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Expression and functions of FGF ligands during early otic development

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ABSTRACT Classical studies have postulated the action of an endomesodermal signal initiating inner ear induction, subsequently followed by a neural tube-derived signal to complete the process of otic placode formation in the surface ectoderm. Members of the Fibroblast growth factor (FGF) gene family have been implicated in these processes. In this review, expression analysis and recent experimental evidence for candidate inner ear FGF ligands during inner ear induction is discussed. Careful examination of the spatiotemporal expression patterns of different FGFs during inner ear induction reveals that the sequential appearance of FGF members in the endoderm and/or mesoderm is followed by expression in the posterior hindbrain in all vertebrate species analysed to date. Experimental manipulations have demonstrated the sufficiency and/or necessity of some FGFs during different steps of inner ear induction *in vitro* and *in vivo*. Combining the advantages of the molecular tools and approaches available in different experimental systems such as zebrafish, chicken or mouse will eventually lead to a complete understanding of how FGFs control inner ear induction in vertebrates.

KEY WORDS: *inner ear, otic placode, fibroblast growth factor, otic induction*

Inner ear induction is already initiated during gastrulation by endomesodermal tissue which comes to underlie competent ectoderm. During neurulation, a second inducing neural signal from the presumptive hindbrain reinforces and maintains inner ear induction. The initial classical embryology experiments, addressing the sufficiency and necessity of different tissues during inner ear induction, have in more recent times been combined with molecular probes and tools to begin to build a molecular framework explaining different steps of inner ear induction (Baker and Bronner-Fraser, 2001; Noramly and Grainger, 2002; Groves, 2005). Members of the Fibroblast growth factor (FGF) gene family are among the prime candidates to control inner ear induction since they show a spatiotemporal expression pattern consistent with playing a role during this process. Secondly, their inductive capacities and necessity during embryonic patterning and the formation of various organ systems underscores their potential to also participate during the early phases of inner ear formation (Reuss and von Bohlen und Halbach, 2003; Bottcher and Niehrs, 2005; Thisse and Thisse, 2005a). In this review the expression patterns of FGFs and the recent experimental evidence for their participation during inner ear induction is reviewed.

Expression of FGFs during inner ear induction

In this first section the spatiotemporal expression patterns of FGFs during inner ear induction in different vertebrate models is described. Following the experimental evidence that a first inductive signal for otic placode formation is present in the endomesoderm we refer to this phase as the initiation of induction (Fig. 1A,D). The second phase of the induction process is initiated by a neural signal from the developing hindbrain and is complete by the onset of placode formation (Fig. 1B,E). Finally, we refer to FGFs expressed at the moment when the otic placode has completed its formation and starts its invagination (Fig. 1C,G). During inner ear induction in the chicken these three phases roughly correspond to the periods before (Fig. 1A) and after (Fig. 1B) the specification of part of the preplacodal domain to form the otic placode and after the commitment to the otic fate (Fig. 1C; Groves and Bronner-Fraser, 2000; Bailey and Streit, 2006)

Chicken FGF expression

During initiation of inner ear induction in chicken embryos FGF8 and FGF19 are the first FGF family members detected at the 0 somite stage (ss, stage HH6 after Hamburger and Hamilton, 1992; Ladher *et al.*, 2000; Ladher *et al.*, 2005). *Fgf8* is expressed in the endoderm whereas *Fgf19* is detected in mesoderm that underlies the preplacodal ectoderm (Fig. 1A). The exact temporal order of expression of both FGFs is at present unclear although one study defines the onset of *Fgf*19 expression slightly later at the 1ss (HH7; Kil *et al.*, 2005). At HH7 also *Fgf3* expression is first detected in the mesoderm where it is coexpressed with *Fgf19* (Fig. 1A; Kil *et al.*, 2005). At this stage *Fgf3*

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transcripts have also been described in the unsegmented hindbrain just anterior to the first somite (Mahmood *et al.*, 1995), although this domain may correspond to the *Fgf3* hybridisation signal from the underlying mesoderm (Kil *et al.*, 2005). Expression of *Fgf8*, *Fgf3* and *Fgf19* are maintained in the endoderm and mesoderm, respectively, during the following phase of induction until 7ss (HH9) at around the stage when the otic placode can first be visualized (Fig. 1B; Ladher *et al.*, 2000; Karabagli *et al.*, 2002; Brown *et al.*, 2003; Kil *et al.*, 2005). During this period, additional expression domains for *Fgf3* and *Fgf19* are now also apparent in the endoderm and developing hindbrain. In the pharyngeal endoderm *Fgf8* is accompanied almost simultaneously by *Fgf3* (5ss) and *Fgf19* (6ss) expression (Mahmood *et al.*, 1995; Wright *et al.*, 2004; Ladher *et al.*, 2005). Even earlier, *Fgf3* is clearly detected in the hindbrain from the 3-4ss onwards and is thus turned on before the earliest described otic placode marker, *Pax2,* is specified at the 4-5ss (Groves and Bronner-Fraser, 2000; Kil *et al.*, 2005). Upon morphogenesis of hindbrain rhombomeres (r) at 7ss, *Fgf3* expression is observed in r4 and r5 (Mahmood *et al.*, 1995). Finally, from the 5ss until 9ss, *Fgf19* is transiently observed in the posterior ventral hindbrain (Ladher *et al.*, 2000; Wright *et al.*, 2004; Kil *et al.*, 2005). Interestingly, expression of FGF family members has not been detected in the preplacodal ectoderm so far (Fig. 1B).

When the otic placode has formed and starts to invaginate, the *Fgf3* expression domain in the hindbrain is maintained but also extends to include r6 at 10ss (Fig. 1C, Mahmood *et al.,* 1995). Likewise, at this stage *Fgf3*, *Fgf8* and *Fgf19* expression is still observed in the pharyngeal endoderm (Hidalgo-Sanchez *et al.*, 2000; Ladher *et al.*, 2000; Adamska *et al.*, 2001; Karabagli *et al.*, 2002; Stolte *et al.*, 2002; Wright *et al.*, 2004). Moreover, the otic placode itself now shows abundant transcripts for *Fgf10,* as well as more weak expression for *Fgf8* (Adamska *et al.*, 2001; Karabagli *et al.*, 2002).

Mouse FGF expression

In mouse embryos *Fgf8* is already detected at embryonic day 7 (E7) in the heart mesoderm underlying the neural plate (Ladher *et al.*, 2005), although the relevance of this *Fgf8* expression domain for otic induction is uncertain (Fig. 1D). At E8 (0ss) *Fgf8* expression is detected in the splachnic mesoderm and at the 3ss all mesenchyme underneath the preplacodal ectoderm shows *Fgf8* transcripts (Ladher *et al.*, 2005). The potential influence of this expression domain on otic induction is underlined by the finding that *Fgf10* expression is also observed in this mesenchyme from the 0ss stage onwards (Fig. 1D; Alvarez *et al.*, 2003; Wright and Mansour, 2003a). During E8, *Fgf10* expression is maintained in the mesenchyme whereas *Fgf8* transcripts are now also transiently observed in the preplacodal ectoderm and the pharyngeal endoderm from the 4 to 8ss (Fig. 1E; Crossley and Martin, 1995; Ladher *et al.,* 2005). Around the same time (3ss), *Fgf3* expression is first detected in the hindbrain and preplacodal ectoderm (Wright and Mansour, 2003a). *Fgf3* expression is initially detected as a stripe in the presumptive posterior hindbrain, but from the 5ss onwards broadens rostrally and reaches the level of r1 (Mahmood *et al.*, 1996; McKay *et al.*, 1996; Alvarez *et al.*, 2003; Wright and Mansour, 2003a; Powles *et al.*, 2004). During this period, *Fgf10* expression is first observed in neural tissue in the ventral part of the posterior hindbrain (Fig. 1E; Alvarez *et al.,* 2003).

As placode formation proceeds, *Fgf8* disappears from the placodal ectoderm, but some transcripts are still observed in the ventral surface ectoderm, pharyngeal endoderm and intervening mesoderm between 8 to 12ss (Fig. 1F; Ladher *et al.,* 2005; Park *et al.,* 2006). Shortly before placode invagination at 10ss, *Fgf3* transcripts are now also seen in the pharyngeal endoderm (Mahmood *et al.*, 1996; McKay *et al.*, 1996). In the neural domain relevant to inner ear induction, *Fgf3* is maintained in the developing hindbrain with strongest expression observed in r5 and r6 while it becomes downregulated in the otic placode as it starts to invaginate around 12ss-13ss (Fig. 1F; McKay *et al.,* 1996; Wright and Mansour, 2003a). During this period *Fgf10* expression is initiated in the invaginating placode and the pharyngeal endoderm, where in the latter case it also accompanies the expression of *Fgf3* and *Fgf8* (Pirvola *et al.*, 2000; Alvarez *et al.*,

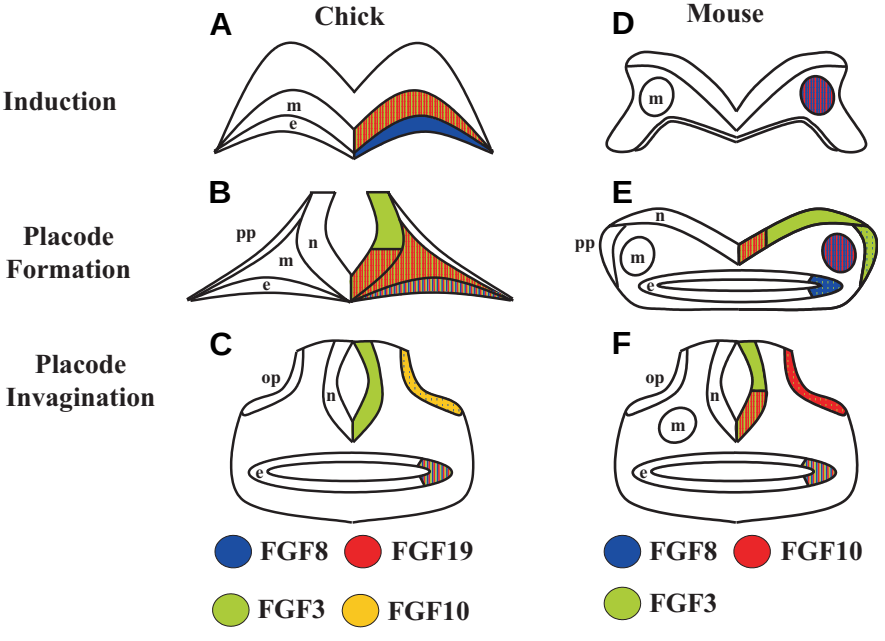

Fig. 1. Expression of FGFs during otic placode induction in chick and mouse. *Schematic sections of embryos taken at the level where inner ear induction takes place in chicken and mouse. The presence of the different FGF members is indicated by the colour code at the bottom of the figure.* **(A,D)** *Expression of* Fgfs *in the endoderm and/or mesoderm is observed during the first phase of induction.* **(B,E)** *A second phase of induction is defined by the onset of* Fgf *expression in neural tissue together with the initiation of otic placode formation in the surface ectoderm.* **(C,F)** *Finally, a third phase is characterized by the completion of placode formation and the initiation of placode invagination, a period when expression of some* Fgfs *expressed during placode induction is still maintained whilst other* Fgfs *initiate their expression in the placode itself or in the endoderm. For details, see text. Abbreviations: e, endoderm; m, mesoderm; n, neural tube; op, otic placode; pp, preplacodal ectoderm.*

2003; Wright and Mansour, 2003a). While *Fgf10* transcripts in the mesenchyme have diminished, *Fgf10* expression in the ventral part of r5 and r6 that flank the invaginating placode is still evident (Alvarez *et al.*, 2003; Wright and Mansour, 2003a).

Mouse FGF15 has been identified as the ortholog of chick FGF19. Unlike *Fgf19*, *Fgf15* is not expressed in the mesoderm but in the neuroectoderm from 0ss throughout all phases of otic induction and placode formation. Moreover, it is detected in the preplacodal ectoderm at 8ss and the pharyngeal endoderm from 13ss onwards (Wright *et al.*, 2004).

Zebrafish, Medaka and *Xenopus* FGF expression

In contrast to the situation in chicken and mouse where several *Fgfs* are expressed in a dynamic manner in the different tissues involved during otic induction, in the zebrafish only two FGF members have been detected during this process. *Fgf3* and *Fgf8* are coexpressed in several tissues implicated in otic induction. At 50% epiboly *Fgf3* is expressed in the germring, at 75% epiboly in the prechordal plate, followed by expression in the anlage of r4 and the paraxial cephalic mesoderm by 80% epiboly and early segmentation stages (Phillips *et al.*, 2001; Maroon *et al.*, 2002; Nechiporuk *et al.*, 2007). *Fgf8* shows a very similar expression pattern to *Fgf3* in the germring and r4, but is not observed in the prechordal plate until the 6ss (Phillips *et al.*, 2001; Maves *et al.*, 2002; Walshe *et al.*, 2002). Weak detection of *Fgf8* in the paraxial cephalic mesoderm has been reported at 80% epiboly followed by stronger expression during early segmentation (Reifers *et al.*, 2000; Thisse *et al.*, 2001; Nechiporuk *et al.*, 2007; Nikaido *et al.*, 2007). The spatiotemporal expression domains of *Fgf3* and *Fgf8* have been graphically outlined in detail by Phillips *et al.* (2001) and Whitfield *et al.* (2002). While no evidence has been obtained for the expression of *Fgf10* and *Fgf19* during early otic induction, both these FGFs have been detected in the otic placode itself (Miyake *et al.*, 2005; Thisse and Thisse, 2005b).

In Medaka, *Fgf3* and *Fgf8* are also coexpressed in a stripe in the posterior hindbrain from the end of gastrulation (stage 18) until the 6ss (stage 21, Hochmann *et al.*, 2007). In *Xenopus*, *Fgf3* expression is present from the late gastrula (Stage 12,5) and throughout neurulation in r3, r4 and r5 (Lombardo *et al.*, 1998). *Fgf8* expression has not been described in the posterior hindbrain during neurulation, but is present as a horseshoe-shaped stripe corresponding to the placodal region of neuroectoderm adjacent to the developing neural tube (Christen *et al.*, 2003; Fletcher *et al.*, 2006).

Functional analysis of FGFs during inner ear induction

The experimental evidence for the involvement of FGF members during early inner ear development is discussed below. Key experiments and their results are summarized in Table 1.

TABLE 1

KEY EXPERIMENTS ADDRESSING FGF FUNCTION DURING INNER EAR INDUCTION

Type of experiment	Outcome	References
Chicken		
Loss-of-function of FGF8 (si RNA at stage 4)	Reduced or absent placode and Pax2 expression	Ladher *et al.,* 2005.
Ectopic FGF8 (beads, stage 5 mesoderm)	Induction of FGF19	Ladher *et al.,* 2005.
Ectopic FGF19 (beads, stage 5 ectoderm, stage 7 non-otic tissue including neural tissue)	Induction of FGF3 and otic markers	Ladher *et al.,* 2000.
Inhibition of FGF receptor signalling (SU5402)	Block of Pax2 expression (until 4ss)	Martin and Groves, 2006.
Loss of FGF3 and FGF19 in posterior hindbrain (RA-deficient quail)	Formation of otic vesicle unaffected	Kil *et al.,* 2005.
Expression of FGF3 and FGF19 in neural tube (RA-deficient quail)	Expansion of otic placode and ectopic otic vesicles	Kil *et al.,* 2005.
Loss-of-function of FGF3 in hindbrain or pharyngeal endoderm (si RNA at stage HH8)	Block of otic vesicle formation	Zelarayan *et al.,* 2007.
Ectopic FGF3 (viral overexpression in surface ectoderm and electroporation in hindbrain at HH8)	Formation of ectopic otic vesicles	Vendrell *et al.,* 2000; Zelarayan *et al.,* 2007.
Mouse		
Ectopic FGF10 (FGF3) (hindbrain, transgenic)	Formation of ectopic otic vesicles	Alvarez *et al.,* 2003.
FGF3 knockout	Reduced size of otic vesicle	Mansour *et al.,* 1993; Alvarez *et al.,* 2003.
FGF10 knockout	Reduced size of otic vesicle	Ohuchi *et al.,* 2000.
FGF15 knockout	Fomation of otic vesicles	Wright *et al.,* 2004.
FGF3/FGF10 double knockout	Loss of otic vesicle or microvesicles	Alvarez *et al.,* 2003; Wright *et al.,* 2003.
FGF3/FGF8 double knockout (FGF8 hypomorph or conditional allele)	Loss of otic vesicle or microvesicles	Ladher *et al.,* 2005; Zelarayan *et al.,* 2007.
Fish and *Xenopus*		
Loss-of-function of FGF3 or FGF8 (morpholino injection, mutants)	Reduced size of otic vesicle and otic marker expression	Phillips *et al.,* 2001; Léger *et al.,* 2002; Maroon *et al.,* 2002; Liu *et al.,* 2003.
Transplantation of wild-type cells in hindbrain of FGF8 mutant at shield stage	Rescue of Pax2 expression	Léger *et al.,* 2002.
Ectopic FGF3 and FGF8 (RA treatment, plasmid)	Formation of ectopic otic vesicles	Lombardo *et al.,* 1998; Phillips *et al.,* 2001; Bajoghli *et al.,* 2004; Phillips *et al.,* 2004; Solomon *et al.,* 2004; Hans *et al.,* 2007.
Loss-of-function of FGF3 and FGF8 (morpholino injections, mutants)	Loss of otic vesicle and otic marker expression	Phillips *et al.,* 2001; Léger *et al.,* 2002; Maroon *et al.,* 2002; Liu *et al.,* 2003.
Inhibition of FGF receptor signalling (SU5402)	Block of Pax2 expression and otic vesicle formation	Léger *et al.,* 2002; Maroon *et al.,* 2002.

Functional analysis of FGFs in chicken inner ear induction

The FGF family members that have been shown to act first during chicken inner ear induction are FGF8 and FGF19. The functional significance of their expression domains in the endoderm and mesoderm, respectively and their interactions have been addressed in two studies by Ladher *et al.* (Ladher *et al.*, 2000; Ladher *et al.*, 2005). FGF8 has been shown to be capable of inducing FGF19 in mesoderm isolated at HH5 (Ladher *et al.*, 2005). Vice versa, electroporation of siRNA directed against *Fgf8* in HH4 embryos results in the loss of *Fgf19* expression at HH7 as well as loss of *Pax2* expression and placodal tissue by HH12-14. Loss of *Pax2* expression can be rescued by FGF19 in explant cultures derived from embryos electroporated with siRNA directed against *Fgf8*. Therefore, *Fgf8* expression in the endoderm is sufficient and necessary for *Fgf19* expression in the mesoderm and suggests that FGF8 acts via FGF19 during otic induction.

The involvement of FGF signalling between 0-4ss (HH6-8) has also been tested by blocking FGF receptors with SU5402 resulting in a loss of some (e.g. *Pax2*) but not all otic markers (Martin and Groves, 2006). Vice versa, FGF2, which is able to activate several isoforms of the four FGF receptors, induces otic markers in isolated non-otic ectoderm at this stage. In contrast, FGF19 by itself is not able to induce otic markers in presumptive otic or non-otic regions at these stages, but does so in the presence of neural tissue (Ladher *et al.*, 2000; Martin and Groves, 2006). *Fgf3* is present in mesodermal and neural tissue during otic induction and is induced by FGF19, but its sufficiency to induce otic markers at these stages has not yet been tested (Ladher *et al.*, 2000; Kil *et al.*, 2005).

Wnt8c is expressed in the neuroectoderm overlying *Fgf19* expressing mesoderm at HH7 and is induced by FGF19 in unspecified stage 5 ectoderm. Wnt8c on the other hand induces *Fgf3* and weak expression of otic markers in isolated presumptive otic ectoderm at HH7, whereas the combination of both Wnt8c and FGF19 together induce strong expression of otic markers (Ladher *et al.*, 2000). From these results it was proposed that FGF19 from the mesoderm and Wnt8c from neural tissue act as synergistic signals for otic induction. The influence of *Fgf3*, *Fgf19* and *Wnt8c* expression on otic induction has also been recently addressed in vitamin A deficient (VAD) quails (Kil *et al.*, 2005). In this experimental system, *Fgf3* and *Fgf19* expression is still present in the mesoderm whereas the posterior hindbrain is lost and expression of *Fgf3*, *Fgf19* and *Wnt8c* has shifted caudally. Since the otic placode is still induced under these conditions, it was concluded that expression of these genes in the posterior hindbrain is not required during otic induction, whereas the expression of *Fgf3* and *Fgf19* in the mesoderm may be necessary. Nevertheless, the posterior hindbrain still has inducing activity stimulating otic placode formation since VAD embryos have a caudally expanded otic placode, possibly due to the posteriorly shifted expression domains of *Fgf3*, *Fgf19* and *Wnt8c* in the neighbouring neural tube (Kil *et al.*, 2005). Taken together, an alternative interpretation of the study by Ladher *et al.* (2000) might involve FGF19 and FGF3, with FGF3 being induced by both FGF19 and Wnt8c as the factors initially responsible for otic induction. However, the sufficiency or necessity for *Fgf3* expression in the mesoderm for otic induction has not yet been addressed. Likewise, the necessity for *Fgf19* during otic induction remains to be analysed by directly blocking its expression in the mesoderm using e.g. siRNA-mediated gene knockdown.

The role of FGF3 during early inner ear development has however been studied at slightly later stages, at HH8 (5ss). At this stage the placodal precursors that are initially specified as lens at HH6 (Bailey *et al.*, 2006) have already acquired otic properties and the specification of the otic placode has occurred as assessed by *Pax2* expression (Groves and Bronner-Fraser, 2000). First, the necessity for *Fgf3* expression in the neural tube for early inner ear information has been addressed by siRNA-mediated knockdown of *Fgf3 in vivo* (Zelarayan *et al.,* 2007). At HH8, strong expression of *Fgf3* is observed in the posterior hindbrain (Mahmood *et al.*, 1995) and knockdown of its expression blocks the transition from the otic placode to the otic vesicle (Zelarayan *et al.,* 2007). These results appear to confirm similar observations made when *Fgf3* expression was blocked in explants at HH10 (Represa *et al.*, 1991). At this stage the otic placode has already completed its formation and is commited to form an otic vesicle (Groves and Bronner-Fraser, 2000). Invagination of the otic placode was blocked by antibodies which were raised against an epitope present in the chicken FGF3 protein (Represa *et al.*, 1991). Similar results were obtained in the presence of anti-sense oligonucleotides directed against *Fgf3*, although the sequences used were based on human *FGF3* and thus contained several mismatches (Mahmood *et al.*, 1995). Nevertheless, there is now considerable evidence that expression of *Fgf3* in the hindbrain from HH8 onwards appears to be required for otic placode invagination. Likewise, results from the ectopic overexpression of *Fgf3* in the surface ectoderm and neural tube at HH8 *in vivo* in the intact embryo support this hypothesis (Vendrell *et al.*, 2000; Zelarayan *et al.,* 2007). These experiments lead to an increase in the size of the endogenous otic placode and in addition, to the formation of ectopic otic placodes and vesicles in a broad area of the surface ectoderm (Vendrell *et al.*, 2000), reflecting a widespread competence to respond to FGF3. Although these experiments do not exclude an indirect action of FGF3 on otic placode formation via other signals, e.g. in the neighbouring mesoderm, they most likely mimic the action of FGF3 from its natural sources at this stage (e.g. the posterior hindbrain) by stimulating the formation of ectopic otic placodes in the competent surface ectoderm. Interestingly, isolated non-otic ectoderm at HH8 and earlier (0-4ss) induces otic but not neural or mesodermal markers upon treatment with FGF2, further suggesting a direct action of the FGF signal on the ectoderm (Martin and Groves, 2006). Finally, knockdown of *Fgf3* in the pharyngeal endoderm at HH8 also blocks placode invagination (Zelarayan *et al.,* 2007). Thus both hindbrain- and endoderm-derived FGF3 is required for this process.

The potency of the FGF3 signal is also underlined by overexpressing FGF2, FGF8 or FGF10 at HH8 in the surface ectoderm of the intact embryo, which fail to induce ectopic placodes (Vendrell *et al.*, 2000; Y. Alvarez and T.S., unpublished observations). However, implantation of FGF2 beads into the mesoderm close to the future otic placode at HH8 *in vivo* results in the formation of small ectopic otic placodes at a low frequency (V. Vendrell and T.S., unpublished observations). Likewise, FGF2 induces small ectopic otic placodes close to the endogenous otic vesicle upon implantation of beads at HH10 to HH11 (Adamska *et*

al., 2001). This activity may again reflect the capacity of FGF2 to activate isoforms of all four FGF receptors and thus also to promote ectopic otic placode formation although to a much lesser extent than FGF3. Both FGF2 and FGF8 beads implanted into the mesoderm also increase otic marker gene expression and the size of the normal otic vesicle (Adamska *et al.*, 2001). These results may reflect a patterning function for FGF signalling in the otic vesicle, which has also been suggested in zebrafish (Leger and Brand, 2002).

Functional analysis of FGFs in mouse inner ear induction

The sufficiency of FGFs to induce ectopic otic placodes from the developing hindbrain has been tested in transgenic mice (Alvarez *et al.*, 2003). Different FGF family members were misexpressed in r3 and overexpressed in r5 from the 3ss stage onwards (Theil *et al.*, 1998; Alvarez *et al.*, 2003). Misexpression of *Fgf3,* whose endogenous onset of expression in the hindbrain coincides with this stage (Wright and Mansour, 2003a) shows only a very limited capacity to induce ectopic otic vesicles (Alvarez *et al.*, 2003). In contrast, misexpression of *Fgf10,* whose endogenous onset of expression in the hindbrain takes place slightly later at the 5ss, leads consistently to the formation of ectopic vesicles next to r3-r5 that also express some otic markers (Alvarez *et al.*, 2003). Interestingly, more recent analysis of these transgenic animals has revealed that ectopic *Fgf8* expression accompanies *Fgf10* misexpressing embryos but not following ectopic expression of *Fgf3*, indicating a positive role for FGF8 during the induction of ectopic otic placodes (Zelarayan *et al.,* 2007). However, misexpression of *Fgf8* in r3 and r5 leads to early embryonic lethality and thus prevents the analysis of transgenic embryos during otic induction (Alvarez *et al.,* 2003 and unpublished observations). Similarly, null mutants for *Fgf8* have revealed an essential role for this gene during gastrulation and consequently show early embryonic lethality (Meyers *et al.*, 1998). Thus, so far it has been difficult to assign a specific role for the early expression pattern of *Fgf8* in the mesoderm and endoderm at E7-E8 during otic induction (Ladher *et al.*, 2005). Mouse mutants carrying a hypomorphic or a conditional allele which is inactivated mosaically from E7 onwards next to *Fgf8* null alleles form otic vesicles (Ladher *et al.,* 2005; Zelarayan *et al.,* 2007). Therefore, so far no evidence has been obtained for a unique requirement for FGF8 during inner ear induction. Tissue-specific inactivation of *Fgf8* in the mesoderm or endoderm may circumvent the early lethality and reveal the unique requirements of FGF8 in these tissues during inner ear induction.

Null mutants for *Fgf3* or *Fgf10* form otic vesicles albeit reduced in size (Ohuchi *et al.*, 2000; Alvarez *et al.*, 2003; Wright and Mansour, 2003b). *Fgf3* mutants may also show a more ventrally localized otic vesicle and alterations in expression of otic markers in a variable manner (Wright and Mansour, 2003a; Ladher *et al.*, 2005). In humans, homozygous mutations in *FGF3* that are likely to result in non-functional proteins are associated with a new form of syndromic deafness characterized by inner ear agenesis (Tekin *et al.*, 2007). Due to the absence of inner ear structures in the patients it has been suggested that inner ear development is disturbed at a very early stage.

Mouse mutants for the FGF receptor 2 IIIb isoform, to which FGF3 and FGF10 bind with high affinity also develop smaller otic vesicles (Pirvola *et al.*, 2000). Finally, although mouse FGF15 is sufficient to induce otic markers in stage 4/5 chicken rostral ectoderm, *Fgf15* null mouse mutant embryos form normal otic vesicles (Wright *et al.*, 2004). Likewise, inner ear phenotypes are absent in null mutants for FGF receptor 4 to which FGF15 binds with high affinity (Weinstein *et al.*, 1998).

In contrast to mouse mutants that lack single members of the FGF gene family, FGF double mutants have been much more informative in demonstrating the roles of FGFs during inner ear induction thus revealing considerable redundancy between family members. Homozygous null mutant embryos for both *Fgf3* and *Fgf10* either entirely lack otic vesicles or show the formation of microvesicles (Alvarez *et al.*, 2003; Wright and Mansour, 2003a). Furthermore, the microvesicles formed are ventralised and more distantly located from the neural tube compared to control embryos (Figure 2A,C; Alvarez *et al.*, 2003; Wright and Mansour, 2003a). Some of the microvesicles express otic genes whereas others show reduced or absent staining of otic markers, including *Pax2* and *Dlx5* (Alvarez *et al.*, 2003; Wright and Mansour, 2003a). Occasionally, development of these microvesicles continues to later stages of inner ear development, where defects in the dorsal

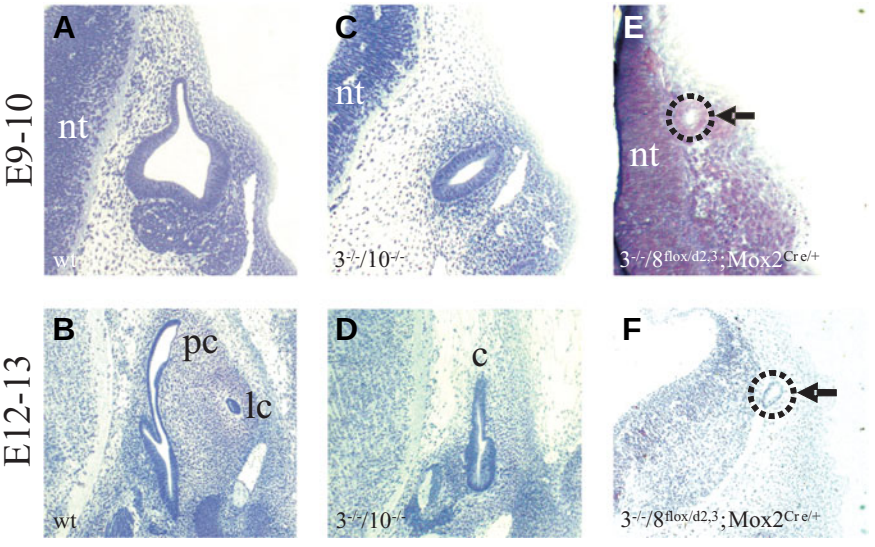

Fig. 2. Inner ear phenotypes of *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ and *Fgf3*$^{-/-}$/*Fgf8*$^{flox/d2,3}$; *Mox2*$^{Cre/+}$ mutant mouse embryos. *Sections through the developing inner ear of wild-type* **(A,B)**, Fgf3$^{-/-}$/Fgf10$^{-/-}$ **(C,D)** *and* Fgf3$^{-/-}$/Fgf8$^{flox/d2,3}$; Mox2$^{Cre/+}$ *mutants* **(E,F)** *around E9-10 and E12-13.* **(A,C)** *Compared to wild-type embryos the otic vesicle is smaller and in a more ventral and distal position from the neural tube (nt) in* Fgf3$^{-/-}$/Fgf10$^{-/-}$ *mutants.* **(B,D)** *During otic vesicle differentiation* Fgf3$^{-/-}$/Fgf10$^{-/-}$ *embryos only form a single semicircular canal (c) whereas the posterior (pc), lateral (lc) and anterior semicircular canals (not shown) are observed in wild-type embryos.* **(E,F)** Fgf3$^{-/-}$/Fgf8$^{flox/d2,3}$; Mox2$^{Cre/+}$ *mutants form microvesicles that are often found in a dorsal position in close proximity to the neural tube (nt).*

vestibular part of the inner ear now become evident (Fig. 2D; Zelarayan *et al.* 2007). Similar defects can also be observed in mutant embryos homozygous null for *Fgf3* and carrying one mutant *Fgf10* null allele (Zelarayan *et al.,* 2007). Placode formation and the expression of otic placode markers has been analysed at E8 in *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ double mutants (Alvarez *et al.*, 2003; Wright and Mansour, 2003a). Placodal tissue and several otic placode markers are still detected in these mutants. However, the dorsal part of the placodal ectoderm fails to form and thus the expression of otic markers including *Pax2* or *Dlx5* is absent in this area or in some cases missing entirely throughout the placode (Alvarez *et al.*, 2003; Wright and Mansour, 2003a).

Double mutants for *Fgf3* and *Fgf8* develop a similar phenotype to *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ double mutants (Ladher *et al.*, 2005; Zelarayan *et al.*, 2007). Mutant embryos carrying a hypomorphic and a null allele for *Fgf8* on a homozygous null *Fgf3* mutant background (*Fgf3*$^{-/-}$/*Fgf8*$^{neo/neo}$) do not form otic vesicles and lack expression of otic markers in the dorsal part of the placodal ectoderm (Ladher *et al.*, 2005). Interestingly, these mutants also show a downregulation of *Fgf10* expression in the mesoderm during inner ear induction, indicating that *Fgf3* and *Fgf8* may be redundantly required for normal levels of *Fgf10* expression. Similar to *Fgf3*$^{-/-}$/*Fgf8*$^{neo/neo}$ mutants, embryos carrying a null allele and a mosaically deleted conditional allele for *Fgf8* on a homozygous null *Fgf3* mutant background (*Fgf3*$^{-/-}$/*Fgf8*$^{flox/d2,3}$; *Mox2*$^{Cre/+}$) also show a severe phenotype that results in the formation of microvesicles which are in an abnormal dorsal position close to the neural tube (Fig. 2E; Zelarayan *et al.,* 2007). These microvesicles show absence or abnormal expression of otic markers. At later stages these microvesicles fail to differentiate or a complete loss of otic tissue is observed (Fig. 2F; Zelarayan *et al.,* 2007). Taken together, comparative phenotyping of *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ and *Fgf3*/*Fgf8* double mutant combinations indicate that the latter mutants have a slightly more severe inner ear phenotype (Ladher *et al.,* 2005; Zelarayan *et al.*, 2007). This suggests a more pronounced role for FGF8 rather than FGF10 during inner ear induction, possibly due to the earlier and more widespread expression of *Fgf8* in several tissues implicated during otic induction. Inactivation of both *Fgf8* and *Fgf10* should result in an even more severe phenotype, because *Fgf8* and *Fgf10* are both expressed before *Fgf3* and are likely to initiate otic induction as early endodermal and/or mesodermal signals.

Examination of the hindbrain of *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ and *Fgf3*$^{-/-}$/*Fgf8*$^{neo/neo}$ mutant embryos showed no changes in hindbrain marker expression, indicating that FGFs do not act indirectly on otic induction by controlling hindbrain patterning as suggested in zebrafish (Wright and Mansour, 2003a; Ladher *et al.*, 2005). Since high-affinity receptors for FGF3 and FGF10 are expressed in the preplacodal ectoderm, at least these two FGFs may act directly to establish expression of otic markers in the future otic placode (Wright and Mansour, 2003a). Interestingly, more recent studies have revealed that *Fgf10* misexpression from the hindbrain is sufficient to rescue otic vesicle development in *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mutant embryos (Zelarayan *et al.*, 2007). This shows that a FGF signal from neural tissue (the hindbrain) is able to reinstruct the placodal ectoderm in these mutants to form an otic vesicle. Since the formation of placodal tissue is only partially affected in *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ mutant embryos the initial steps of placode induction could be maintained by the expression of *Fgf8* which is present in various tissues during early otic induction (Ladher *et al.*, 2005). *Fgf3* and *Fgf10* thus possibly reinforce and maintain inner ear induction initiated by *Fgf8*.

Functional analysis of FGFs in zebrafish, medaka and *Xenopus* inner ear induction

The effects of a loss of FGF3 or FGF8 on inner induction in zebrafish has been tested in mutants and by morpholino knockdown experiments. Using both approaches, a reduction in size of the otic vesicle combined with reduced or loss of expression of otic markers has been observed (Phillips *et al.*, 2001; Leger and Brand, 2002; Maroon *et al.*, 2002; Liu *et al.*, 2003). The size of the otic vesicle in *Fgf8* morphants appears slightly smaller than in *Fgf3* morphants, possibly due to a non-redundant requirement for FGF8 during hindbrain patterning influencing otic induction (Wiellette and Sive, 2004). The central role of the hindbrain during inner ear induction is also underlined by the fact that only wild-type hindbrain cells rescue *Pax2* expression in *Fgf8* mutant embryos in cell transplantation experiments (Leger and Brand, 2002). Furthermore, ectopic expression of *Fgf3* or *Fgf8* induces otic markers and formation of ectopic otic vesicles in zebrafish, Medaka and *Xenopus* (Lombardo *et al.*, 1998; Phillips *et al.*, 2001; Bajoghli *et al.*, 2004; Hans *et al.*, 2004; Phillips *et al.*, 2004; Solomon *et al.*, 2004). Initially, an indirect way for ectopic expression of both *Fgf3* and *Fgf8* was chosen by treating wild-type zebrafish with retinoic acid (Phillips *et al.*, 2001). This leads to an expansion of the expression domains of *Fgf3* and *Fgf8* and of the otic marker *Pax8* and results in the formation of ectopic otic vesicles. However, retinoic acid treatment may also have more pleiotropic effects in these experiments since the neural plate is posteriorized and the hindbrain is expanded (Phillips *et al.*, 2001). More recently, experiments were used to demonstrate that FGF-dependent otic induction by retinoic acid may also occur without perturbing patterning of the neural plate (Hans *et al.*, 2007). Similarly, plasmid-mediated misexpression of *Fgf3* or *Fgf8* at the 8 cell stage leads to ectopic or expanded expression of the otic markers *Pax8*, *Pax2a* and *Dlx3b* without expansion of the neural plate (Phillips *et al.*, 2004; Solomon *et al.*, 2004). To better control the timing of ectopic FGF expression during otic induction in embryos, heat-inducible promoters have been successfully used for *Fgf8* in Medaka and more recently also in zebrafish (Bajoghli *et al.*, 2004; Hans *et al.*, 2007). In the latter case, early misexpression of *Fgf8* until midgastrula stages was shown to actually reduce the amount of otic tissue, probably due to its effects on dorsoventral patterning that negatively affect expression of *Foxi1* and *Dlx3b* that are required as competence factors for FGF-dependent otic induction (see below). In contrast, larger otic vesicles are obtained when *Fgf8* misexpression is carried out between the end of gastrulation and early segmentation stages, presumably due to the induction of a larger area of competent ectoderm to acquire an otic fate (Hans *et al.,* 2007).

The redundant requirements for FGF3 and FGF8 for otic placode formation have been demonstrated in several studies (Phillips *et al.*, 2001; Leger and Brand, 2002; Maroon *et al.*, 2002; Liu *et al.*, 2003). All studies agree that zebrafish mutants or morphants lacking both FGF3 and FGF8 show a severe loss or absence of otic tissue and markers. However, some discrepancies exist on the presence or absence of the earliest marker

indicating otic fate, *Pax8*, in *Fgf3/Fgf8* double mutants. Whereas most studies report a severe reduction or absence of this marker (Phillips *et al.*, 2001; Leger and Brand, 2002; Liu *et al.*, 2003) one study reports the maintenance of normal *Pax8* expression in about half of the double mutant embryos (Maroon *et al.*, 2002). In a related experiment, these authors also demonstrate unchanged expression of *Pax8* upon blocking FGF receptor signalling with the inhibitor SU5402. In contrast, Leger and Brand (2002) note absence of *Pax8* expression using even lower concentrations of SU5402 than those reported by Maroon *et al.* (2002). More consistently, both studies report absence of the otic marker *Pax2* upon SU5402 treatment before segmentation, but differ again on the effects of SU5402 on *Pax2* maintenance at later stages (Leger and Brand, 2002; Maroon *et al.*, 2002). The remaining otic tissue in some *Fgf3/Fgf8* double mutants has been shown to consist of a few scattered placodal cells only, indicating that the capacity to form a placodal epithelium has been lost (Liu *et al.*, 2003). In the cases of double mutant embryos where *Pax8* expression is not detected and morphological signs of otic placode formation are not observed one may conclude that a complete loss of otic placode induction has been achieved.

Loss of FGF3 and FGF8 also affects the development of the posterior hindbrain (Maves *et al.*, 2002; Walshe *et al.*, 2002) where targets of FGF receptor signalling are downregulated in the hindbrain and otic region (Maroon *et al.*, 2002). Therefore, the hindbrain region where *Fgf3* and *Fgf8* are coexpressed plays an essential role for inner ear induction in zebrafish. However, both FGFs are also expressed in other tissues known to be involved during inner ear induction, such as the cephalic paraxial mesoderm (Mendonsa and Riley, 1999; Phillips *et al.*, 2001; Thisse *et al.*, 2001; Nechiporuk *et al.*, 2007). Zebrafish mutants or morphants for *one-eyed pinhead* (*oep*) that lack mesoendodermal tissue underlying the otic placode show a loss of *Fgf3* and *Fgf8* in their mesoendodermal domains of expression (Phillips *et al.*, 2001; Leger and Brand, 2002; Nechiporuk *et al.*, 2007). In both cases hindbrain expression of *Fgf3* and *Fgf8* was not affected during inner ear induction. However, while one study reported normal expression of otic markers including *Pax8* (Leger and Brand, 2002), Phillips *et al.* (2001) showed that expression of this otic marker was reduced, indicating a possible requirement for FGF3 and/or FGF8 for inner ear induction outside of the hindbrain. Therefore, at present it is still unclear which expression domains of *Fgf3* and *Fgf8* are required for inner ear induction in zebrafish.

The model that envisages FGF signalling cooperating with *Wnt8* during otic placode induction, originally suggested in chicken has been analysed in more detail in zebrafish (Ladher *et al.*, 2000; Phillips *et al.*, 2004). Ectopic expression of *Fgf3* or *Fgf8* was shown to be sufficient to induce ectopic otic placodes in the absence of *Wnt8*. However, global ectopic expression of *Wnt8* also induced ectopic otic tissue, but this effect was shown to depend on the expansion of *Fgf3* and *Fgf8* expression domains. Finally, otic induction and expression of *Fgf3* and *Fgf8* was delayed in *Wnt8* morphants. Since vice versa, *Wnt8* expression in the hindbrain is also reduced in *Fgf3/Fgf8* double mutants, the existence of a positive feedback loop has been postulated, that guarantees timely expression of *Fgf3* and *Fgf8* in the hindbrain which then act directly on preplacodal cells to induce the otic fate (Phillips *et al.*, 2004).

Several studies have analysed the interaction of FGF3 and FGF8 signalling with transcription factors expressed during inner ear induction in the preplacodal surface ectoderm. *Foxi1* has been shown to be required for the induction of *Pax8* expression mediated by FGF signalling (Hans *et al.*, 2004; Solomon *et al.*, 2004; Hans *et al.*, 2007). Foxi1 has therefore been termed a competence factor for FGF3 and FGF8 that permits the acquirement of otic fate by preplacodal cells, as assessed by *Pax8* expression (Nissen *et al.*, 2003; Hans *et al.*, 2004; Solomon *et al.*, 2004; Hans *et al.*, 2007). On the other hand, *Pax8* morphants have more profound defects during inner ear induction in a *Fgf8* mutant background than in the presence of *Fgf3* morpholinos, indicating once again a more dominant role for FGF8 compared to FGF3 (Wiellette and Sive, 2004; Mackereth *et al.*, 2005). A second pair of competence factors for FGF signalling, *Dlx3b* and *Dlx4b*, have been shown to be required for the proper initiation of *Pax2a* expression at a later stage (Hans *et al.*, 2004; Mackereth *et al.*, 2005). *Sox9a* expression has also been shown to depend on FGF signalling via the expression of *Pax8 and* later the maintenance of *Sox9a* expression depends on *Pax2a* (Hans *et al.*, 2004). Finally, zebrafish *Atoh1b*, a homologue of *Atoh1* that is necessary for hair cell differentiation in the mouse (Woods *et al.*, 2004) has recently been shown to be required in the preplacodal ectoderm in zebrafish (Millimaki *et al.*, 2007). *Atoh1b* is coexpressed with *Pax8* in preplacodal ectoderm during early segmentation and requires FGF signalling. More detailed schemes of the interaction of transcription factors and FGF signalling during otic induction can be found in the studies of Hans *et al.*, (2004), Solomon *et al.* (2004) and Millimaki *et al.* (2007).

In *Xenopus*, expression of a dominant negative FGF receptor has been shown to reduce *Sox9* expression (Saint-Germain *et al.*, 2004). Morpholinos directed against *Sox9* lead to the absence of *Pax8* expression and otic vesicles are not formed. This lead to the suggestion that *Sox9* may be upstream of *Pax8* but may also be explained by a positive feedback loop between both genes to maintain each others expression (Liu *et al.*, 2003; Hans *et al.*, 2004).

Summary and outlook

The expression studies of different FGF members during inner ear induction confirm their sequential presence in endomesodermal and neural tissue during inner ear induction. Direct evidence for the necessity of a single FGF member during the initiation of inner ear induction (before otic specification) only currently exists for FGF8 in the chicken endoderm (Ladher *et al.*, 2005). Moreover, the sufficiency and necessity of FGF8 to induce FGF19 in the overlying mesoderm indicates that this event represents an important step during chicken inner ear induction. However, at present direct evidence for the necessity of FGF19 for inner ear induction is lacking. Since *Fgf3* is induced by FGF19 and both are coexpressed in the mesoderm (Ladher *et al.*, 2000; Kil *et al.*, 2005) the necessity and sufficiency of FGF3 in the mesoderm for the early phase of inner ear induction is certainly worth testing.

The significance of the early mesodermal and/or endodermal expression of *Fgf8* together with *Fgf10* in mouse or *Fgf3* in zebrafish during inner ear induction has not yet been directly addressed. Tissue-specific inactivation of *Fgf8* or *Fgf10* in a *Fgf3* homozygous null mutant background during this phase will provide useful information on the necessity of these expression

domains for otic induction in the mouse. The second phase of inner ear induction (after otic specification and before otic commitment) is clearly defined by the conserved expression of *Fgf3* in the developing hindbrain in all vertebrates. Since in the mouse, *Fgf3* expression is not present in endoderm or mesoderm at or before this stage, the effects on inner ear induction seen in *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ and *Fgf3/Fgf8* double knockout mice have to be attributed entirely to the loss of hindbrain *Fgf3* expression. Interestingly, at this stage *Fgf10* and *Fgf19* which are initially expressed in the mesoderm are now present in the ventral part of the hindbrain in mouse and chicken, respectively, from where they may participate in otic induction (Ladher *et al.*, 2000; Alvarez *et al.*, 2003). Knockdown of *Fgf3* in chick hindbrain interferes with placode invagination but more severe phenotypes may be obtained upon inactivation at an earlier stage (Zelarayan *et al.,* 2007).

Fgf3/Fgf8 double mutants in zebrafish have a more severe phenotype than both *Fgf3*$^{-/-}$/*Fgf10*$^{-/-}$ and *Fgf3/Fgf8* double mutants in mouse. While the zebrafish mutants often completely lack placodal tissue (Phillips *et al.*, 2001; Leger and Brand, 2002), mouse mutants usually still form some placodal ectoderm or microvesicles (Alvarez *et al.*, 2003; Wright and Mansour, 2003a; Ladher *et al.*, 2005). These phenotypes may be caused by a complete loss of *Pax8* expression in the zebrafish double mutants, whereas this marker is reduced but still present in the double mouse mutants. In contrast, *Pax2* expression which is activated after *Pax8* expression during otic development, is more consistently absent from placodal tissue in both zebrafish and mouse FGF double mutants. The residual *Pax8* expression at (and possibly before) E8 in mouse FGF double mutants may be sufficient for the formation of some placodal tissue. However, by analogy to the zebrafish, absence of *Pax2* and *Dlx* genes then may also lead to the loss of responsiveness to FGF signalling in the murine otic placode and thus to the formation of microvesicles or complete absence of otic tissue at later stages.

It has been suggested that the ventral part of the placodal ectoderm, which continues to express some otic genes in FGF double mouse mutants, may contribute to the epibranchial placodes (Groves, 2005). In zebrafish it has recently been shown that Sox3 defines the common primordium of the otic and epibranchial placodes (Sun *et al.*, 2006; Nikaido *et al.*, 2007). This *Sox3*-positive primordium then segregates into a *Pax2a*-positive medial area and a *Pax2a*-negative lateral area, giving rise to the otic and epibranchial placodes, respectively. Disruption of FGF signalling in FGF zebrafish mutants or by using SU5402 leads to a loss of *Sox3* expression and a failure to form the epibranchial placodes (Sun *et al.*, 2006; Nechiporuk *et al.*, 2007; Nikaido *et al.*, 2007). It will thus be interesting to further define the differential requirements for the induction of the otic placode versus the epibranchial placode, for example by examining expression of *Sox3* and the formation of the epibranchial placode in mouse FGF double mutants.

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