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Differential requirements for FGF3, FGF8 and FGF10 during inner ear development

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Abstract

FGF signaling is required during multiple stages of inner ear development in many different vertebrates, where it is involved in induction of the otic placode, in formation and morphogenesis of the otic vesicle as well as for cellular differentiation within the sensory epithelia. In this study we have looked to define the redundant and conserved roles of FGF3, FGF8 and FGF10 during the development of the murine and avian inner ear. In the mouse, hindbrain-derived FGF10 ectopically induces FGF8 and rescues otic vesicle formation in *Fgf3* and *Fgf10* homozygous double mutants. Conditional inactivation of *Fgf8* after induction of the placode does not interfere with otic vesicle formation and morphogenesis but affects cellular differentiation in the inner ear. In contrast, inactivation of *Fgf8* during induction of the placode in a homozygous *Fgf3* null background leads to a reduced size otic vesicle or the complete absence of otic tissue. This latter phenotype is more severe than the one observed in mutants carrying null mutations for both *Fgf3* and *Fgf10* that develop microvesicles. However, FGF3 and FGF10 are redundantly required for morphogenesis of the otic vesicle and the formation of semicircular ducts. In the chicken embryo, misexpression of *Fgf3* in the hindbrain induces ectopic otic vesicles in vivo. On the other hand, *Fgf3* expression in the hindbrain or pharyngeal endoderm is required for formation of the otic vesicle from the otic placode. Together these results provide important insights into how the spatial and temporal expression of various FGFs controls different steps of inner ear formation during vertebrate development.

Keywords: Fibroblast growth factor; Otic vesicle; Otic placode; Mouse; Chicken

Introduction

Induction of the otic placode is controlled by signals from the endoderm, mesoderm and neural ectoderm. Whereas the endoderm and mesoderm contain the initial signals for placode induction, the neural ectoderm (i.e. the adjacent hindbrain) is thought to complement these signals by directing later stages such as placode maintenance and its invagination to form the otic vesicle. The otic vesicle then undergoes a complex process of morphogenesis and differentiation leading to the formation of the mature inner ear containing sensory epithelia innervated by the cochleovestibular ganglion. In the auditory sensory epithelium sound is transduced by inner and outer sensory hair cells which are embedded between supporting cells. Members of the fibroblast growth factor (FGF) family are key signals during multiple stages of inner ear development required for otic placode induction, otic vesicle formation and its subsequent morphogenesis and differentiation leading to formation of the mature inner ear. During the early stages of inner ear deve-

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lopment, placode induction and vesicle formation, FGF3 appears to play a highly conserved role across different vertebrates, including the mouse, chicken and zebrafish (Baker and Bronner-Fraser, 2001; Noramly and Grainger, 2002, Whitfield, 2002, Brown et al., 2003, Riley and Phillips, 2003, Barald and Kelley, 2004, Groves, 2005; Schlosser, 2006 and summarized below).

In mouse, the restricted expression of FGF3 in the developing hindbrain during and prior to otic placode induction (Wilkinson et al., 1988) implicated FGF3 as the inducer of the inner ear. This notion was reinforced by a similar expression pattern in chick and by the demonstration that antisense oligonucleotides and antibodies, directed against human FGF3, blocked the formation of the otic vesicle in chick embryo explants (Represa et al., 1991). Later studies did question if the oligonucleotides used in this study were able to specifically target *Fgf3* expression since they contained several mismatches to the chicken *Fgf3* cDNA sequence (Mahmood et al., 1995) and indicated that further experiments were required to resolve the inductive role of *Fgf3* in the chick. There appeared to be some considerable doubt to this inductive role in mouse as well since analysis of the two different *Fgf3* mutant alleles generated to date revealed that otic vesicles are formed during development (Mansour et al., 1993; Alvarez et al., 2003). Recently, it has been shown that FGF3 is redundantly required together with FGF10 to direct the expression of otic marker genes in the developing otic placode of $Fgf3^{-/-}/Fgf10^{-/-}$ double mutants, and such mutants fail to form the otic vesicle or only develop microvesicles (Alvarez et al., 2003; Wright and Mansour, 2003).

Interestingly, FGF10, and to a much lesser degree FGF3, induces the formation of ectopic otic vesicles when misexpressed in the developing mouse hindbrain during the period of inner ear induction (Alvarez et al., 2003). In addition, loss-of-function mouse mutants for FGF10 develop smaller sized otic vesicles and fail to develop the semicircular ducts of the vestibular system (Ohuchi et al., 2000, 2005; Pauley et al., 2003). Recently, mouse mutant embryos carrying a hypomorphic and a null allele for FGF8 on an FGF3 homozygous mutant background ($Fgf3^{-/-}/Fgf8^{H/-}$) have been shown to develop a very similar phenotype to $Fgf3^{-/-}/Fgf10^{-/-}$ double mutants (Ladher et al., 2005). Unfortunately, the use of a hypomorphic allele for FGF8 does not allow the demonstration of exactly when FGF8 is redundantly required together with FGF3 for otic vesicle formation. During inner ear induction, *Fgf8* is expressed in the endoderm and mesoderm underlying the future otic placode and in the preplacodal ectoderm where the placode will develop (Crossley and Martin, 1995; Ladher et al., 2005). During later development, *Fgf8* expression is also observed in the inner hair cells (IHCs) of the cochlear sensory epithelium (Pirvola et al., 2002; Shim et al., 2005) where it is postulated to control the formation of neighboring supporting cells called pillar cells (PCs; Mueller et al., 2002; Shim et al., 2005).

A conserved role in inner ear induction in chick embryogenesis was suggested by the antibody/oligonucleotide blocking experiments of Represa et al. mentioned above (Represa et al., 1991). This was further supported by overexpression of *Fgf3* in the surface ectoderm of chicken embryos, leading to the formation of ectopic placodes and vesicles expressing otic markers (Vendrell et al., 2000). However, these experiments were performed at a stage when *Fgf3* expression is not observed in the surface ectoderm, but rather is found in the hindbrain adjacent to the area where the otic placode develops (Mahmood et al., 1995; Kil et al., 2005). Thus, the exact role of hindbrain FGF3 expression in inner ear development has not been addressed.

In the present study we further define the sufficiency and unique or redundant requirements of several FGF family members for different steps of vertebrate inner ear development. Thus, hindbrain-derived FGF10 rescues otic vesicle formation in FGF mouse mutants deficient for this process and is able to induce ectopic *Fgf8* expression. On the other hand, misexpression of *Fgf3* leads to ectopic expression of otic markers in the hindbrain. Inactivation of *Fgf8* during different timepoints of mouse development does not affect otic vesicle formation but affects differentiation of PCs in the cochlear sensory epithelium. However, FGF8 is redundantly required with FGF3 for otic vesicle formation during, but not after inner ear placode induction. The combined loss of FGF3 and FGF8 during inner ear induction is more detrimental for otic vesicle formation than noted in the previously reported combined inactivation of FGF3 and FGF10 (Alvarez et al., 2003; Wright and Mansour, 2003). However, FGF3 and FGF10 are crucially involved in the morphogenesis of the vestibular system. In the chicken, *Fgf3* expression in the hindbrain is able to induce ectopic otic vesicles, whereas its inactivation impairs invagination of the otic placode to form the otic vesicle. A similar phenotype is also observed upon blocking *Fgf3* expression in the pharyngeal endoderm. The central role of FGF3 acting alone or together with other FGF family members in different tissues and cell types of the developing embryo to control inner ear formation and differentiation is discussed.

Materials and methods

Transgenic mice

The following mouse lines used in this study have been described previously: $Fgf3^{-/-}$ and $Fgf10^{-/-}$ knockout mutants and transgenic mice expressing *Fgf3* or *Fgf10* under the control of the *EphA4* enhancer (Alvarez et al., 2003), mutants carrying a conditional ($Fgf8^{flox}$) or a null allele ($Fgf8^{d2,3}$) for *Fgf8* (Meyers et al., 1998), mouse lines in which *cre* has either been targeted to the *Foxg1* (*BF-1*; Hebert and McConnell, 2000), or *Mox2* (Tallquist and Soriano, 2000) locus, transgenic mice which express *lacZ* under the control of *Fgf3* regulatory sequences (Powles et al., 2004) and the ROSA26 Cre reporter strain (Soriano, 1999).

Histology, RNA in situ hybridization, β-galactosidase staining and paint-fillings of inner ears

Preparation of histological sections stained with Toluidine Blue O, β-galactosidase staining, RNA whole-mount in situ hybridization and the sectioning of stained embryos have been described previously (Alvarez et al., 2003). Sections from embryos stained for β-galactosidase activity were counterstained with hematoxylin and eosin. The riboprobes corresponding to chicken *Fgf3* (Aragon et al., 2005) and murine *Fgf8* have been described (Crossley and Martin, 1995). All other riboprobes used in this study have been