the mesenchyme of both the dorsal (vestibular) and ventral (cochlear) part of the inner ear (Figs. 1F,G). Receptor expressions were homogeneous, except that, in the cochlea, most *Fgfr2(IIIc)* transcripts were confined to the outer ring of the otic capsule, destined for the temporal bone, whereas the mesenchyme adjacent to the cochlear

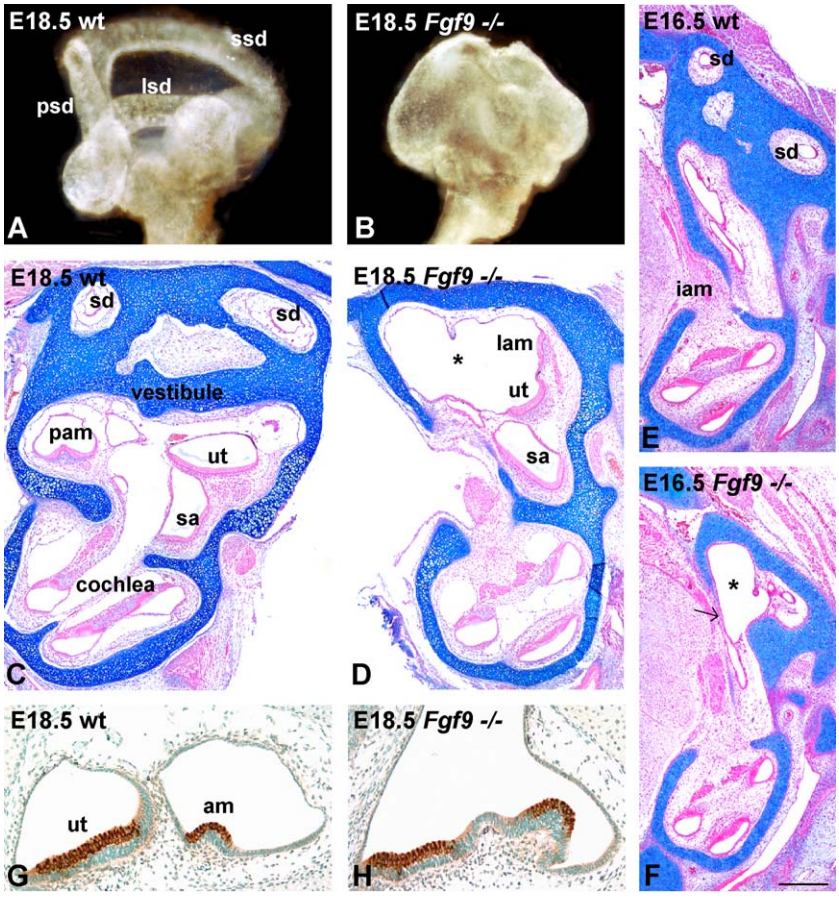

Fig. 2. Disturbed inner ear development in *Fgf9* null mutant mice at late embryogenesis. (A,B) Paint-filled whole mounts of E18.5 inner ears show that the three semicircular ducts seen in wild-type embryos (A) are not formed in the mutants (B). (C,D) Alcian Blue-stained sections of E18.5 inner ears show that the vestibular part of the otic capsule is hypoplastic in the mutants. In addition, semicircular ducts are missing and replaced by a dilated cavity (asterisks). (E,F) Alcian Blue-stained sections through E16.5 heads show the hypoplasia of the vestibular capsule of the mutants. Note also the enlargement of the internal acoustic meatus and thinning of a medially located domain of the vestibular capsule (arrow). (G,H) Myosin-immunostained sections prepared at E18.5 show that despite the absence of semicircular ducts, vestibular sensory epithelia appear normal in the mutants. Myosin marks the hair cells of the inner ear. Abbreviations: wt, wild type; ssd, superior semicircular duct; psd, posterior semicircular duct; lsd, lateral semicircular duct; sd, semicircular duct; ut, utricle; sa, saccule; pam, ampulla associated with the posterior semicircular duct; lam, ampulla associated with the lateral semicircular duct; am, ampulla; iam, internal acoustic meatus. Scale bar in F, 250 μm (A, B); 140 μm (C, D); 160 μm (E, F); 70 μm (G, H).

epithelium was largely devoid of this expression (Fig. 1G). Low levels of *Fgfr1(IIIc)* and *Fgfr2(IIIc)* were detected in the otocyst epithelium, most distinctly *Fgfr1(IIIc)* in the ventromedial domain (Fig. 1F) and in the greater epithelial ridge of the cochlear duct (data not shown; Pirvola et al., 2002). The epithelial fusion plates were negative for these receptors (Figs. 1F,G). *Fgfr1(IIIc)* and *Fgfr2(IIIc)* expressions persisted in the mesenchyme of the late-embryonic inner ear, but at lower levels than during otocyst stages (data not shown; Pirvola et al., 2000). Similar to *Fgf9*, *Fgfr2(IIIb)*, encoding the main receptor for Fgf10 (Igarashi et al., 1998; Lu et al., 1999), was expressed in the nonsensory epithelium of the otocyst (Fig. 1H; Pirvola et al., 2000). Thus, *Fgfr2(IIIb)* expression was largely non-overlapping with the distribution of *Fgfr1(IIIc)* and *Fgfr2(IIIc)* transcripts.

Fgf9-deficient mice show a malformed inner ear

Based on these expression data, we sought to determine the in vivo functions of Fgf9 in the developing inner ear. Mice homozygous for a targeted deletion of *Fgf9* have been generated and described to exhibit male-to-female sex reversal and severe lung hypoplasia (Colvin et al., 2001a,b). The mutants die at birth. As shown in paint-filled whole mounts of the inner ears at birth, the three semicircular ducts seen in normal mice were not formed in the mutants and were replaced by a large cavity (Figs. 2A,B). Histological analyses made at late embryogenesis confirmed these observations. Superior and posterior semicircular ducts were absent in all mutants analyzed (Figs. 2C–F). A rudimentary lateral duct was present in about 40% of the mutants (Fig. 2F); the other individuals show complete absence of this duct. Although semicircular ducts were absent, the three ampullary sensory epithelia, which are normally connected to these ducts, were present and showed a normal complement of hair cells as evidenced by myosin VI-immunostaining (Figs. 2G,H). Myosin VI marks the differentiating and mature hair cells of the inner ear (Hasson et al., 1997). Likewise, saccule appeared morphologically normal as well the sensory epithelium of utricle, which, however, was not separated into an individual chamber, but occupied the same dilated cavity together with the ampullary sensory epithelia (Figs. 2G,H). The loss of *Fgf9* did not affect endolymphatic duct development (data not shown).

In addition to the absence of semicircular ducts, another distinct feature of the *Fgf9*-deficient ears was the severe hypoplasia of the vestibular component of the otic capsule, as demonstrated by Alcian Blue staining, which specifically stains cartilage (Figs. 2C–F). Alcian Blue stained the cartilaginous outer domain of the otic capsule, which develops into the temporal bone. In addition to the general hypoplasia of the vestibular capsule, *Fgf9* inactivation caused a prominent thinning of the cartilaginous domain situated between the vestibular epithelium and the brainstem. This defect led to an increased size of the internal acoustic meatus, which conveys the 7th and 8th cranial nerves through the temporal bone (Figs. 2E,F). In contrast to the vestibular part of the otic capsule, the capsule around the cochlear duct appeared normal in the mutants (Figs. 2C–F).

The length of the cochlear duct and the cytoarchitecture of the sensory and nonsensory cochlear epithelia appeared normal in *Fgf9* null mutants, as analyzed at birth. However, abnormalities were seen in the mesenchyme of mutant cochleas, as evidenced by failures in the development of scala vestibuli, a fluid-filled chamber located above the Reissner's membrane (Figs. 3A,B). Scala vestibuli is formed by mesenchymal cavitation (Sher, 1971). At birth, in normal cochleas, a distinct scala vestibuli has been formed in the basal coil, but not yet in the later-differentiating upper turns. Scala vestibuli of age-matched mutants was enlarged, had an aberrant shape, and contained mesenchymal cells forming irregular trabeculae (Figs. 3A,B). In normal mice at birth, two mesenchymal cell layers are attached to the Reissner's membrane, but in age-matched mutants this cellular organization was disturbed (Figs. 3C,D). Taken together, although dysmorphogenesis caused by the loss of *Fgf9* is milder in the cochlea as compared to the vestibule, phenotypic analysis coupled with the expression of *Fgf9* and its receptors point to the role of Fgf9 signaling in epithelial–mesenchymal interactions in both parts of the developing inner ear.

We next investigated the developmental stage that is affected by *Fgf9* inactivation and that leads to the abnormal otic capsule and the absence of semicircular ducts. The observations made at E14.5 of the absence of these ducts

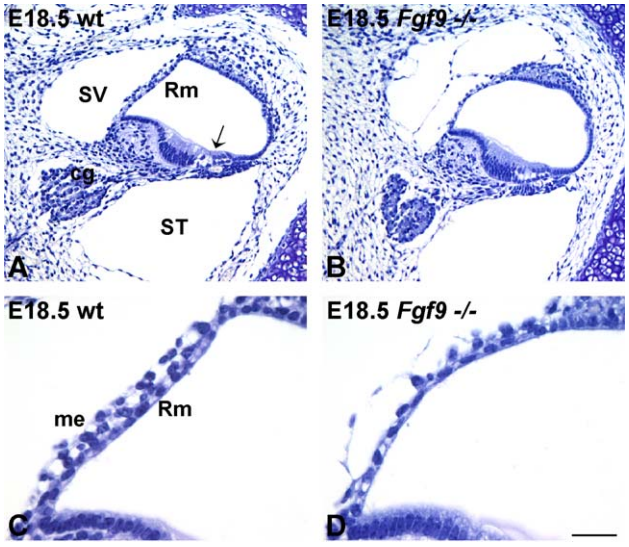

Fig. 3. Defects in the development of the cochlea of *Fgf9* null mutants at E18.5, as shown by hematoxylin staining. (A,B) Scala vestibuli of the mutants is enlarged and contains irregular mesenchymal trabeculi. The auditory sensory epithelium (arrow) of the mutants appears normal. (C,D) Higher magnification shows that the two mesenchymal cell layers normally attached to the Reissner's membrane are disorganized in the mutants. Abbreviations: wt, wild type; SV, scala vestibuli, Rm, Reissner's membrane; ST, scala tympani; cg, cochlear ganglion; me, mesenchyme. Scale bar in D, 60 μm (A, B); 15 μm (C, D).

and the small size of the vestibular component of the otic capsule, as shown by Alcian Blue-staining (Figs. 4A,B), suggested an early origin of the defects. In normal otocysts at E12.0, central regions of the walls of the vertical and horizontal outpocketings represent the primordial semicircular ducts. These epithelial domains are nearly in contact with one another at E12.0 (Fig. 4C) and fuse half a day later to generate fusion plates (Fig. 4D). At E12.0 and E12.5, mutant otocysts appeared otherwise normal, but the outpocketings were dilated, without any attempt of the opposing walls to approach one another (Figs. 4E,F). These results indicate that the initiation of semicircular duct development is blocked in *Fgf9* null mutants due to the inhibition of fusion plate formation. All the mutants analyzed lacked the formation of the fusion plates giving rise to the superior and posterior semicircular ducts. Lateral fusion plate was formed in some of the mutants, these findings being consistent with the observations made at late embryogenesis of a rudimentary lateral duct in about 40% of the mutants. Strikingly, at E12.0 and E12.5, in addition to the defects in the development of the epithelial fusion plates, volume of the mesenchyme around the dorsal part of the otocyst was clearly reduced in the mutants, as shown by hematoxylin staining (Figs. 4C–F) and *Sox9* in situ hybridization (Figs. 4G,H). *Sox9* is a precocious marker for the differentiation of mesenchymal cells into chondrocytes

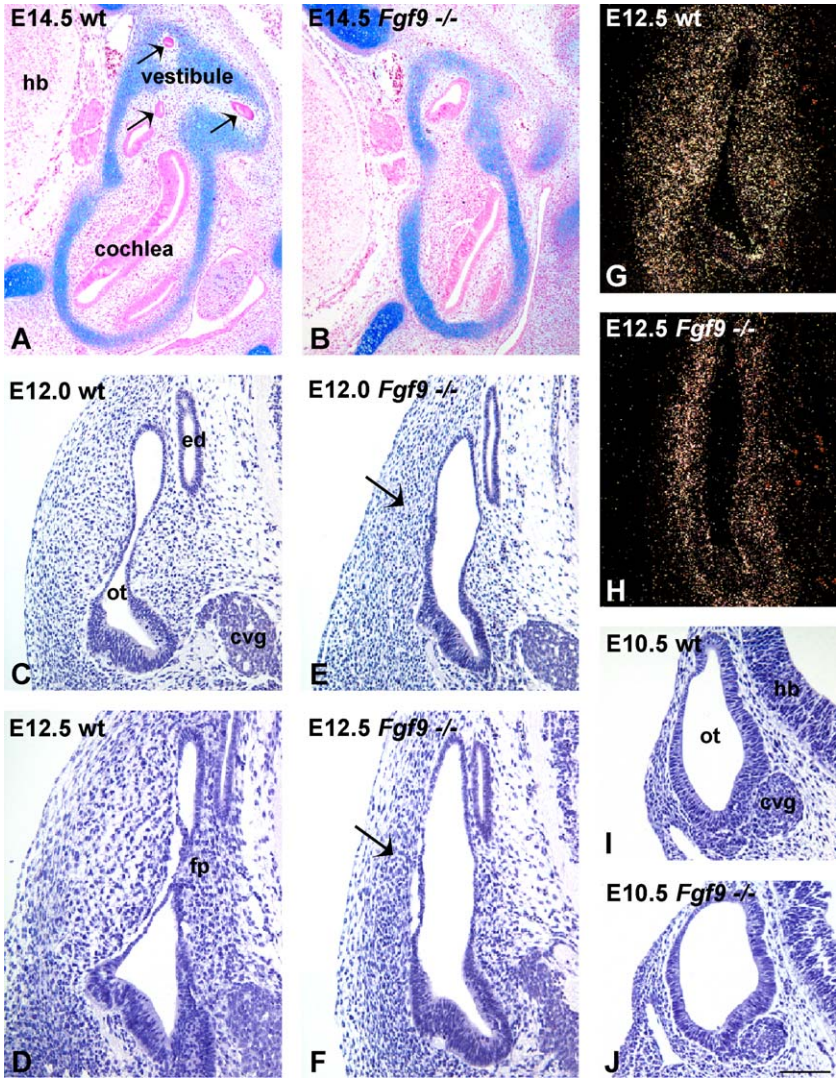

Fig. 4. Defects at the early stages of inner ear development in *Fgf9* null mutants. (A,B) Alcian Blue staining shows that chondrogenesis has been initiated in the inner ears of both controls and mutants at E14.5. Already at this stage, the mutants show prominent hypoplasia of the vestibular mesenchyme, which is associated with the absence of semicircular ducts (arrows in control specimen). (C,D) As shown by hematoxylin staining, in controls, the opposing walls of the outpocketing giving rise to the superior semicircular duct are approaching one another at E12.0 and the corresponding fusion plate is formed by E12.5. (E,F) In the mutants, the walls of the outpocketing generating the superior semicircular duct remain dilated and do not fuse. Note reduced mesenchymal volume around the dorsal (vestibular) part of the otocyst (arrows). (G,H) At E12.5, *Sox9* is strongly expressed in the otic mesenchymal cells of both mutants and controls, but the *Sox9*-positive area is more restricted in mutants. Control and mutants sections are adjacent to D and F, respectively. (I,J) Inner ears of mutants and controls do not show morphological differences at E10.5. Abbreviations: wt, wild type; ot, otocyst; cvg, cochleovestibular ganglion; ed, endolymphatic duct; fp, fusion plate; hb, hindbrain. Scale bar in J, 120 μm (A, B); 60 μm (C–F, I, J); 80 μm (G, H).

(Akiyama et al., 2002; Bi et al., 1999). Despite these distinct phenotypic changes at E12, inner ears of E10.5 mutants appeared morphologically normal; these results suggest that Fgf9 does not play a major role at the earliest stages of inner ear development (Figs. 4I,J).

What might be the mechanism used by Fgf9 to regulate fusion plate formation? As assayed by the TUNEL method, no differences were observed in the extent of apoptosis between mutant and control inner ears between E10.5 and E18.5 (data not shown). In contrast, at E12.0 and E12.5, clear differences were seen in proliferative activity, based on the S-phase-specific BrdU staining and on the phospho-histone H3-staining, which marks the M-phase of the cell cycle. Differences in proliferative activity were evident in the condensing otic mesenchyme, but not in the otic epithelium (Figs. 5A–F). In controls, intense proliferation was seen throughout the otic capsule (Fig. 5A). *Fgf9* mutants showed a modest reduction in proliferation in the mesenchyme surrounding the ventral part of the otocyst, which develops into the cochlea (Fig. 6). Proliferation was prominently declined in the mesenchyme around the dorsal part of the otocyst, destined for the vestibule (Fig. 5B). Specifically, numbers of BrdU- and phospho-histone H3-positive cells were reduced in the mesenchymal area next to the dilated epithelial outpocketings (Figs. 5C–F and 6), these results being consistent with the observations of reduced mesenchymal volume in this area. Together, decreased mesenchymal proliferation in *Fgf9* mutants appears to inhibit approaching of the opposing walls of the otocyst, resulting in the absence of fusion plates.

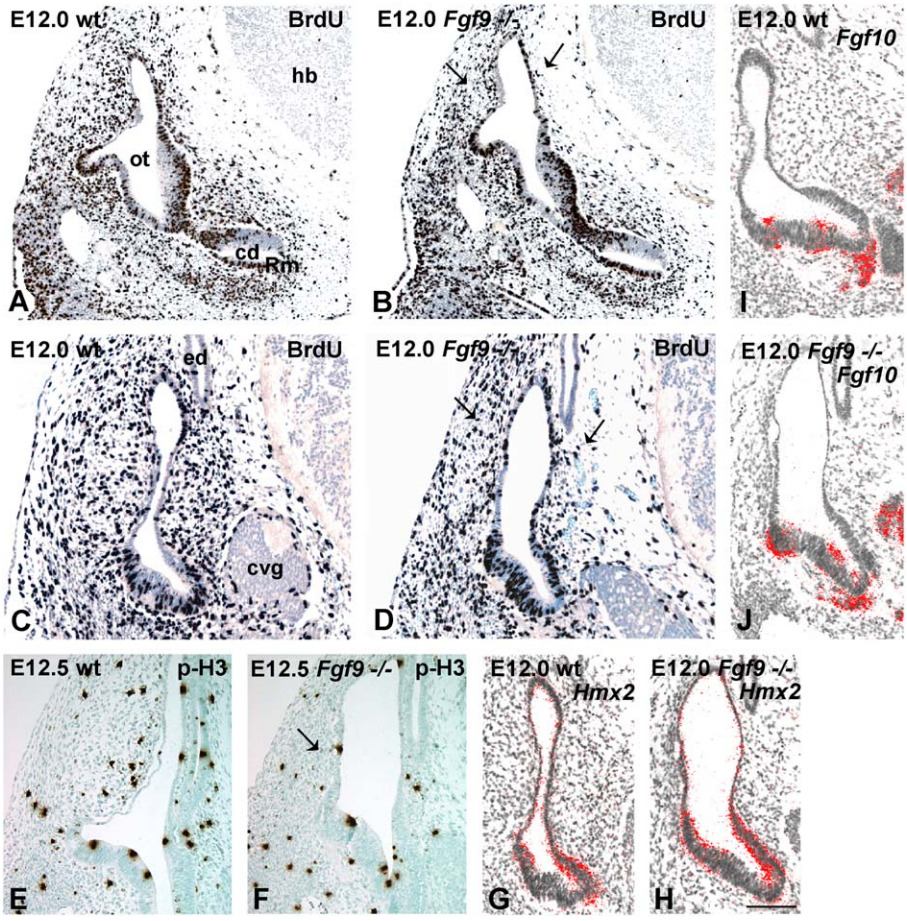

Fig. 5. Defects in proliferative activity in the precartilage otic mesenchyme of *Fgf9* null mutants (A–F). *Hmx2* (G,H) and *Fgf10* (I,J) expression in the otocysts of controls and *Fgf9* mutants. (A) BrdU labeling shows intense proliferation throughout the otic mesenchyme of a control embryo at E12.0. (B) In a littermate mutant, proliferation is clearly reduced (arrows) in the vestibular mesenchyme. Note also the decreased mesenchymal volume and the dilated primordia of the epithelial semicircular ducts. (C,D) As shown at higher magnification in the anterior part of the inner ear at E12.0, in the mutant, BrdU labeling is decreased (arrows) in the mesenchyme next to the dilated outpocketing, which gives rise to the superior semicircular duct. Note also the reduced mesenchymal volume. (E,F) Phospho-histone H3-immunostaining shows, in the mutant at E12.5, decreased numbers of mitotic profiles (arrow) in the otic mesenchyme next to the primordia of the semicircular ducts. Note also the reduced mesenchymal volume. (G,H) As shown by radioactive in situ hybridization, *Hmx2* is expressed in the otic epithelium of both controls and mutants at E12.0. (I,J) In situ hybridization shows similar expression pattern of *Fgf10* in the thickened regions of the otic epithelium of mutants and controls at E12.0. The *Fgf10*-positive epithelial patches give rise to the inner ear sensory epithelia and to the neurons of the cochleovestibular ganglion. The migrating neuroblasts account for the expression seen in the mesenchyme. Abbreviations: wt, wild type; ot, otocyst; cd, cochlear duct; ed, endolymphatic duct; cvg, cochleovestibular ganglion; hb, hindbrain; Rm, Reissner's membrane. Scale bar in H, 110 μm (A, B); 60 μm (C–F); 70 μm (G–J).

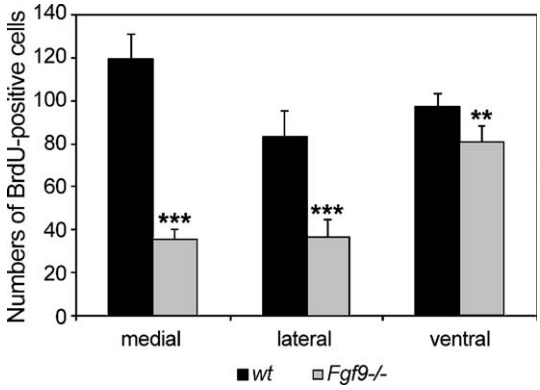

Fig. 6. Quantification of BrdU-positive cells in the lateral and medial regions of the vestibular mesenchyme (fields analyzed are next to the epithelial walls of the presumptive superior semicircular duct) and in the ventral region of the cochlear mesenchyme (next to the Reissner's membrane) of *Fgf9* mutants and littermate controls at E12.0. Each bar presents average from six to eight inner ears. Two sections per inner ear were analyzed and one field of each region was analyzed per section. Error bar represents standard deviation. Fields analyzed for a given region (lateral, medial, ventral) are exactly from the same level of the inner ear. *Sox9* in situ hybridization was used on adjacent sections to distinguish the otic mesenchyme from the non-otic mesenchyme. The decline in proliferation is very clear in the vestibular areas of mutants as compared to controls (***, $P < 0.001$). Differences are significant also in the cochlear ventral region (**, $P < 0.01$). Student's t test was used for statistical analysis.

Differences in the extent of mesenchymal proliferation were not anymore obvious at E14.5 (data not shown), at the stage when the mutant inner ears showed the distinct absence of semicircular ducts.

Our results suggest that the epithelial Fgf9 signals through its receptors present in the adjacent mesenchyme. We did not find detectable levels of *Fgfr1(IIIc)* and *Fgfr2(IIIc)* in the epithelial primordia of the semicircular ducts. To confirm that the effect of Fgf9 on semicircular duct development is not due to direct patterning changes within the epithelium, we compared the expression of two homeobox genes, *Hmx2* (also termed as *Nkx5.2*) and *Dlx5*, in *Fgf9* mutants and controls. *Hmx2* and *Dlx5* are expressed in the vestibular part of the otocyst and the knockout mice completely lack semicircular ducts (*Hmx2*) or show only a rudimentary lateral duct (*Dlx5*) (Acampora et al., 1999; Depew et al., 1999; Hadrys et al., 1998; Rinkwitz-Brandt et al., 1995; Wang et al., 2001). As analyzed between E12.0 and E14.5 (Figs. 5G,H), *Hmx2* and *Dlx5* were expressed in a similar fashion in the control and mutant otocysts, suggesting that the expression of these transcription factors is independent of *Fgf9*. Netrin1 is a laminin-related protein. Similar to *Fgf9* inactivation (this study), targeted disruption of *Netrin1* leads to the absence of semicircular ducts (Salminen et al., 2000). In both cases, fusion plate formation is blocked. In situ hybridizations performed at E12.0, E12.5, E15.5, and E18.5 showed that *Netrin1* is expressed in the otic epithelium of *Fgf9* mutants (data not shown). Together, these data showing that the expression of epithelial genes known to regulate semicircular duct formation is unaffected in *Fgf9* mutant mice suggest that the direct downstream targets of Fgf9 signaling reside within the otic mesenchyme.

Based on the knowledge that Fgf9 signaling can modulate *Fgf10* expression during organogenesis (Colvin et al., 2001a), we studied whether the normal expression of *Fgf10* in the sensory patches and neurogenic region of the otocyst (Pirvola et al., 2000) is maintained in the mutants. As analyzed at E12.0 and E18.5, we found unaltered *Fgf10* expression in the mutants and, thus, this expression in the inner ear seems to be independent of Fgf9 signaling (Figs. 5I,J).

Discussion

Epithelial–mesenchymal interactions based on Fgf9 signaling in the embryonic inner ear

In the present study, we give evidence of an epithelial Fgf signal, Fgf9, controlling inner ear morphogenesis. Our results suggest that, during otocyst stages, Fgf9 is involved in interactions between the otic epithelium and the surrounding mesenchyme where its receptors are expressed. This is in contrast to two other *Fgfs*, *Fgf10* and *Fgf3*, which are also expressed in the otocyst epithelium, but which signal through their common receptor, *Fgfr2(IIIb)*, also present in the epithelium (De Moerlooze et al., 2000; Mansour et al., 1993; Pauley et al., 2003; Pirvola et al., 2000).

The mesenchyme around the otocyst condenses and forms the otic capsule, the shape of which follows the contour of the cochleovestibular membranous (epithelial) labyrinth. These general observations suggest an intimate crosstalk between the otic epithelium and the mesenchyme. In vitro experiments support this suggestion (Frentz and Van de Water, 1991), but the molecular nature of epithelial–mesenchymal interactions in the inner ear is poorly understood. Recently, signaling by bone morphogenetic proteins has been suggested to regulate the growth of the otic capsule in the chick (Chang et al., 2002). The present study shows that *Fgf9* null mutants at birth have a severely hypoplastic vestibular component of the otic capsule. This defect appears to be due to reduced mesenchymal proliferation at early developmental stages. We found that Fgf9 stimulates mesenchymal proliferation mainly before E14.5. At this stage, the null mice, lacking semicircular ducts, showed a markedly undersized vestibular capsule, but did not show significant differences in the extent of mesenchymal proliferation as compared to controls. At E10.5, at the onset of *Fgf9* expression, we did not see morphological differences between the inner ears of controls and mutants. However, reduction in the volume of the otic mesenchyme and decrease in proliferative activity become very clear by E12. Because Alcian Blue staining, marking chondrogenic regions, is first detected in the otic mesenchyme at E13.5

(current study; McPhee and Van de Water, 1985), we conclude that Fgf9 has most prominent effect on the growth of the otic mesenchyme at the precartilage stages. This suggestion is in line with previous data showing that one of the receptors through which Fgf9 signals, *Fgfr2(IIIc)*, is strongly expressed in the precartilage mesenchymal condensations in the mouse embryo (Finch et al., 1995) and is essential for skeletal development (Eswarakumar et al., 2002; Yu et al., 2003). *Sox9* was strongly expressed in the otic mesenchyme of both controls and mutants, but the *Sox9*-positive area was more restricted in the mutants, this being consistent with the observations of reduced mesenchymal volume. *Sox9* is a master regulator of chondrogenesis (Akiyama et al., 2002; Bi et al., 1999). Strong *Sox9* expression in the otic mesenchymal cells of *Fgf9* mutants suggests that Fgf9 does not play a major role in their differentiation. Prominent *Sox9* expression in the mutants also suggests that it is not a downstream target for Fgf9 signaling. A candidate target gene is the POU-domain transcription factor *Brn4*, which is widely expressed in the otic mesenchyme and causes defects in otic capsular development when inactivated (Phippard et al., 1998, 1999). However, the cellular mechanisms underlying this phenotype have not been shown and, thus, it is at the present unknown whether *Brn4* is involved in the Fgf9-induced proliferative pathway in the otic mesenchyme.

Reciprocal epithelial–mesenchymal interactions in the developing inner ear

During embryogenesis, *Fgf9* expression and function in the inner ear show parallels to organs such as the lung, testis, and tooth (Colvin et al., 2001a,b; Kettunen et al., 1998). In all these tissues, the epithelial Fgf9 stimulates mesenchymal proliferation. In many developing organs, Fgf signaling is involved in reciprocal interactions between the epithelium and mesenchyme. This appears to be the case in the inner ear as well. Our results suggest that the initiation of semicircular duct development is blocked in *Fgf9* null mice due to decreased mesenchymal proliferation. In normal development, a force originating from the mesenchyme is thought to drive together the opposing walls of the otocyst, leading to the formation of fusion plates, which give rise to semicircular ducts. Our study suggests that this force is, at least partly, generated by mesenchymal expansion through cell proliferation. In *Fgf9* null mutants, the superior and posterior semicircular ducts were always absent, but a rudimentary lateral duct was present in about half of the specimens. These findings imply for some degree of heterogeneity in the molecular mechanisms underlying the initiation of development of the three semicircular ducts.

In addition to mesenchymal proliferation, other mechanisms, especially those related to the extracellular matrix (ECM), are likely to contribute to fusion plate formation. *Netrin1* is expressed in the nonsensory areas of the otocyst epithelium and its inactivation blocks the formation of fusion plates (Salminen et al., 2000). Because Netrin1 is a laminin-related protein, it has been speculated that localized defects at the level of the basement membrane and ECM contribute to the abnormal phenotype of *Netrin1*-deficient mice (Salminen et al., 2000). Based on the similarities in the inner ear phenotype of *Netrin1* and *Fgf9* null mutants, a possibility is that these genes belong to the same genetic pathway. However, the unaltered expression of *Netrin1* in the inner ears of *Fgf9* mutants suggests that Fgf9 is not an upstream regulator of *Netrin1*. It has been experimentally shown in the early inner ear of *Xenopus* that enzymatic destruction of glycosaminoglycans (GAGs) blocks the inward movement of the opposing otic epithelial faces, which normally generate fusion plates (Haddon and Lewis, 1991). Localized production of GAGs by these epithelial domains has also been demonstrated (Haddon and Lewis, 1991). Thus, accumulation of GAGs in the ECM may provide a driving force for fusion plate formation. In this respect, it is interesting that GAGs are important modulators of Fgf activity (reviewed by Ornitz, 2000; Pellegrini, 2001) and, therefore, the effect of GAGs may at least partially be exerted through regulation of Fgf9 activity.

Interestingly, in *Fgf9* null mutants, similarities can be seen in the underlying cellular mechanism causing failures in the formation of the inner ear's semicircular ducts (this study) and the lung's airways (Colvin et al., 2001a). In both cases, the early epithelial outpocketings remain dilated due to reduction in the amount of surrounding mesenchyme. In the lung and limb bud, reciprocal cross-talk between these compartments by Fgfs is well-established. In these organs, Fgf10 is a key mesenchymal signal and Fgf9, Fgf8, Fgf4, and/or Fgf17 are epithelial signals (Colvin et al., 2001a,b; Min et al., 1998; Sekine et al., 1999; Sun et al., 2002). Further evidence for Fgf-based reciprocal signaling comes from the lungs of *Fgf9* null mice in which *Fgf10* expression in the mesenchyme is reduced (Colvin et al., 2001a). The present findings suggest that reciprocal epithelial–mesenchymal interactions operate in the developing inner ear as well. However, in contrast to organogenesis of many other tissues and to the stages of otic placode induction when *Fgf10* is transiently expressed in the mesenchyme next to the placode (Alvarez et al., 2003; Wright and Mansour, 2003), *Fgf10* is expressed in the otic epithelium rather than in the surrounding mesenchyme at the otocyst stages. Thus, Fgf10 signaling appears not to be involved in epithelial–mesenchymal interactions at the otocyst stages. However, in addition to the force generated by the expanded otic mesenchyme, a diffusible mesenchymal signal is likely to be involved in driving the opposing otic epithelial walls together. The identity of this putative signal is at the moment unknown. Although *Fgf10* is not expressed in the mesenchyme surrounding the otocyst,

one possibility was that Fgf9 signaling regulates epithelial *Fgf10* expression through intricate signaling loops between the epithelium and mesenchyme. However, our expression studies on *Fgf9* mutants do not support this possibility.

The present data show that, in the inner ear, *Fgf9* null mutation has strongest effect on the development of the vestibule, but defects are also seen in the cochlea. Consistent with the observations of *Fgf9* expression in the developing Reissner's membrane and its receptors in the adjacent mesenchyme, the normal attachment of mesenchymal cells to the Reissner's membrane failed in *Fgf9* mutants and resulted in an enlarged and trabeculated scala vestibuli. Thus, in addition to the function at the prechondrogenic stages, Fgf9 has a role in the later-developing inner ear, in the remodeling of the cochlear mesenchyme. The remodeling process continues postnatally (Sher, 1971), but the lethality of *Fgf9* mutants at birth prevented us from following disturbances in this process at later stages. The observed defects in the interaction between adjacent epithelial and mesenchymal cell sheets suggest that the extracellular properties such as cell adhesion and cell migration are affected, and, thus, speak for the involvement of the ECM in Fgf9 signaling during cochlear development. Taken together, our results on the cochleas of *Fgf9* mutants support the conclusions made in the vestibule that Fgf9 signaling is involved in epithelial–mesenchymal interactions. The observations of unaltered expression of *Dlx5* and *Hmx2* in the otocysts of *Fgf9* mutants suggest that these epithelial transcription factors do not serve as downstream targets for *Fgf9*. These observations further support our suggestion that the direct targets for the otic epithelium-derived Fgf9 signal reside in the mesenchyme.

In conclusion, the present study provides genetic evidence for the role of Fgf9 signaling in inner ear morphogenesis, specifically in the growth of the otic capsule and the initiation of semicircular duct formation. Our data point to the importance of cross-talk between the epithelium and mesenchyme in global shaping of the inner ear. In this respect, Fgf9 signaling in the inner ear shows parallels to many other developing organs. Our future studies will aim at identifying the downstream targets for Fgf9 signaling in the otic mesenchyme and at placing *Fgf9* in the network of otic epithelial genes known to affect semicircular duct formation.

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