Requirements for FGF3 and FGF10 during inner ear formation

Yolanda Alvarez[1,*], Maria Teresa Alonso[1,2,*], Victor Vendrell[1,*], Laura Cecilia Zelarayan[1], Pablo Chamero[1,2], Thomas Theil[3], Michael R. Bösl[1], Shigeaki Kato[4], Mark Maconochie[5], Dieter Riethmacher[1] and Thomas Schimmang[1,†]

[1]Center for Molecular Neurobiology Hamburg, University of Hamburg, Falkenried 94, D-20251 Hamburg, Germany
[2]Instituto de Biología y Genética Molecular, Universidad de Valladolid y Consejo Superior de Investigaciones Cientificas, Departamento de Bioquímica, Biología Molecular y Fisiología, Facultad de Medicina, E-47005 Valladolid, Spain
[3]Institute for Animal Developmental and Molecular Biology, Heinrich-Heine-University, D-40225 Düsseldorf, Germany
[4]Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-Ku, Tokyo 113, Japan
[5]School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK
*These authors contributed equally to this work
[†]Author for correspondence (e-mail: schimman@epos.zmnh.uni-hamburg.de)

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Summary

Members of the fibroblast growth factor (FGF) gene family control formation of the body plan and organogenesis in vertebrates. FGF3 is expressed in the developing hindbrain and has been shown to be involved in inner ear development of different vertebrate species, including zebrafish, *Xenopus*, chick and mouse. In the mouse, insertion of a neomycin resistance gene into the *Fgf3* gene via homologous recombination results in severe developmental defects during differentiation of the otic vesicle. We have addressed the precise roles of FGF3 and other FGF family members during formation of the murine inner ear using both loss- and gain-of-function experiments. We generated a new mutant allele lacking the entire FGF3-coding region but surprisingly found no evidence for severe defects either during inner ear development or in the mature sensory organ, suggesting the functional involvement of other FGF family members during its formation. Ectopic expression of FGF10 in the developing hindbrain of transgenic mice leads to the formation of ectopic vesicles, expressing some otic marker genes and thus indicating a role for FGF10 during otic vesicle formation. Expression analysis of FGF10 during mouse embryogenesis reveals a highly dynamic pattern of expression in the developing hindbrain, partially overlapping with FGF3 expression and coinciding with formation of the inner ear. However, FGF10 mutant mice have been reported to display only mild defects during inner ear differentiation. We thus created double mutant mice for FGF3 and FGF10, which form severely reduced otic vesicles, suggesting redundant roles of these FGFs, acting in combination as neural signals for otic vesicle formation.

Key words: Fibroblast growth factor, Otic vesicle, Hindbrain, Mouse

Introduction

The formation of the vertebrate inner ear has been postulated to comprise several stages (Groves and Bronner-Fraser, 2000; Baker and Bronner-Fraser, 2001; Rinkwitz et al., 2001; Noramly and Grainger, 2002). Initially, a signal from the endomesoderm underlying the presumptive otic field initiates the induction of the otic placode. This process appears to be complemented by a signal originating from hindbrain neural tissue next to the developing placode to complete formation of the otic vesicle. Subsequently, the otic vesicle undergoes a series of morphogenetic processes, leading to the formation of distinct subcompartments, including the cochlea, the different parts of the vestibular system and the endolymphatic sac. Simultaneously, cellular differentiation leads to the generation of specialised cell types, including cochleovestibular neurons and sensory hair cells that permit correct functioning of the mature sensory organ.

Owing to their gene expression patterns and various experimental manipulations, several members of the fibroblast growth factor (FGF) gene family, including FGF2, FGF3, FGF8, FGF10 and FGF19 have been implicated in different stages of inner ear formation (Baker and Bronner-Fraser, 2001; Rinkwitz et al., 2001; Noramly and Grainger, 2002). Among these, FGF3 in particular has been the earliest candidate postulated to play a role during early inner ear development. Initially, it was proposed on the basis of its expression pattern in the developing hindbrain next to the forming inner ear placode and vesicle in mice, thus consistent with a role in inner ear induction (Wilkinson et al., 1988). Furthermore, this early hindbrain expression pattern is fundamentally conserved between different vertebrate species including avians (Mahmood et al., 1995), amphibians (Lombardo et al., 1998a) and fish (Phillips et al., 2001). This idea gained further support by experiments in which antibodies and antisense oligonucleotides, presumably directed against FGF3, blocked otic vesicle formation in chicken explants (Represa et al., 1991), although later studies have questioned the conclusions that were drawn from these experiments (Mahmood et al., 1995). Further doubt for a role in otic vesicle formation was derived from the generation of *Fgf3* mutant mice, where a

neomycin resistance (*neo*^{r}) gene was inserted into the coding region of this gene via homologous recombination in order to prevent its expression (Mansour et al., 1993). The analysis of *Fgf3* homozygous mutant mice showed that formation of the otic vesicle was unaffected, arguing against an early role of FGF3 during inner ear development. However, defects affecting the morphogenesis and differentiation of the inner ear were described, such as a loss of the endolymphatic duct leading to hydrops of the inner ear, a lack of the posterior semicircular canal and cochlear sensory neurons, and behaviors characteristic of inner ear defects. Importantly, only 50% of homozygous mutants were recovered after birth, and only very few of these animals survived to adulthood. Moreover, the inner ear phenotype described had reduced penetrance and expressivity. This could be explained by either a non-uniform genetic background, the existence of parallel signaling pathways, leaky expression of the mutant allele, or any combination of the above. Therefore, the consequences of a loss of FGF3 function for mouse inner ear development may not have been fully explored. Indeed, a role for FGF3 during early inner ear development has gained further support following its overexpression in chicken embryos, which leads to the formation of ectopic vesicles expressing otic marker genes (Vendrell et al., 2000). In the same species, FGF3 expression is also induced by another FGF family member, FGF19, which together with WNT8C and possibly FGF3 itself, act as synergistic signals to induce otic development (Ladher et al., 2000). Next to FGF3, FGF2 and FGF8 have also been shown to induce ectopic otic structures and/or expression of genes marking otic identity (Lombardo and Slack, 1998b; Adamska et al., 2001; Léger and Brand, 2002). Several recent studies have reported the requirement for both FGF3 and FGF8 function for proper formation of the otic placode and vesicle in zebrafish, demonstrating the synergistic role of a combination of different FGF family members acting in a redundant fashion during this process (Phillips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003). In mice, FGF3 has been suggested to share redundant functions with FGF10 during tooth morphogenesis (Kettunen et al., 2000). As the expression of FGF3 and FGF10 also partially overlap during otic morphogenesis, and they have been suggested to play roles in forming parallel signaling pathways (Pirvola et al., 2000), experiments examining functional relationships between these and other FGF ligands are required to dissect the complexity of FGF inputs into otic vesicle formation.

In the present study we address the potential role of FGF3 and other FGF family members, including FGF2, FGF8 and FGF10 to act as neural signals during murine inner ear formation. We also generate a new mutant allele for *Fgf3* and find that, unexpectedly, mice that lack the *Fgf3*-coding region show no apparent inner ear defects. Ectopic expression of different FGFs to anterior regions of the developing hindbrain reveals that FGF10 acts as a potent inducer of ectopic vesicles with otic character, thereby indicating its capacity to function as a neural signal during inner ear formation. A role in normal otic vesicle formation is also supported by its endogenous expression in the hindbrain next to the developing inner ear placode and vesicle. Finally, by the analysis of double mutant mice, we confirm that FGF3 and FGF10 act as redundant signals during otic vesicle formation. A similar analysis of double mutant mice for FGF3 and FGF10 has been reported recently (Wright and Mansour, 2003).

Materials and methods

Generation of transgenic mice

A genomic fragment containing the FGF3-coding region and a 3′ located 3.2 kb genomic fragment (Peters et al., 1989) were used for construction of the targeting vector (see Fig. 1A). After electroporation of ES cells (line E14.1), DNA from G418-resistant clones was digested with *Eco*RI and analysed by Southern blot using a 5′ external probe (see Fig. 1B). Two clones showing homologous recombination were injected into blastocysts to generate chimeric mice. Heterozygous *Fgf3*$^{cDNAneo/+}$ mice were mated with deleter-cre mice (Holzenberger et al., 2000) to remove the *Fgf3* cDNA and the neomycin cassette. Cre-mediated excision was confirmed by Southern blot and PCR (see Fig. 1B,C). The primer pairs used for the analysis of the wild-type, targeted and knock-out allele had the following sequences: wild-type (5′ primer, ATGGGCCTGATCTGGCTTCT-GCT; 3′ primer, TCCACCGCAGTAATCTCCAGGATGC), cDNAneo (5′ primer, ACGACGGGCGTTCCTTGCGCAGCTGTG; 3′ primer, CCTGGTACTATGGTGCTA), knockout (5′ primer, CATCTCC-ACCTCTCTCCAG; 3′ primer, CCTGGTACTAT GGTGCTA).

Mice were bred into a mixed 129×C57/Bl6 background. Genotyping of adult mice and embryonic offspring revealed the presence of genotypes at the expected Mendelian ratios. FGF10 knockout mice were genotyped by PCR according to Sekine et al. (Sekine et al., 1999). After crossing of double heterozygous mice for FGF3 and FGF10, all combinations of the expected genotypes were recovered at the expected Mendelian ratios at E9 and E10. However, no *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mutant embryos were recovered at E11 or E12.

For the generation of transgenic mice expressing FGFs in the hindbrain a plasmid containing the *EphA4* r3/r5 enhancer and a *lac*Z reporter gene was used (see Fig. 3A). Murine FGF cDNAs were amplified by PCR and cloned into the *Ehe*I site of the vector. Transgenic mice were generated and identified by PCR or β-galactosidase staining, as described previously (Theil et al., 1998). Ectopic expression of FGFs was verified by RNA in situ hybridisation. Levels of transgene expression were estimated by RNA in situ hybridization and β-galactosidase staining. Maximal levels of transgene expression were found to be similar between embryos expressing different transgenes.

Histology and RNA in situ hybridization analysis

Embryos were isolated and fixed in 4% paraformaldehyde (PFA) at 4°C between 1 and 3 days and dehydrated through a graded ethanol series. Embedding was performed using the Kulzer Histotechnique using Technovit 7100 (Heraeus). Microtome sections (6 μm) were stained with Toluidine Blue O and mounted on slides with Eukitt (Sigma). For the preparation of adult inner ears, mice were perfused with 4% PFA and isolated inner ears were decalcified using trichloroacetic acid. After a postfixation in 4% PFA inner ears were processed as described above.

RNA whole-mount in situ hybridization was essentially performed as described by Conlon and Rossant (Conlon and Rossant, 1992) using digoxigenin- and fluorescein-labelled riboprobes, which were detected by using alkaline phosphatase-coupled antibodies. For double detection, NBT/BCIP (purple) staining was always carried out first, and the antibody was stripped in 0.1 M glycine-HCl (pH 2.2). The embryos were then incubated with the other antibody and stained with INT/BCIP (red). For histological examination, embryos were postfixed in 4% PFA, embedded in gelatin and sectioned at 30 μm on a vibratome or embedded in Tissue-Tek (Sakura) and sectioned at 10 μm on a cryostat. For whole-mount RNA in situ hybridization the following probes were used: *Dlx5* (Acampora et al., 1999; Depew et al., 1999), *Sox9* (Ng et al., 1997), *Lmx1* (Failli et al., 2002), *Pax2*

(Rinkwitz-Brandt et al., 1996), *kreisler*/*Mafb* (Giudicelli et al., 2003), lunatic fringe (Morsli et al., 1998) and full-length cDNA for *Fgf3* (kindly provided by Clive Dickson). *Fgf10* expression analysis was performed using a probe corresponding to nucleotides 12-547 (kindly provided by Rosanna Dono) and a full-length cDNA (Invitrogen). As negative controls to confirm specificity and fidelity of *Fgf10* expression, *Fgf10*$^{-/-}$ mutant embryos were also used.

Results

Generation of mice lacking the entire *Fgf3*-coding region

Previous analyses of the role of FGF3 during mouse inner ear development have been based on *Fgf3*$^{neo/neo}$ mutant mice in which the *neo*^{r} gene had been inserted into exon 1b of the *Fgf3*-coding region by homologous recombination (Mansour et al., 1993). Mice homozygous for this mutation showed defects in tail formation and differentiation of the inner ear. However, the analysis of these mice has been complicated by the fact that fewer than 50% of the expected homozygous mutants were recovered postnatally, and that the observed inner ear phenotype showed variation in both penetrance and expressivity. The latter results may be explained by leaky expression of the mutant *Fgf3* allele as such expression could not entirely be excluded (Mansour et al., 1993). Therefore, to define further the in vivo function of FGF3 and to avoid any potential interference caused by remnants of its coding region and/or selectable marker genes and/or heterologous promoters we decided to generate a new mutant *Fgf3* allele devoid of all *Fgf3*-coding sequences. The sequences contained in exons 1b, 2 and 3 of the *Fgf3* gene were replaced by a cDNA encoding *Fgf3* and a *neo*^{r} gene flanked by loxP sites via homologous recombination (Fig. 1A). Subsequently, the cDNA and the *neo*^{r} gene were removed by Cre-mediated deletion between the external loxP sites present in the targeted locus to create heterozygous *Fgf3*$^{+/-}$ animals (Fig. 1A; see Materials and methods). Fidelity of the targeting event in embryonic stem cells and subsequent Cre-mediated excision was demonstrated by Southern blots and PCR (Fig. 1B,C; see and Materials and methods). By crossing heterozygous *Fgf3*$^{+/-}$ mice we produced homozygous *Fgf3*$^{-/-}$ mutant embryos that lack *Fgf3* expression as assayed through whole-mount RNA in situ hybridisation (Fig. 1D).

FGF3 mutant mice are viable and show tail defects, but normal inner ears

In contrast to *Fgf3*$^{neo/neo}$ mutants (Mansour et al., 1993), *Fgf3*$^{-/-}$ mice lacking the entire coding region for *Fgf3* were found be viable and fertile and showed no abnormal behavior. The most striking phenotype of *Fgf3*$^{-/-}$ mutants was their shortened, thickened and curved tail (Fig. 2A). This phenotype was first observed at day 11 of embryonic development (E11) and has also been described in *Fgf3*$^{neo/neo}$ mice (Mansour et al., 1993). To analyse in more detail any inner ear phenotypes in *Fgf3*$^{-/-}$ mice, we performed a histological analysis of developing ears from these mutants from the otic vesicle stage until adulthood, focusing especially on those structures that had been described as defective in *Fgf3*$^{neo/neo}$ mutant mice. At E10.75 otic vesicles of *Fgf3*$^{-/-}$ mutants appeared slightly smaller compared with age-matched wild-type littermates (Fig. 2B,C). However, all of the inner ears of *Fgf3*$^{-/-}$ mutant animals examined (*n*=60) were otherwise found to have an apparently

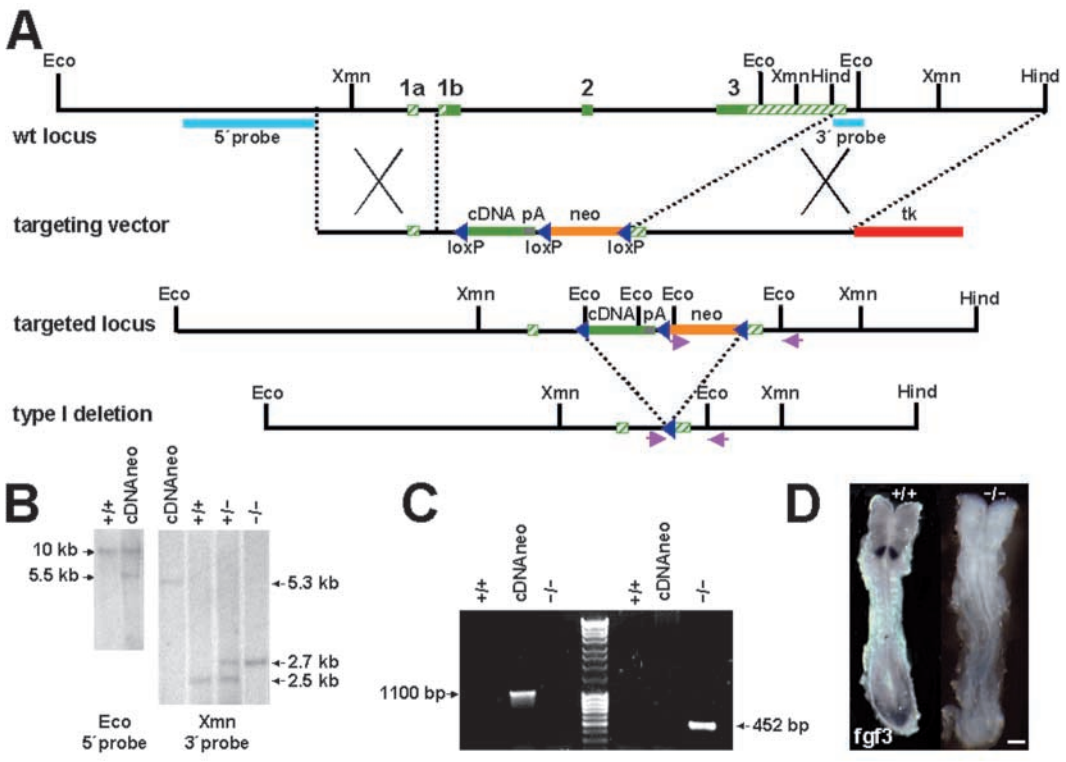

Fig. 1. Deletion of the *Fgf3*-coding region in mice. (A) The genomic locus with the exons and coding regions of the *Fgf3* gene indicated. The coding region was replaced by a FGF3 cDNA and a *neo*^{r} gene flanked by loxP sites by homologous recombination. The introduced sequences were removed by Cre-mediated excision between the external loxP sites. (B) Southern blot analysis was carried out using the probes indicated in A to detect the correct targeting event in ES cells and subsequent deletion of the introduced sequences. (C) PCR analysis using primer pairs indicated by pink arrows in A demonstrating the presence and absence of specific products amplified from the targeted locus and after generation of the knock out allele. (D) Whole-mount RNA hybridisation analysis of *Fgf3* expression in the hindbrain of an E8.5 *Fgf3*$^{-/-}$ mutant embryo and a wild-type littermate. Scale bar: 100 μm.

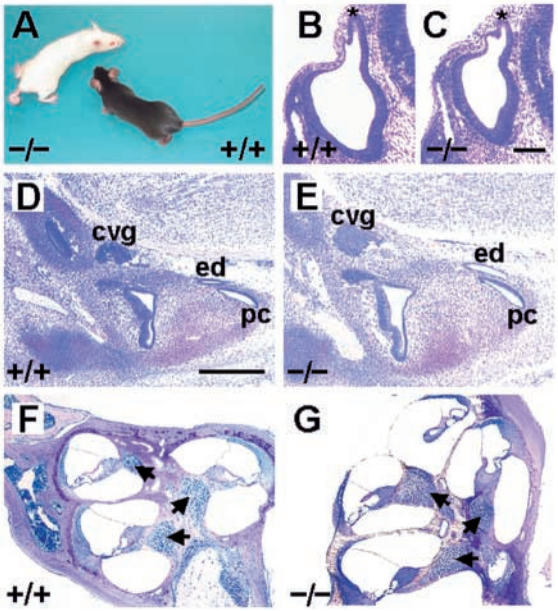

Fig. 2. Tail and inner ear phenotype of *Fgf3*$^{-/-}$ mutants. (A) *Fgf3*$^{-/-}$ adult mutants show a shortened, thickened and kinked tail. (B,C) Sections through the otic vesicle of a wild-type and a *Fgf3*$^{-/-}$ mutant littermate at E10.75. The endolymphatic duct is indicated by an asterisk. Note the reduced size of the otic vesicle in the mutant embryo compared with the wild-type control animal. (D,E) Transverse sections through the inner ear at E13.5. The cochleovestibular ganglion (cvg), endolymphatic duct (ed) and posterior semicircular canal are indicated (pc). (F,G) Sections through the cochlea of wild-type and *Fgf3*$^{-/-}$ adult mutant littermates. The cochlear ganglion is indicated by arrows. Scale bars: in C, 200 μm for B,C,F,G; in D, 200 μm for D,E.

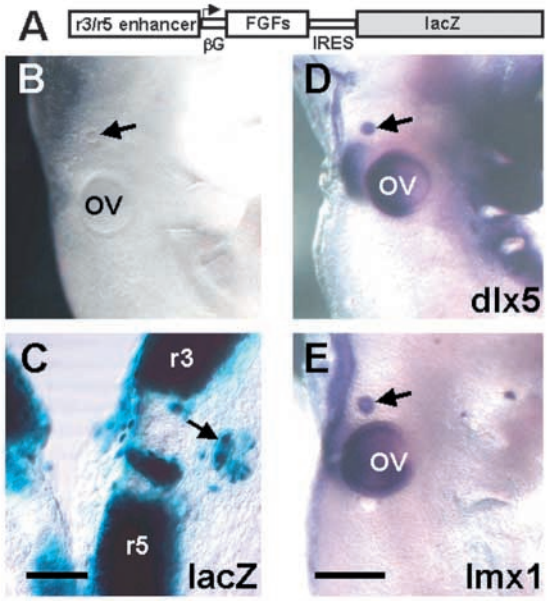

Fig. 3. Ectopic expression of FGFs and formation of ectopic otic vesicles. (A) Construct for the generation of transgenic mice. An *Epha4* r3/r5 enhancer in combination with a *β*-globin minimal promoter is used to drive expression of FGFs and an IRES-*lac*Z reporter gene in the hindbrain. (B) Formation of an ectopic vesicle (arrow) anterior to the otic vesicle (OV) in an FGF10 transgenic embryo. (C) Section through the hindbrain of an FGF10 transgenic embryo stained for *lac*Z. An ectopic vesicle (arrow) has formed next to rhombomere (r) 4. (D,E) Expression of *Dlx5* and *Lmx1* in ectopic vesicles (arrows) of FGF10 transgenic mice. Scale bars: in C, 80 μm for C and 200 μm for B,D; in E, 200 μm for E.

normal morphology, including presence of the endolymphatic duct, the posterior semicircular canal and cochlear ganglia (Fig. 2B-E). Adult homozygous mutants showed a normal Preyer's reflex and revealed no obvious structural abnormalities of the cochlea or the vestibular system (Fig. 2F,G and data not shown). Therefore, we found no evidences that deletion of the *Fgf3*-coding region has consequences on viability or on function of the inner ear.

Ectopic expression of FGF3 and FGF10 leads to formation of ectopic vesicles with otic characteristics

The lack of an inner ear phenotype in *Fgf3*$^{-/-}$ mutants suggested the involvement of other FGF gene family members during early inner ear development. Considerable evidence exists demonstrating that the hindbrain is a source for FGFs, which act as neural signals during formation of the otic placode and vesicle (Represa et al., 1991; Philipps et al., 2001; Léger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003). We were thus interested to examine the potential involvement of FGF2, FGF3, FGF8 and FGF10 during this process, as all these ligands have been implicated in formation of the otic vesicle in different vertebrates (Represa et al., 1991; Lombardo et al., 1998a; Lombardo et al., 1998b; Adamska et al., 2001; Ohuchi et al., 2000; Vendrell et al., 2000; Léger and Brand, 2002). In order to test the capacity of these factors to act as a hindbrain-derived neural signal for otic development in mammals, we used a misexpression approach by ectopically overexpressing FGFs to anterior regions of the developing murine hindbrain. The vertebrate inner ear forms adjacent to a region encompassing the posterior of rhombomere (r) 4 through r6 of the developing hindbrain. An enhancer of the *Epha4* gene has previously been used to successfully direct ectopic expression of genes to r3 and r5 (Theil et al., 1998). The timing and spatial domain of expression driven by this enhancer coincides with formation of the otic placode and vesicle, and thus offers a useful tool to test the capacity of different FGF genes to function as hindbrain-derived neural signals for formation of the murine inner ear in an embryologically appropriate location. FGF misexpression constructs also included a β-globin minimal promoter and an internal ribosomal entry site with a *lac*Z reporter gene, and these constructs were used to generate embryos for transient analysis and for the generation of stable transgenic lines (Fig. 3A). Transgenic embryos were extensively analysed between E9.5 and E10.5, a time when otic vesicle formation is normally complete. The most striking phenotype was obtained through ectopic expression of FGF10 (three independent lines). In 85% of the embryos analysed (n=67) we observed the formation of small ectopic vesicular structures which formed next to r3, r4 and r5 (Fig. 3B). Analysis of sections revealed that these structures had a morphology resembling small rudimentary otic vesicles (Fig. 3C). The otic character of these vesicles was confirmed by the expression of the *Dlx5*, *Lmx1* and *Sox9* genes (Fig. 3D,E; data

not shown), which are expressed in the inner ear placode and vesicle (Ng et al., 1997; Giraldez, 1998; Acampora et al., 1999; Depew et al., 1999; Failli et al., 2002). However, the ectopic vesicles failed to express the otic markers *Pax2* (Rinkwitz-Brandt et al., 1996) and lunatic fringe (*Lfng*) (Morsli et al., 1998), showing that they do not have the capacity to undergo the complete early developmental program required for otic vesicle formation (data not shown). This observation was also confirmed by a lack of further morphological differentiation beyond the otic vesicle stage (data not shown). In contrast to the misexpression experiments using FGF10, we obtained only one FGF3 transgenic line, which produced some ectopic structures in very few embryos from breeding of this line (three out of 63 embryos). Upon analysis of additional independent transient transgenic embryos, we found only one embryo (1 out of 15) that showed ectopic vesicles comparable with those produced in FGF10 transgenic mice (data not shown). Two independent lines ectopically expressing FGF2 were produced, but after breeding we failed to identify any embryos that showed formation of ectopic vesicular structures. Finally, for FGF8 misexpression constructs we failed to produce transgenic lines or transgenic embryos. Whether this was due to pleiotropic effects of the misexpression construct leading to reduced embryonic viability was not determined. In conclusion, these studies demonstrated that FGF10 in particular (with FGF3 to a lesser extent) shows a strong capacity to act as a potent hindbrain-derived neural signal that directs formation of ectopic vesicles with an otic character in misexpression experiments.

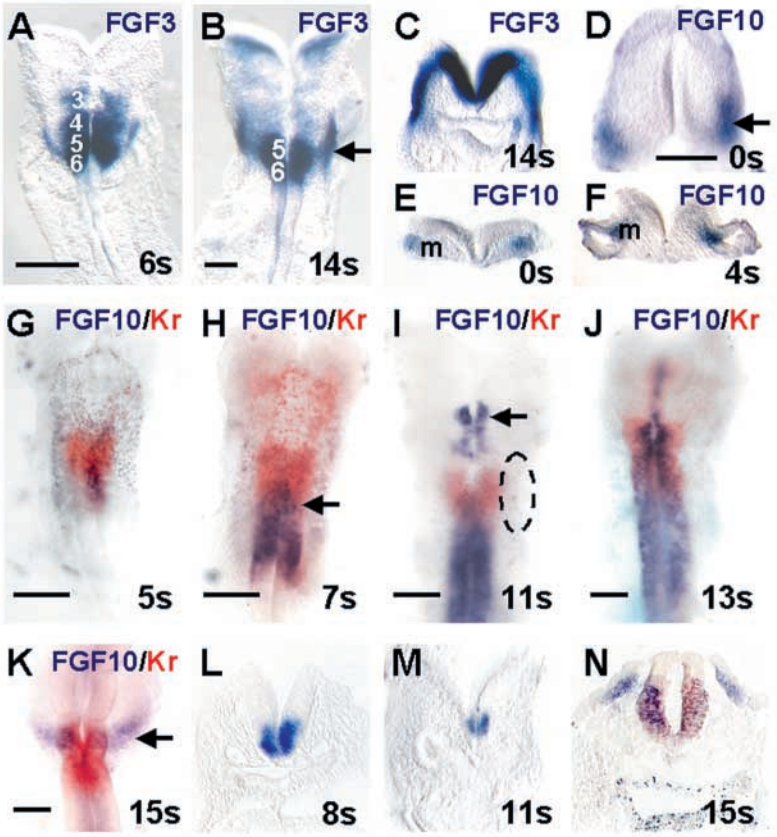

Fig. 4. Expression of *Fgf3* and *Fgf10* during inner ear formation. (A-C) Expression of *Fgf3* was detected at the stages indicated by whole-mount in situ hybridization. The position of the prospective rhombomeres and rhombomeres are indicated. The arrow in B indicates the level of the transversal section shown in C, where expression in the hindbrain and otic placode can be observed. (D-N) Expression of *Fgf10* was detected by whole-mount RNA in situ hybridisation at the stages of development indicated. (D-F) Expression of *Fgf10* is observed in the anterior mesenchyme (m). The arrow in D indicates the level of the transversal section shown in E. A transverse section at this level in a four-somite stage embryo shows that *Fgf10* expression is localized to ventral anterior mesenchyme (F). (G-N) Expression of *Fgf10* in the hindbrain. To facilitate the localisation of *Fgf10* expression relative to the position of rhombomeres 5 and 6, a probe for the *Mafb* gene was used in G-K. (L-N) Transverse sections at the levels indicated by arrows in H,I,K, respectively, show expression of *Fgf10* restricted to the ventral part of the hindbrain. The broken line in I indicates the position the otic placode. In K and N, *Fgf10* expression is also detected in the anterior part of the otic cup. Scale bars: in A, 200 μm for A; in B, 200 μm for B; in D, 50 μm for D; in G, 200 μm for G; in H, 200 μm for H; in I, 200 μm for I; in J, 200 μm for J; in K, 200 μm for K.

Expression of FGF3 and FGF10 during inner ear formation

Before and during otic placode formation, *Fgf3* is detected in the developing neuroectoderm in a broad domain that extends from the midbrain-hindbrain boundary down to r6, with the highest expression levels present in r4, r5 and r6 next to the developing otic placode (Fig. 4A) (Mahmood et al., 1996; McKay et al., 1996). Subsequently, during formation of the otic pit and vesicle, *Fgf3* transcripts are concentrated in r5 and r6 (Fig. 4B,C) (Mahmood et al., 1996; McKay et al., 1996). As FGF10 showed a strong and reproducible capacity to induce ectopic otic vesicles after overexpression in the developing hindbrain, we were interested to analyse its endogenous expression pattern during formation of the otic placode and vesicle. Between the 0 somite (s) to 4 s stage, *Fgf10* was expressed in the anterior and ventral mesenchyme (Fig. 4D-F). From the 5 s stage onwards, we detected a very dynamic expression in the developing hindbrain next to the area where the otic placode and vesicle develops (Fig. 4G-K). To facilitate the detailed localisation of *Fgf10* during early inner ear development in the developing hindbrain, we performed double in situ hybridisation of *Fgf10* with a probe for the *Mafb* gene, which is expressed at the level of r5 and r6 during formation of the hindbrain (Giudicelli et al., 2003), next to where the otic placode and vesicle are formed. Before formation of the otic placode at the 5 s and 7 s stage *Fgf10* was expressed in a domain largely posterior to the anteriormost extent, but overlapping with posterior *Mafb* expression (Fig. 4G,H). After formation of the otic placode at the 10 s and 11 s stage, this domain extended further posteriorly down the neural tube to the level of the fifth somite, and anteriorly maintained its overlapping expression with *Mafb* in r6 (Fig. 4I). During this developmental timepoint, an additional domain of *Fgf10* expression was detected in the anterior hindbrain. At the 13 s stage expression of this anterior domain and the posterior

neural tube domain were both being downregulated but *Fgf10* expression was still maintained in r6 and furthermore, now also extended into r5 (Fig. 4J). Some two somites later (15 s) *Fgf10* expression was detected in r5 and the anterior part of the invaginating otic cup (Fig. 4K). Analysis of sections at these stages showed that *Fgf10* expression was restricted to the neural tissue of the ventral hindbrain (Fig. 4L-N and data not shown). Therefore, *Fgf10* expression in the developing hindbrain coincides spatially and temporally with the formation of the murine otic placode and/or vesicle in the neighboring ectoderm, and also coincides with some of the endogenous areas of *Fgf3* hindbrain expression.

Otic vesicle formation is severely affected in double mutant mice for FGF3 and FGF10

The expression of FGF3 and FGF10 in the developing hindbrain during formation of the otic placode and vesicle suggested their potential involvement in functioning as neural signals during this process. However, as shown in the present study, FGF3 mutant mice carrying a deletion of the coding region of the gene show an apparently normal formation of the inner ear (Fig. 2) or in the second mutant allele $Fgf3^{neo/neo}$ display defects that affect only the differentiation of the otic vesicle (Mansour et al., 1993). Likewise, FGF10 mutant mice form otic vesicles, although their size appears reduced and later differentiation of the inner ear is affected (Ohuchi et al., 2000; Pauley et al., 2003). To explore the possibility that FGF3 and FGF10 may act as redundant factors during early inner ear development, we created mice that were doubly mutant for these genes. No $Fgf3^{-/-}/Fgf10^{-/-}$ mutant embryos were recovered later than E10 and we thus concentrated our analysis on stages between E8 and E10. All $Fgf3^{-/-}/Fgf10^{-/-}$ mutant embryos examined showed a severe loss of otic tissue (n=26; Fig. 5). At E8 $Fgf3^{-/-}/Fgf10^{-/-}$ embryos showed a severe reduction or absence of otic marker genes including *Dlx5* and *Pax2* (Fig. 5A-D). At E9 and E10, $Fgf3^{-/-}/Fgf10^{-/-}$ mutants had formed reduced sized otic vesicles (Fig. 5E-P). These vesicles were found in a more ventral position compared with controls and showed a complete lack of the cochleovestibular ganglion (Fig. 5E,F). The inner ear phenotype showed variable expressivity between mutant embryos and also between the two vesicles of the same embryo. In the most affected embryos, the tiny vesicles formed also showed a dramatic reduction or absence of otic marker genes, including *Pax2*, *Dlx5* and *Sox9* (Fig. 5I-N and data not shown). However, in less affected embryos, these genes, and also the otic marker *Lfng*, could be detected in their normal domains of expression (Fig. 5G,H,O,P and data not shown). Therefore, although formation of the otic placode and vesicle is severely disturbed in $Fgf3^{-/-}/Fgf10^{-/-}$ mutants, expression of otic genes and thus initial morphogenesis of the inner ear is observed in a subset of embryos.

Discussion

Inner ear phenotypes of FGF3, FGF10 and FGF receptor 2 mouse mutants

FGF3 has been proposed to act as the hindbrain-derived inducer of the vertebrate inner ear (Wilkinson et al., 1988; Represa et al., 1991). However, in the present study, we have not found any evidence that mice with a complete deletion of the *Fgf3* gene show any of the severe inner ear defects previously described in *Fgf3* mutant mice (Mansour et al., 1993). How can the lack of an inner ear phenotype in $Fgf3^{-/-}$

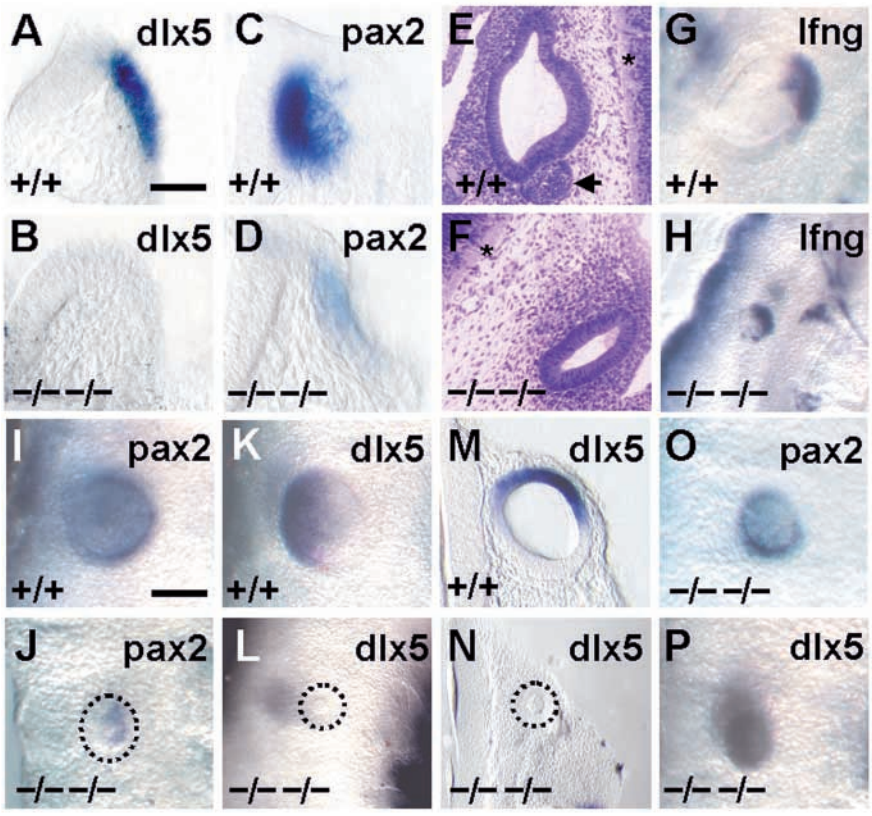

Fig. 5. Defects in inner ear formation in *Fgf3* and *Fgf10* double mutant mice. (A-D) Sections at the level of the otic placode at E8 (eight somites) and the invaginating placode (E8.75) which have been hybridized with the indicated probes. Note the absence of *Dlx5* staining in B. At E8.75, the otic placode in the mutant embryo has only initiated its invagination and shows very weak *Pax2* expression (D), whereas strong expression is detected in the otic cup formed in the wild-type embryo (C). (E,F) Toluidin Blue stained sections through the otic vesicle of a wild-type and a $Fgf3^{-/-}/Fgf10^{-/-}$ mutant littermate at E10.75. Note the absence of the cochlear ganglion in the mutant (indicated by an arrow in the wild-type animal) and a more ventralized position of the vesicle relative to the border of the neural tube (marked by asterisks). (G-P) Expression of the indicated otic marker genes by whole-mount RNA in situ hybridisation in wild-type embryos (G,I,K) and $Fgf3^{-/-}/Fgf10^{-/-}$ mutant littermates at E9.5 (H,J,L,O,P). (M,N) Sections corresponding to the embryos shown in K,L. The punctated circle in J,L,N indicates the circumference of the residual otic tissue formed in the mutants. Note the complete absence of *Dlx5* staining in the vesicle of the mutant animal in L,N. Scale bars: in A, 40 μm for A-D; in I, 100 μm for E-P.

mutants be explained? One major reason appears to be functionally redundant functions of FGF3 with FGF10. FGF3 and FGF10 have been shown to be co-expressed in the developing inner ear in several structures (Pirvola et al., 2000) (this study) and both factors are able to bind with high affinity to the IIIb isoform of FGF receptor 2 (FGFR2-IIIb) (Ornitz et al., 1996; Igarashi et al., 1998). Importantly, in the developing inner ear FGF10 is more intensely and widely expressed when compared with FGF3 (Pirvola et al., 2000) (this study). Specifically, high levels of expression of FGF10 are observed in the neurogenic region of the inner ear and the developing endolymphatic duct. Defects in these structures have been reported in FGFR2-IIIb mutant mice (Pirvola et al., 2000), but not in FGF10 mutant animals (Ohuchi et al., 2000; Pauley et al., 2003) thus indicating that FGF3 and FGF10 function mutually compensate for each other in these areas in *Fgf10*$^{-/-}$ and *Fgf3*$^{-/-}$ mutants, respectively. Indeed, *Fgf3* is also expressed in the neurogenic region (McKay et al., 1996; Pirvola et al., 2000) (N. Powles and M. Maconochie, unpublished), and the absence of a cochleovestibular ganglion in *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mutants further supports functional redundancy between these factors. However, *Fgf3* expression has not been detected in the endolymphatic duct (McKay et al., 1996; Pirvola et al., 2000). In this context, it has been postulated that FGF3 may control formation of the endolymphatic duct via its expression in the neighbouring hindbrain (Mansour et al., 1993; McKay et al., 1996). Remarkably, we also now describe expression of FGF10 in the hindbrain next to developing inner ear (Fig. 4), which may thus also influence the morphogenesis of the inner ear via this source.

Both FGFR2-IIIb and FGF10 mouse mutants develop smaller otic vesicles and show defects during further morphogenesis and differentiation (Ohuchi et al., 2000; Pirvola et al., 2000; Pauley et al., 2003). However, as the phenotype of FGFR2-IIIb mutants is more severe than the one observed in the single FGF10 knockout mice, other FGF ligands are required to control inner ear development via this receptor isoform. Additionally, the IIIc isoform of FGFR2 may also be involved during inner ear formation, as well as the ligands binding this isoform, because hypomorphs affecting all FGFR2 isoforms (Xu et al., 1998) show an otic vesicle that is even smaller than the one observed in FGFR2-IIIb mouse mutants (Pirvola et al., 2000). Finally, the severity of the inner ear phenotype observed in some of the *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ double mutants may only be explained by the interaction of FGF3 and FGF10 with additional FGF receptors next to FGFR2. In this context, it is noteworthy that FGFR1-IIIb has also been shown to act as a functional receptor for FGF3 (Ornitz et al., 1996) and FGF10 (Beer et al., 2000).

A key question raised by our results is the explanation underlying the phenotypic differences observed between the *Fgf3*$^{-/-}$ mutants described in this study and more severe phenotypes noted in *Fgf3*$^{neo/neo}$ animals (Mansour et al., 1993). In the latter strain, a postnatal loss of homozygous mutants and inner ear phenotypes had been reported. *Fgf3*$^{neo/neo}$ mutants show defects during formation of the endolymphatic duct and the cochlearvestibular ganglion that are also observed in FGFR2-IIIb mutant mice (Mansour et al., 1993; DeMoerlooze et al., 2000; Pirvola et al., 2000). Therefore, the most likely reason for the differences observed between *Fgf3*$^{-/-}$ and *Fgf3*$^{neo/neo}$ mice is that in the latter mutants, the compensatory mechanisms present in *Fgf3*$^{-/-}$ mutants (see above) are not active. Thus, variations between the genetic background of these different *Fgf3* mutant strains may well underlie the observed phenotypic differences. However, we have started to backcross mice carrying the deletion of *Fgf3* onto the Bl6 background but have so far not observed any differences from the phenotypes described in the present article. Alternatively, the contrast of this phenotype with that observed in *Fgf3*$^{neo/neo}$ mutants may be explained by the presence of the *neo*^{r} gene in the *Fgf3*$^{neo/neo}$ locus, which may influence the expression and function of neighboring genes (Lewandoski, 2001). This may then lead to the inner ear defects and/or a reduction of viability of *Fgf3*$^{neo/neo}$ mutants. To clarify this issue further we are at present creating mice in which the *Fgf3* gene has been replaced by the *neo*^{r} gene.

Consequences of ectopic expression of FGFs in the hindbrain on inner ear development

Several studies have suggested an important role for FGFs as hindbrain-derived signals controlling inner ear induction (Represa et al., 1991; Phillips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003). To address the capacity of different FGFs to direct the formation of the inner ear, we used a gain-of-function approach by ectopically expressing FGFs in the anterior hindbrain. This aim was achieved for FGF2, FGF3 and FGF10 by expressing them under the control of the *Epha4* enhancer that drives expression in r3 before and during formation of the otic placode and vesicle. Using this enhancer, we were unable to obtain transgenic mice that ectopically express FGF8. However, unlike in zebrafish (Phillips et al., 2001), FGF8 is not expressed in the hindbrain of mice (Lin et al., 2002) and is therefore unlikely to influence mouse otic development via this tissue. Nevertheless, FGF8 may still participate in inner ear development because it is transiently expressed in the otic placode of chicks (Adamska et al., 2001) and the preplacodal ectoderm in mice (Crossley and Martin, 1995). Although we obtained transgenic mice ectopically expressing FGF2, no phenotypic changes could be observed. Moreover, so far we have found no evidence for localized expression of FGF2 within the hindbrain near the otic region (Vendrell et al., 2000) and in addition, FGF2 mouse mutants show no defects during inner ear development (Dono et al., 1998). Therefore, unlike in *Xenopus* and chick embryos, FGF2 does not appear to influence otic development in mice. However, it is important to note that in the latter cases FGF2 was applied via beads implanted into the mesenchyme of the embryos, which may explain the different experimental outcomes. By contrast, we found that ectopic expression of FGF3 and FGF10 in r3 of transgenic embryos resulted in the formation of ectopic vesicles with otic character. However, the capacity of FGF10 to direct the development of these vesicles was much stronger than for FGF3. Possibly, this difference could be explained by an overlap of ectopic FGF10 expression with endogenous FGF3 expression in r3 (Mahmood et al., 1995; McKay et al., 1996), which may result in a more potent combined signal to induce ectopic vesicles in FGF10 transgenic embryos. Vice versa, owing to its endogenous expression restricted to the posterior part of the hindbrain, an overlap between FGF10 expression (Fig. 4) and ectopic FGF3 expression in r3 in FGF3

transgenic embryos does not occur during otic induction and thus may lead to a much weaker single signal for the formation of ectopic vesicles. To further address the potential cooperativity between FGF3 and FGF10, we have created double transgenic embryos containing both misexpression transgenes, but have not obtained an increased frequency of ectopic vesicle formation compared with single transgenic embryos (Y.A. and T.S., unpublished). In summary, our results show that expression of FGF10 (and to a lesser extent FGF3) in the hindbrain is sufficient to direct the formation of ectopic vesicles expressing otic markers. Similar results have been obtained in zebrafish, where ectopic otic vesicles are observed upon anterior expansion of both FGF3 and FGF8 expression in the hindbrain (Phillips et al., 2001). As suggested earlier (Léger and Brand, 2002), these results indicate that hindbrain tissue by itself may contain signals sufficient to direct the formation of the early inner ear.

Control of inner ear formation by FGFs in vertebrates

Both mesoderm and neural tissue contribute to the formation of the inner ear placode and vesicle. However, at present there is a lack of information about which molecular signals are necessary or sufficient to execute this developmental program. Our present results demonstrate that both FGF3 and FGF10 are necessary for formation of the otic vesicle in mice. Interestingly, FGF10 expression is observed in the mesoderm and hindbrain during embryonic development. At the 0 s stage, FGF10 was detected in anterior mesenchyme, which may correspond to an area where the future otic placode will be formed in the overlying ectoderm. Therefore, mesenchymal expression of FGF10 has been suggested to act as an inductive signal for inner ear formation (Wright and Mansour, 2003). However, shortly after this stage (4 s) and before the otic placode has formed, mesenchymal FGF10 expression is observed in a more ventral position (Fig. 4F), which will give rise to pharyngeal mesoderm (Kelly et al., 2001). Importantly, FGF3 is not detected in the anterior mesenchyme, but is co-expressed with FGF10 in r5 and r6 of the developing hindbrain before and during otic placode induction (see Fig. 4) (McKay et al., 1996; Mahmood et al., 1996). Additionally, FGF10, and to a lesser extent FGF3, are sufficient to induce the formation of ectopic vesicles with otic characteristics, when they are expressed ectopically in the developing hindbrain (see above). The co-expression of both genes in the developing murine hindbrain thus suggest that they may act as redundant neural signals during inner ear formation. A similar scenario is apparent in the zebrafish, where FGF3 and FGF8 are co-expressed in r4 and have been shown to control inner ear formation in a redundant fashion (Phillips et al., 2001; Maroon et al., 2002; Léger et al., 2002; Liu et al., 2003).

Loss of FGF3 and FGF8 expression in the zebrafish leads to a failure to induce the otic placode or vesicle (Phillips et al., 2001; Maroon et al., 2002; Léger and Brand, 2002; Liu et al., 2003). In this context, it was also proposed that FGF3 and FGF8 are responsible for epithelial organization of placodal cells to form the otic vesicle (Liu et al., 2003). In addition to these morphological observations, a reduction or loss of otic marker gene expression, including members of the *Pax*, *Dlx* and *Sox* transcription factor gene families was described (Phillips et al., 2001; Maroon et al., 2002; Léger and Brand, 2002; Liu et al., 2003). Interestingly, a differential dependence of transcription factors on the expression of FGF3 and FGF8 has been demonstrated (Liu et al., 2003). Specifically, expression of *sox9a* and *pax2.1* the zebrafish orthologues of mouse *Sox9* and *Pax2* are severely affected in zebrafish mutants for both FGF3 and FGF8 (Phillips et al., 2001; Maroon et al., 2002; Léger and Brand, 2002; Liu et al., 2003). In contrast to these zebrafish mutants, we consistently observe the presence of small otic vesicles in *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mouse mutants, showing that the capacity to organise an otic epithelium is still maintained in these mutants. We have examined expression of *Pax2*, *Dlx5* and *Sox9* in these vesicles and found a severe reduction or absence of expression in the most affected *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mutants. In a very recent publication, similar results have been reported for *Fgf3*$^{neo/neo}$/*Fgf10*$^{-/-}$ mutant embryos (Wright and Mansour, 2003). However, in contrast to the latter study in less affected vesicles of *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$, normal patterns of otic marker gene expression could be observed, indicating that proper inner ear morphogenesis had been initiated. As discussed above, the inner ear phenotypes observed in *Fgf3*$^{neo/neo}$ versus *Fgf3*$^{-/-}$ mutants are also likely to underlie the subtle differences found between the phenotypes of *Fgf3*$^{neo/neo}$/*Fgf10*$^{-/-}$ and *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ animals. The inner ear phenotypes of *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mouse mutants can clearly be considered less severe compared to the zebrafish mutants lacking FGF3 and FGF8. A further difference between zebrafish and mouse mutants may be also present in the hindbrain. Whereas the zebrafish mutants show a loss of hindbrain markers (Maves et al., 2002; Walshe et al., 2002), including a complete absence of *Mafb* expression in r5 and r6, we have observed an unaltered expression of this gene in the hindbrains of *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mouse mutants (Y.A., V.V. and T.S., unpublished). This indicates that although the inner ear defects caused by the absence of FGF genes in zebrafish and mouse are rather similar, there are different consequences on the hindbrain development in these species. A less severe defect in the hindbrain of *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mice may thus also explain the reduced severity of the otic phenotype. Alternatively, FGF3 and FGF10 may act in a completely different mode by directly signaling to and/or within the otic ectoderm.

Conflicting evidence exists on the inhibition of FGF signaling during inner ear induction by using an inhibitor for FGF receptors. Whereas Léger and Brand (Léger and Brand, 2002) reported a complete block of inner ear formation and otic marker genes, Maroon et al. (Maroon et al., 2002) still observed the presence of *pax8* expression which is considered as one of the first steps of otic placode induction in vertebrates. Therefore, the initial steps of inner ear development including formation of the otic placode may be independent of FGF signaling. Interestingly, recent results have shown that the zebrafish forkhead-related transcription factor *foxi* modulates FGF signaling required for inner ear formation (Nissen et al., 2003). Although its expression is independent of FGF signaling, *foxi* interacts with FGF3 and FGF8 by maintaining their expression (Nissen et al., 2003). Furthermore, inner ear formation may involve additional FGF family members. In chick, FGF4 is expressed in a region which will give rise to r4-r6 (Shamim et al., 1999; Shamim and Mason, 1999) and thus has been suggested as an additional hindbrain-derived signal because of its early co-expression with FGF3 (Mahmood et al.,

1995; Maroon et al., 2002). In mice, expression of FGF15, the orthologue of chicken FGF19, has also been observed in the hindbrain next to the developing inner ear (McWhirther et al., 1997). In chicks, FGF3, FGF4 and FGF19 are all expressed in the mesoderm underlying the prospective otic territory (Mahmood et al., 1995; Shamim and Mason, 1999; Ladher et al., 2000). The participation of the endomesoderm in inner ear induction has also been suggested by the analysis of zebrafish *one-eyed-pinhead* mutants (Mendonsa and Riley, 1999; Phillips et al., 2001). However, in a different study of these mutants, it was concluded that otic induction can largely proceed normally in the absence of cephalic endomesoderm and that signals from the hindbrain are sufficient for inner ear induction (Léger and Brand, 2002).

On the basis of the phenotype observed in *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mutants, we suggest that these FGF signals reinforce and/or maintain early inner ear induction and then subsequently participate in patterning of the otic vesicle. The potential involvement of other FGFs in the endomesoderm and hindbrain, and any redundant functions shared with FGF3 or FGF10 will now similarly have to be further addressed during inner ear induction in mice.

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