Expression and Function of FGF10 in Mammalian Inner Ear Development

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We have investigated the expression of FGF10 during ear development and the effect of an FGF10 null mutation on ear development. Our in situ hybridization data reveal expression of FGF10 in all three canal crista sensory epithelia and the cochlea anlage as well as all sensory neurons at embryonic day 11.5 (E11.5). Older embryos (E18.5) displayed strong graded expression in all sensory epithelia. FGF10 null mutants show complete agenesis of the posterior canal crista and the posterior canal. The posterior canal sensory neurons form initially and project rather normally by E11.5, but they disappear within 2 days. FGF10 null mutants have no posterior canal system at E18.5. In addition, these mutants have deformations of the anterior and horizontal cristae, reduced formation of the anterior and horizontal canals, as well as altered position of the remaining sensory epithelia with respect to the utricle. Hair cells form but some have defects in their cilia formation. No defects were detected in the organ of Corti at the cellular level. Together these data suggest that FGF10 plays a major role in ear morphogenesis. Most of these data are consistent with earlier findings on a null mutation in FGFR2b, one of FGF10’s main receptors. Developmental Dynamics 227:203–215, 2003. © 2003 Wiley-Liss, Inc.

Key words: FGF10, inner ear, development morphogenesis, histogenesis, posterior vertical canal

INTRODUCTION

Fibroblast growth factors (FGFs) represent an ancient class of secreted, extracellular signaling molecules. A single ligand is known for insects and at least 22 ligands are known in mammals (Ornitz and Itoh, 2001; Venter et al., 2001). These ligands share 35% amino acid identity over a core region of approximately 120 base pairs and are expressed in distinct spatial and temporal patterns in many developing organs (Ornitz and Marie, 2002). In contrast, only four FGF receptor kinase genes are known in vertebrates (Venter et al., 2001). FGF receptor genes show tissue-specific alternative splicing and act as membrane-bound tyrosine kinase receptors. These receptors are activated by many FGFs that have various binding specificities with the receptors (Ornitz et al., 1996). Variability of ligand receptor interaction is also enhanced by alternative spliceforms of FGF—receptors have unique ligand binding properties resulting in a multitude of spatial and temporal interactions that depend on the concentration and specificity of both receptor and ligand. Despite this complex interaction, some functions of ligands and their receptors appear to be highly conserved across phyla. For example, the insect FGF ligand branchless is essential for tracheal formation (Sutherland et al., 1996), whereas the mammalian FGF10 ligand is essential for lung and limb formation (Min et al., 1998; Sekine et al., 1999). FGFs play multiple roles in cell–cell signaling. They are involved in pattern formation in the central nervous system (Lee et al., 1997; Wang and Zoghbi, 2001) and regulate proliferation, differentiation, migration, and neurite growth (Saffell et al., 1997; Hossain and Morest, 2000; Ornitz and Marie, 2002). Given this wide range of functions, it is not surprising that FGFs and their receptors play a critical role in the development of the vertebrate ear.
The ear develops from an ectodermal thickening into a three-dimensional organ that provides the cellular and extracellular substrate for mechanical sensory transduction of hearing and balance. To perceive these signals, hair cells convert mechanical energy into electric signals and primary neurons conduct this information to the brain (Fritzsch and Beisel, 2001; Fekete and Wu, 2002). FGFs play a role in ear induction (Mansour et al., 1993; Ladher et al., 2000; Vendrell et al., 2000), ear growth and morphogenesis (Pirvola et al., 2000; Adamska et al., 2001; Phillips et al., 2001; Whitfield et al., 2002), and formation and differentiation of hair cells (Pirvola et al., 2002), as well as supporting cells (Colvin et al., 1996). However, the analysis of function of FGF ligands and receptors in the ear is complicated by the apparent complex spatial and temporal expression of many FGFs and FGFRs in the ear (Pickles, 2001) and the possibility that FGF function may not be conserved across species (Noramly and Grainger, 2002). For example, it remains unclear how the two or more ligands for the FGFR2b isoform that are so crucial for ear development...
(Pirvola et al., 2000) are expressed and interact with this and other receptors.

Previous work has shown the necessary role of one of the known ligands for FGFR2b in the ear, FGF3 (Mansour, 1994). It appears that ear formation is even more compromised in FGF3/10 double null mutants (Wright and Mansour, personal communication), suggesting that those two ligands are the main factors that regulate ear morphogenesis through FGFR2b. FGF10 has the highest affinity for the b isoform of FGFR2 (Ohuchi et al., 2000; Yu et al., 2000). However, FGF3,7,10, and 22 are considered to be paralogs of an ancestral chordate FGF gene (Satou et al., 2002) and the roles of FGF7 and 22 in the ear are essentially unknown.

Based on these data, we hypothesized that FGF10 should be involved in ear morphogenesis and formation of sensory hair cells and possibly sensory neurons. Here, we show that FGF10 is indeed essential for the morphogenesis of the posterior vertical canal and its sensory epithelium but not the sensory neurons innervating this canal epithelium. We also show a milder effect of the FGF10 null mutation on other vestibular sensory organs. We suggest that a redundancy of the other FGF ligands accounts for the less-severe effect of FGF10 null mutation in other vestibular epithelia.

RESULTS

Expression of FGF10 in the Developing Ear

Unpublished observations suggest the expression of FGF10 in the ectoderm before otocyst formation (E8), and this expression may play a role in otic placode induction (Noramly and Grainger, 2002). By using in situ hybridization, we followed FGF10 expression from E9.5 onward (Fig. 1A); the time the otocyst first appears in development. At E9.5, the expression was concentrated in the lower half of the otocyst and did not extend to the presumptive endolymphatic duct. One day later, the ex-
pression had changed and strong expression was present on the anterior pole of the otocyst as well as in delaminating cells of the forming cochleovestibular ganglion (Fig. 1D). In addition, a second, less-extensive area of expression became visible at the posterior pole of the otocyst.

At E11.5 (Fig. 1B,C,E,F), the expression was highly focused, showing strong expression in three distinct patches. By comparison with later stages, these three patches were identified as the future epithelia for the three canal cristae. In addition, FGF10 was highly expressed in the delaminating sensory neurons of the forming cochlear and vestibular ganglion. Less-prominent expression in the utricle and cochlea could be visualized only after the ganglion was surgically detached. All sites of expression became stronger at later stages, and these structures were described in part elsewhere (Pirvola et al., 2000).

The pattern of expression was analyzed in more detail for each sensory epithelium at P0, a terminal point for our analysis of the FGF10 null mutant mice because these lungless animals die at birth. At this stage, FGF10 was expressed in all sensory epithelia but showed variable patterns in each sensory epithelium. For example, in the cochlea, FGF10 was expressed most strongly in the greater epithelial ridge. It was only faintly expressed around outer hair cells and showed no detectable expression in Claudius cells or the cochlear lateral wall (Fig. 2C,D). The expression around outer hair cells was very faint and was undetectable in sections (Pirvola et al., 2000). Within the greater epithelial ridge, FGF10 showed a continuous increase in expression, being highest at the region adjacent to the inner hair cells. The highest expression level in the cochlea was similar to that in sensory neurons, suggesting that these cells may be a major source of FGF10 in the ear throughout development. Both the utricle and saccule showed an interesting pattern that indicated a higher concentration near the neural side, falling off toward the abneural side (Fig. 2B). This pattern was inversely related to that of the greater epithelial ridge of the cochlea. However, all other sensory epithelia lacked the clear step in expression intensity seen in the area between the inner and outer hair cells. All crista organs retained their strong FGF10 signal (Fig. 2A) with only a limited indication of a gradient falling off toward the abneural side.

In summary, FGF10 expression showed a diffuse up-regulation in the lower half of the developing ear that changed over only 2 days into strong expression in the canal cristae and somewhat less-intense expression in all sensory neurons and all other epithelia. The cochlea, utricle, and saccule showed a delayed up-regulation that displayed prominent gradients, falling off gradually or stepwise from either the neuronal to the abneural side or the reverse, in each sensory epithelium.

![Fig. 4.](image-url) These epoxy resin embedded, thick sections show the sensory epithelia of embryonic day 19.5 FGF10 null mutant ears (A–E) and littermate wild-type control (F). The coronal sections (A–D) show that the horizontal crista (HC) is in its own small recess, whereas the anterior vertical crista (AC) is inside the utricular space and becomes confluent with the utricle (U). Moreover, the utricle forms a recess (B) never found in control animals. The sagittal sections illustrate this confluence of utricular and anterior vertical cristae in the mutant (E) compared with the control wild-type littermate (F). Scale bars = 100 μm in A–F. (Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)
Ears in FGF10 Null Mutants

E18.5, FGF10 null mutant ears displayed severe dismorphogenesis with complete absence of all aspects of the posterior canal system: there was no posterior crista, no posterior canal, and no trace of any myelinated nerve fiber to the area of the posterior canal (Fig. 3A,B). A blind-ending stub was formed instead of the horizontal canal, and the anterior canal was largely continuous with the utricle without forming a recognizable canal. The short canal emerging from the horizontal crista extended vertically toward the cochlea rather than horizontal, toward the posterior canal. In addition, the anterior and horizontal cristae were much smaller and barely segregated from the utricle.

Fig. 5. These confocal (A,B) and scanning electron microscopy images (C,D) illustrate the dismorphogenesis of the horizontal canal and the disorientation of the anterior vertical cristae and its continuity with the utricle in newborn (P0, A,B) and embryonic day 18.5 (E18.5; C,D) FGF10 null mutant. Note that the anterior crista (AC) is virtually continuous with the utricle in the FGF10 null mutant (B,D) but separated by nonsensory epithelium in the control littermate (A,C). Note also the formation of the nonsensory cruciate eminence (CE) in the control animal (C) and the change in shape of the remaining anterior cristae in the FGF10 null mutant (B,D). The shape of the horizontal crista (HC) is different in the FGF10 null mutant (B) and the control littermate (A). Close-up in B shows the proximity of bulbed kinocilia (red anti-tubulin Alexa 568 fluorescence) of anterior canal crista with nonbulbed kinocilia of the utricle as well as the position of the kinocilium with respect to the green phallidin-Alexa 488-positive stereocilia. Scale bar = 100 μm in A-D, 10 μm in the insert.
tent with the overall size reduction of the FGF10 ear, the saccule and cochlea sensory epithelia also appeared somewhat smaller but otherwise unchanged.

Our serial histologic sections revealed no trace of a posterior crista. In fact, the utricle ended blindly as suggested by our dissected ears (Fig. 4A). The anterior crista was smaller in FGF10 null mice and barely segregated from the utricle. In contrast to the wild-type control (Fig. 4F), the anterior crista was a partially continuous group of hair cells on the anterior end of the utricle in the FGF10 null mutant (Fig. 4C–E). The anterior canal formed a blind-ending structure that was largely continuous with the utricle over most of its extent. The horizontal canal formed a small, blind-ending extension, with the crista clearly separated from the utricle (Fig. 4A–D). The presence of a cupula rather than otoconia suggests that these hair cells represent the crista of the anterior and horizontal canal. In most specimens examined, the utricle formed a central recess rather than a flat sheet of cells (Fig. 4B).

Histologically, hair cells appeared normal in all remaining sensory organs. However, closer examination using tubulin immunocytochemistry combined with phalloidin staining for actin revealed that hair cells in the canal epithelia had larger bulbs at their tips in FGF10 null mutant mice (Fig. 5A,B). These flat mounts also showed close proximity of cells with different sized bulbous tips near the anterior crista, suggesting an incomplete segregation of the anterior crista from the utricle (insert Fig. 5B). Flattening the remaining vestibular epithelia also revealed some size and shape differences (compare Fig. 5A,B) and resulted in cracks of the wrinkled and misshapen mutant epithelia. The polarity of hair cells, as determined by the position of the kinocilium with respect to stereocilia seemed to be identical in the mutant and their wild-type littermates. However, the mutant epithelia showed a somewhat greater variety of odd polarities in individual hair cells.

**Scanning Electron Microscopy and Transmission Electron Microscopy Observations**

We next examined the appearance of the remaining vestibular organs by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM revealed the continuity of the anterior crista with the utricle (Fig. 5C,D). The anterior crista was very different from control mice. In control mice, the anterior crista consisted of two hemicristae separated by a hair cell-free cruciate eminence (Fig. 5C). The anterior crista was transversally orientated with respect to the utricle. In contrast, no cruciate eminence was visible in the FGF10 null mutant crista. In addition, the remaining crista appeared to be 90 degrees rotated and was longitudinally rather than transversally orientated as in control animals (Fig. 5D). The horizontal crista also showed some shape changes, but the absence of a cruciate eminence made those changes less conspicuous.

The TEM analysis showed normally developed hair cells in the five remaining sensory epithelia, including well-developed inner and outer hair cells (Fig. 6B). However, we found many vestibular hair cells, in particular in the canal cristae that displayed disorganized apical specializations such as blobbing of the stereocilia (Fig. 6A).

In summary, our morphologic analysis showed that the most severe effects of the FGF10 null mutation are in the canal system. Canals are either absent (posterior canal) or severely disorganized (anterior and horizontal canal). In addition to these morphogenetic effects, entire sensory epithelia are either missing (posterior canal crista) or were smaller and disoriented (anterior and horizontal canal). Remaining hair cells of vestibular sensory epithelia showed some aberrations in the
formation of apical stereocilia and enlarged bulbs on the kinocilia.

Development of Innervation

We found no myelinated fibers extending toward the area of the missing posterior canal crista in late FGF10 null embryos. This absence could be related to the complete loss of all components of the posterior canal system, including primary sensory neuron formation. Alternatively, primary sensory neurons and their fibers could develop initially, with innervation lost in later embryos, possibly a result of the absence of neurotrophins (Bianchi et al., 1996) that would have been produced by the missing crista. We investigated these alternative possibilities by using E11.5 and E12.5 embryos (Fig. 7). Our data showed that the initial formation of sensory neuron projection for the posterior canal was normal at E11.5 (Fig. 7A,B). Within 1 day, afferent fibers invaded all vestibular sensory organs in control littermates (Fig. 7C,E). In FGF10 null mutants, there was a less-extensive fiber supply to the anterior and horizontal crista (Fig. 7D) and the fibers to the posterior crista showed no signs of invading the apparently absent posterior crista (Fig. 7F). One day later, fibers to the posterior crista disappeared and the pattern of innervation was much like that at E18.5 (Fig. 8C). Thus, sensory neurons for all canals appear to form normally, but they become lost to the posterior crista and likely downsized to the anterior and horizontal crista. These patterns of innervation are consistent with the loss of the posterior crista (and concomitant loss of trophic signals) and reduced size of the anterior and horizontal cristae.

Comparison With FGFR2b

FGF10 and FGF3 are two known ligands for FGFR2b. FGF3 null mutants have not been investigated in detail with respect to their innervation (Mansour et al., 1993) and only a few sensory neurons appear to survive in FGFR2b null mutants (Pirvola et al., 2000). Moreover, the presence of nerve fibers as well as the degree of development past the otocyst stage varied considerably. We wanted to understand in more detail the relative role played by two of the known ligands of FGFR2b.

We compared the projection of the few remaining sensory fibers in the FGFR2b null mutants with a control ear and the ear of the FG10 null mutants (Fig. 8A–C). In most FGFR2b mutants, no innervation to the otocyst could be detected by tubulin or neurofilament immunocytochemistry in either whole-mount otocysts (this study) or sectioned material (Pirvola et al., 2000). Typically, only cystic vesicles with barely distinct endolymphatic duct formed. Four of the more differentiated ears of FGFR2b null mutants showed, despite reduction in size, a differentiation into a somewhat elongated sack. Those ears also displayed acetylated tubulin-positive fibers that extended toward the posterior half of the otocyst (Fig. 8B).

Comparison of the topology and overall distribution of these fibers suggested that they may represent a bundle running toward the area of the undifferentiated posterior crista and multiple fibers extending toward an area that topologically compares with the cochlea (Fig. 8B). In striking contrast, the fiber bundle to the posterior crista were always absent in similar aged FGF10 null mutant mice (Fig. 8C). These data suggest that at least the FGF10 effects on the posterior canal are not entirely mediated by FGFR2b but that another FGF receptor may also play a role. Indeed, some signaling by means of FGFR1b is known (Yu et al., 2000).

DISCUSSION

FGF Expression Patterns and Their Implications

Among the expression patterns of FGFs thus far described for the vertebrate ear (Mansour et al., 1993; McKay et al., 1996; Colvin et al., 1999; Pirvola et al., 2000; Noramly and Grainger, 2002), FGF10 is characterized by progressive restriction to sensory neurons and sensory epithelia and intricate patterns of expression within sensory epithelia. In contrast, other FGFs show different expression patterns. For example, FGF8 shows an early expression, followed by down-regulation, and is again up-regulated later to display expression in the forming inner hair cells (Pirvola et al., 2002). This inner hair cell expression appears to mediate FGFR3-related pillar cell development (Colvin et al., 1996). In contrast, FGF3 is up-regulated early but shows less expression in the differentiating ear around E11, before any localization to specific sensory epithelia is possible (McKay et al., 1996). FGF9 appears to be expressed in a pattern somewhat similar to FGF10 in the cochlea (Colvin et al., 1999). Only scant data exist on the spatiotemporal expression of other FGFs in the developing otocyst. A recent study suggested that most FGFs are expressed in the ear at one time or another (Pickles, 2001). However, these data have not been extended to a detailed spatiotemporal expression pattern analysis by using in situ hybridization.

Understanding the possible functional redundancies and interactions between these FGFs and their ligands requires a more detailed in situ hybridization analysis of many more of the FGFs, as was recently performed for facial development (Bachler and Neubuser, 2001). Such multiple in situ hybridizations with various probe are especially needed for FGF 3,7,10, and 22, as those FGFs might have redundant signaling capacity (Ornitz and Itoh, 2001; Satou et al., 2002).

The expression pattern and intensity of FGF10 in situ hybridization suggests that FGF10 plays a major role in canal crista development, development of the greater epithelial ridge, as well as development of the sensory neurons. The function of FGF10 in primary sensory neurons might be difficult to assess owing to the alleged presence of FGF1 and 2 in sensory neurons (Hossain and Morrest, 2000). Despite the paucity of topologic expression data concerning redundancy of FGFs that could signal like FGF10, little redundancy seems to exist in the crista organs, in particular the posterior crista. Most importantly, FGF3 expression is concentrated in the anterior pole of the otocyst, likely overlapping with the
Fig. 7. These DiI confocal images show the detailed pattern of afferent innervation of the developing vestibular system at embryonic day 11.5 (E11.5) and E12.5. A,B: The initial formation of fiber outgrowth to the canal cristae is similar in the control and FGF10 null mutant littermate. Note the closer spacing of posterior crista sensory neurons (PCS) to the posterior crista (PC) in the mutant compared with the control animal, owing to the smaller size of the mutant ear. One day later, afferents invade all crista sensory epithelia in the wild-type (WT; C,E) but only reach sparingly into the anterior (AC) and horizontal (HC) cristae in the FGF10 null mutant (D). F: Fibers still extend to the absent posterior crista of the FGF10 null mutant but show numerous side branches to nonsensory areas of the developing ear. Arrows in A indicate orientation. Scale bars = 100 μm in A–F.

Fig. 8. These whole-mount ears compare the pattern of innervation as revealed with DiI in a postnatal day 0 (P0) control animal with an embryonic day 18.5 (E18.5) FGF10 null mutant and immunocytochemistry for acetylated tubulin of an E18 FGFR2b null mutant. Note that the cochlea innervation is rather normal in the FGF10 null mutant (compare A and C). Utricle (U), saccule (S), and horizontal crista (HC) also receive rather normal innervation. However, the innervation to the anterior crista (AC) is reduced in the FGF10 null mutant (compare A and C), and there is no innervation to a posterior crista (PC) in the FGF10 null mutant. In the FGFR2b null mutant, there is some innervation in a pattern consistent with a cochlear projection (C). There is also a fiber bundle to the posterior aspect, consistent with a posterior crista-like innervation in the FGFR2b null mutant (compare A and B). ED, endolymphatic duct. Scale bars = 100 μm in A–C.
anterior expression domain of FGF10 around E10. Thus, FGFR3 could provide redundant signaling with FGF10 in the anterior part of the otocyst during initial formation of the otocyst but not in the posterior part as it is not expressed there.

**FGF10 and Morphogenesis of the Ear**

FGF10 binds with high affinity to FGFR2b and with lesser affinity to other FGF receptors (Yu et al., 2000). Null mutations of FGF10 appear to mimic null mutants of FGFR2b (Ohuchi et al., 2000). Of the receptor null mutations thus far analyzed for the ear (FGFR1, FGFR2b, FGFR3), only FGFR2b shows major morphogenetic defects (Pirvola et al., 2000), whereas FGFR1 and FGFR3 show only histologic defects (Colvin et al., 1996; Pirvola et al., 2002). Consistent with the affinity of FGF10 for FGFR2b and the morphogenetic defects of FGFR2b, FGFR1 null mutants show morphogenetic defects as well. However, the FGF10 null ear is much further developed than the FGFR2b null mutant ear (Fig. 8). This finding suggests that other ligands also signal through FGFR2b and, thus, may partially compensate for FGF10 loss. Clearly, FGF3 expression in the anterior aspect of the otocyst (Mansour et al., 1993) could compensate for the absence of FGF10 in this area. However, no expression of FGFR3 is to be expected in the posterior canal. In the absence of other known FGFs expressed in this area, we suggest that absence of FGF10 causes dismorphogenesis of the posterior canal system by means of lack of signals through FGFR2b. This finding does not suggest that all effects of FGF10 are only mediated through FGFR2b, as certain features in the receptor mutant and the ligand mutant are mutually exclusive and suggest that FGF10 signals through at least one other FGF receptor or FGFR2 isoform (Fig. 8).

FGFs are known to interact with bone morphogenetic proteins (BMPs; Adamska et al., 2001; Revest et al., 2001; Horner et al., 2002), and BMP signaling interference results in dismorphogenesis of the semicircular canals (Chang et al., 1999; Gerlach et al., 2000). Moreover, in nervous tissue, FGFs antagonize the antineural function of BMPs (Dzie del Corral and Storey, 2001; Scully and Rosenfeld, 2002). Thus, in the absence of FGF10, no proneural signal for the formation of posterior canal hair cells might be established, resulting in lack of posterior crista formation as well as reduction of the anterior and horizontal crista. Further studies are required to show whether and how much expression of BMP-4 or BMP-4 signaling is affected and how this relates to the number of remaining hair cells in FGF10 null mutant mice. Likewise, future studies need to evaluate how the reduction or absence of cristae affects canal formation as the latter may depend on organized formation of a crista. In Otx1 null mutants, no organized horizontal crista (Fritzhch et al., 2001) or horizontal canal forms (Cantos et al., 2000).

**FGF10 and Histogenesis of the Sensory Epithelia**

Given the already established role of FGF receptors in inner ear histogenesis (Colvin et al., 1996; Pirvola et al., 2002), it is not surprising to find a role for FGF10 in the histogenesis of the remaining vestibular sensory epithelia. However, in contrast to previous work that showed effects on the differentiation of specific cellular components of the cochlea, FGF10 has a major effect on the overall orientation and form of the sensory epithelia. FGF10 null mutant mice have smaller anterior cristae. In addition, these cristae appear to be rotated 90 degrees compared with their wild-type littermates. The differentiation of sensory hair cells, known to be mediated by the bHLH gene Math1 (Bermingham et al., 1999; Fritzhch et al., 2002) and the Pou domain factor Bmph3c (Xiang et al., 1998, 2003), is largely unaffected in FGF10 null mutant mice. However, many vestibular hair cells had more or less severe alterations in their sensory cilia in the remaining canal cristae (Figs. 5, 6), whereas cochlear hair cells were normal, including their apical specializations (Fig. 6).

In addition, the cruciate eminence, a prominent nonsensory structure of the anterior and posterior crista (Fig. 5), is apparently absent or transformed in FGF10 null mice. The absence or presence of the cruciate eminence requires further elucidation by using GATA3 as a molecular marker for the cruciate eminence (Karis et al., 2001). Apparently, even the initial formation of the posterior canal crista is completely abrogated in FGF10 null mutants, whereas the formation of hair cell and supporting cells is reduced in the anterior and horizontal crista. How FGF10 interacts with the molecular processes that regulate hair cell and supporting cell formation (Zine et al., 2001; Pirvola et al., 2002) to downsize the numbers of hair cells and supporting cells remains unknown. Data on FGFR1 suggest that this may come about by regulating precursor proliferation (Pirvola et al., 2002). Such ideas are consistent with the overall stunted growth of FGF10 null mutant ears (Figs. 3, 7). Detailed analysis of cell proliferation and cell death is needed to further specify the FGF10 effects.

The interesting expression pattern of FGF10 in the developing greater epithelial ridge (GER) of the cochlea is not related to any recognizable phenotype in the FGF10 null mutant embryos. These data are consistent with recent findings on the expression of FGFR1b null mice, a major binding partner in the cochlea. These mice live to adult life and show no hearing deficits. This finding suggests that the FGF10/FGFR1b interaction is not sufficient to generate a cochlear phenotype. What function the strong and highly patterned expression of FGF10 plays in the GER awaits further analysis by using conditional mutants of the FGF10, comparable to a recent study on FGFR1b (Pirvola et al., 2002).

**FGF10 and Neurogenesis of the Ear**

FGF10 and other FGFs are expressed in the delaminating sensory neurons and, thus, could play a role in their differentiation. In addition, the early expression of FGF10 in the area of the ear from which sensory neurons delaminate (Fritzhch et al., 2002)
could possibly influence formation of sensory neurons as much as hair cell/supporting cell clones. In contrast to this possible outcome, FGF10 null mutant mice seem to form rather normal complements of sensory neurons. However, two points require further discussion: the reduced innervation of the anterior and horizontal canal and the rapid loss of innervation to the posterior canal.

Clearly, the initial formation of sensory neurons to the posterior canal shows hardly any overt reduction and fairly normal targeting (Fig. 7A,B). However, within only 2 days, all afferent fibers to the posterior canal are lost (Figs. 7, 8). These data suggest that formation of the posterior crista afferents and their initial targeting is fully independent of the posterior crista. However, consistent with losses of fibers to the posterior crista in neurotrophin and neurotrophin receptor mutant mice (Fritzsch et al., 1995; Bianchi et al., 1996), there is loss of these fibers over a similar period in the FGF10 null mutant mice. We propose that this loss in FGF10 null mutants occurs for reasons similar to the losses in brain-derived neurotrophic factor (BDNF) or trkB null mutants: lack of trophic support resulting from the lack of formation of the posterior crista. Further analysis with specific markers for the forming posterior crista such as BMP4 (Morsli et al., 1998; Fekete and Wu, 2002) and BDNF (Farinas et al., 2001) are needed to detail these suggestions.

In contrast to the effect of Otx1 on the horizontal canal, where only canal formation is affected (Cantos et al., 2000; Fritzsch et al., 2001), FGF10 appears to affect both canal morphogenesis and formation of the posterior crista. Further information on gene(s) that regulate formation of sensory neurons is needed to understand the differences between both model systems of canal dismorphogenesis.

The effects of loss of FGF10 on the pattern of anterior and horizontal crista innervation is more complex. First, from the onset there is an apparent reduction of sensory fibers growing toward these cristae (Fig. 7), suggesting that, in those endorgans, formation of hair cell clones and sensory neurons may be linked (Ma et al., 2000; Fekete and Wu, 2002). Such a link was recently proposed for all but the posterior canal crista (Fritzsch et al., 2002). Moreover, the initial pattern of innervation is retained for these sensory epithelia, suggesting that there is a target-mediated phase of sensory neuron loss. A quantitative evaluation of sensory neurons projecting fibers to the remaining canal cristae is needed to support or refute this idea.

Comparison of FGF10 With That of FGFR2b and FGFR1b Mutants

Recent data (Yu et al., 2000) have demonstrated a high affinity of FGF10 for the FGFR2b receptor isoform. FGF10 also binds with high affinity to FGFR1b (Pirvola et al., 2002). Others have shown that FGF10 null mutants affect organ development in a manner similar to an FGFR2b null mutation (Ohuchi et al., 2000). Neither the FGFR1b null mutant nor the FGF10 null mutant show major morphogenetic or histogenetic defects in the cochlea, an area of high co-expression. These data suggest that other FGFs and FGFRs can compensate for this specific ligand/receptor pair in the cochlea. More data on expression patterns of other FGF ligands and receptors are needed to firmly establish these suggestions and, if such expression is shown to exist, double null mutants will be needed to show effects. In addition, transgenic expression of suggested redundant FGF(s) under FGF10 promoter control is needed to verify the signal redundancy as recently shown for inner ear neurotrophins (Coppola et al., 2001; Farinas et al., 2001).

SUMMARY AND CONCLUSION

As expected, based on previous null mutations of FGFs or FGFRs, FGF10 plays a major role in ear morphogenesis, ear histogenesis, and, probably indirectly, in sensory neuron survival. It is possible that the differential effects of FGF10 null mutation in various sensory organs is related to partial overlap with the presence of other FGFs, thus providing in some but not all endorgans signal redundancy to counteract the antineural activity of BMPs. It is also likely that the dismorphogenesis of the canals is related to variations in BMP-mediated signaling owing to the absence of FGF10.

EXPERIMENTAL PROCEDURES

In Situ Hybridization

We analyzed the expression of FGF10 by using a FGF10 digoxigenin (Dig)-labeled riboprobe produced from a pBluescript SKII+ - FGF10 cDNA clone (GenBank accession no. NM 008002, representing nucleotides 11-597) was kindly provided by B. Hogan (Vanderbilt University Medical Center, Nashville, TN). Both sense and antisense Dig-labeled probes were generated following the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN). The whole-mount in situ hybridization followed essentially a protocol described elsewhere (Judice et al., 2002) with minor modifications. Briefly, whole embryos (E9.5-E10.5), dissected ears (E11.5-E14.5), or dissected parts of ears (P0) were defatted, digested with protease K, fixed in 4% paraformaldehyde, washed, and hybridized overnight with riboprobe. The tissues were treated with RNaseA to digest unbound probe, washed, and incubated overnight with alkaline phosphatase-labeled anti-Dig Fab fragments. After several washings, the tissues were incubated in a nitroblue phosphate/5-bromo, 4-chloro, 3-indolil phosphate (NBTBCIP) detection solution until the areas in the ear showed discernible reaction product. The time in reaction solution varied to ensure complete detection, even for weak signal. After the reaction for alkaline phosphatase detection, ears or ear parts were mounted flat in glycerol and viewed in a Nikon Eclipse 800 microscope using DIC to indicate boundaries. Images were grabbed and further processed with ImagePro and Coreldraw software. At least six ears were analyzed for each time point.
Breeding of FGF10 Null Mice

FGF10 heterozygotic founder pairs were generously provided by Dr. W.S. Simonet (Amgen, Inc., Thousand Oaks, CA). Generation of the null mutant mice was done in general as outlined elsewhere (Min et al., 1998). Genotyped FGF10 heterozygotic animals were bred to C57 mice and the offspring genotyped by using the following primers: FGF10KO-5' (5'-CACCAAGAACG-GAGCCGATG-3'), FGF10WT-5' (5'-CATGTCCTAGCCTTTCCCG-3'), and FGF10com-3' (5'-ACTCTTTGCG-CTCTATCTAG-3'). The amplification protocol was a initial step of 2 min at 94°C; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; and a final step at 72°C for 7 min. The expected product sizes for the wild-type allele was 900 bp (FGF10WT-5' / H11032), FGF10 heterozygotic founder pairs were generously provided by Dr. W.S. Simonet (Amgen, Inc., Thousand Oaks, CA). Generation of the null mutant mice was done in general as outlined elsewhere (Min et al., 1998). Genotyped FGF10 heterozygotic animals were bred to C57 mice and the offspring genotyped by using the following primers: FGF10KO-5' (5'-CACCAAGAACG-GAGCCGATG-3'), FGF10WT-5' (5'-CATGTCCTAGCCTTTCCCG-3'), and FGF10com-3' (5'-ACTCTTTGCG-CTCTATCTAG-3'). The amplification protocol was a initial step of 2 min at 94°C; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; and a final step at 72°C for 7 min. The expected product sizes for the wild-type allele was 900 bp (FGF10WT-5' and FGF10com-3' primer set) and for the targeted null allele was 1,000 bp (FGF10KO-5' and FGF10com-3' primer set).

Genotyped animals were bred and FGF10 null mice were obtained at the expected Mendelian frequency (1 in 4). FGF10 null mutant embryos can be easily phenotyped by the lack of limbs and lungs, as previously described (Min et al., 1998). For embryos, timed pregnant females were killed by cervical dislocation and the embryos were dissected and either immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer or perfusion-fixed (E9.5–E12.5) or perfusion fixed (E13.5–E18.5) with 4% paraformaldehyde in 0.1 M phosphate buffer. All procedures were approved by the Creighton IACUC committee.

Light Microscopy, TEM, and SEM

The ears of two E18.5 FGF10 null embryos and two wild-type littermates each were dissected and processed for either light microscopy and TEM or SEM. Briefly, ears for scanning microscopy were fixed with 1% OsO4, dissected, critical point tried, mounted, and sputter coated. Ears were viewed with a Hitachi SEM.

For light microscopy and transmission electron microscopy, parafomaldehyde perfusion-fixed ears were post-fixed in 1% OsO4, dehydrated in a graded alcohol series, and embedded in epoxy resin. One ear each of an E18.5 and a P0 FGF10 embryo were processed for 2-µm serial sections. Serial sections were counterstained with Toluidine blue, cover-slipped, viewed, and photographed with a Nikon Eclipse 800. Selected epithelia of the other ear were cut ultrathin. Sections were collected on a copper mesh, counterstained with uranyl and lead, and viewed in Phillips CM10 TEM.

Fiber Tracing

Two ears each of aldehyde-fixed E11.5, E12.5, E14.5, and E18.5 FGF10 null mutants and the same number of littermates were used for fiber tracing by using Di inserted into the brainstem (Fritzsch and Nichols, 1993). After appropriate diffusion times, ears were dissected, mounted with glycerol, and viewed by using confocal microscopy. Z-axis stacks were collapsed to generate single whole images of the entire innervation.

In addition, fibers were also visualized by using tubulin immunocytochemistry as previously described (Fritzsch et al., 1997). Briefly, defatted ears were incubated with primary antibodies for acetylated tubulin (Sigma) followed by an horseradish peroxidase–conjugated secondary antibody. Diaminobenzidine was used as a substrate to detect the fibers. Two ears of newborn FGF10 null mutants and control littermates were immunolabeled for acetylated tubulin (Sigma) to visualize the kinocilia by using Alexa 568–conjugated secondary antibody (Molecular Probes). These ears were also labeled for actin by using Alexa 488–conjugated phalloidin (Molecular Probes) to visualize the stereocilia. These ears were whole-mounted in glycerol and viewed with a Bio-Rad Radiance 2000 confocal system mounted on a Nikon Eclipse 800.

FGFR2b Null Mutant Ears

To better understand the possible molecular interactions between FGF10 and its possible receptors, we also investigated ears of the FGFR2b null mutant mice previously described (Pirvola et al., 2000). We examined a total of 6 ears at E18.5 by using acetylated tubulin immunocytochemistry.

Viewing Whole Inner Ears

To further characterize the ear defects, two ears of two E19.5 embryos were dissected after OsO4 staining. By using this stain, the entire ears were dissected out of the cartilage and imaged as whole ears, as previously described (Retzius, 1884). Images of dissected ears were grabbed with a monochrome CCD camera on an Olympus ZSH dissecting scope and further processed by using CorelDraw software.

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