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; Thissie and Thissie, 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 Fgf19 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...
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’s expression can be observed 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 splanchic 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 9ss 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 12sss (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-13sss (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.](image)
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; Thiss 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; Thiss and Thiss, 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>
<thead>
<tr>
<th>Type of experiment</th>
<th>Outcome</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Chicken</strong></td>
<td></td>
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<tr>
<td>Loss-of-function of FGF8 (si RNA at stage 4)</td>
<td>Reduced or absent placode and Pax2 expression</td>
<td>Ladher et al., 2005.</td>
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<tr>
<td>Ectopic FGF8 (beads, stage 5 mesoderm)</td>
<td>Induction of FGF19</td>
<td>Ladher et al., 2005.</td>
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<tr>
<td>Ectopic FGF19 (beads, stage 5 ectoderm, stage 7 non-otic tissue including neural tissue)</td>
<td>Induction of FGF3 and otic markers</td>
<td>Ladher et al., 2000.</td>
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<tr>
<td>Inhibition of FGF receptor signalling (SU5402)</td>
<td>Block of Pax2 expression (until 4ss)</td>
<td>Martin and Groves, 2006.</td>
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<td>Loss of FGF3 and FGF19 in posterior hindbrain (RA-deficient quail)</td>
<td>Formation of otic vesicle unaffected</td>
<td>Kil et al., 2005.</td>
</tr>
<tr>
<td>Expression of FGF3 and FGF19 in neural tube (RA-deficient quail)</td>
<td>Expansion of otic placode and ectopic otic vesicles</td>
<td>Kil et al., 2005.</td>
</tr>
<tr>
<td>Loss-of-function of FGF3 in hindbrain or pharyngeal endoderm (si RNA at stage HH8)</td>
<td>Block of otic vesicle formation</td>
<td>Zelarayan et al., 2007.</td>
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<tr>
<td>Ectopic FGF3 (viral overexpression in surface ectoderm and electroporation in hindbrain at HH8)</td>
<td>Formation of ectopic otic vesicles</td>
<td>Vendrell et al., 2000; Zelarayan et al., 2007.</td>
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<tr>
<td><strong>Mouse</strong></td>
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<td>Ectopic FGF10 (FGF3) (hindbrain, transgenic)</td>
<td>Formation of ectopic otic vesicles</td>
<td>Alvarez et al., 2003.</td>
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<tr>
<td>FGF3 knockout</td>
<td>Reduced size of otic vesicle</td>
<td>Mansour et al., 1993; Alvarez et al., 2003.</td>
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<td>FGF10 knockout</td>
<td>Reduced size of otic vesicle</td>
<td>Ohuchi et al., 2000.</td>
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<td>FGF15 knockout</td>
<td>Formation of otic vesicles</td>
<td>Wright et al., 2004.</td>
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<td>FGF3/FGF10 double knockout</td>
<td>Loss of otic vesicle or microvessicles</td>
<td>Alvarez et al., 2003; Wright et al., 2003.</td>
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<tr>
<td>FGF3/FGF8 double knockout (FGF8 hypomorph or conditional allele)</td>
<td>Loss of otic vesicle or microvessicles</td>
<td>Ladher et al., 2005; Zelarayan et al., 2007.</td>
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<td><strong>Fish and Xenopus</strong></td>
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<tr>
<td>Loss-of-function of FGF3 or FGF8 (morpholino injection, mutants)</td>
<td>Reduced size of otic vesicle and otic marker expression</td>
<td>Phillips et al., 2001; Léger et al., 2002; Maroon et al., 2002; Liu et al., 2003.</td>
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<tr>
<td>Transplantation of wild-type cells in hindbrain of FGF8 mutant at shield stage</td>
<td>Rescue of Pax2 expression</td>
<td>Léger et al., 2002.</td>
</tr>
<tr>
<td>Ectopic FGF3 and FGF8 (RA treatment, plasmid)</td>
<td>Formation of ectopic otic vesicles</td>
<td>Lombardo et al., 1998; Phillips et al., 2001; Bajoghli et al., 2004; Philips et al., 2004; Solomon et al., 2004; Hans et al., 2007.</td>
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</tr>
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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 Fgfg3 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 committed 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 (Repesa et al., 1991). Similar results were obtained in the presence of anti-sense oligonucleotides directed against Fgf3, although the sequences used were based on humans 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 (Adam ska 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 et al., 2007). Therefore, so far no evidence has been provided that FGFs will have a role for the early expression pattern of Fgf8 during the induction of ectopic otic placodes (Zelarayan et al., 2007). How- ever, misexpression of Fgf10, 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). 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

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). Interestingly, more recent analysis of these transgenic animals has revealed that ectopic Fgf8expression 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). How- ever, misexpression of Fgf10 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 Fgf10 in the mesoderm and endoderm at E7-E8 during otic induction (Ladher et al., 2005). Mouse mutants carrying a hypomorphic or a condition- al 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 inac- tivation of Fgf8 in the mesoderm or endoderm may circumvent the early lethality and reveal the unique requirements of FGF8 in these tis- sues 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 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-/-/Fgf8flox/d2,3; Mox2Cre/+ mutant mouse embryos. Sections through the developing inner ear of wild-type (A,B), Fgf3-/-/Fgf10-/- (C,D) and Fgf3-/-/Fgf8flox/d2,3; Mox2Cre/+ 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-/-/Fgf8flox/d2,3; Mox2Cre/+ 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 Dlx5/6 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/neos) 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 Fgf8 and Fgf10 may be redundantly required for normal levels of Fgf10 expression. Similar to Fgf3+/-Fgf8+/-neo/neos mutants, embryos carrying a null allele and a mosaically deleted conditional allele for Fgf8 on a homozygous null Fgf3 mutant background (Fgf3+/-Fgf8+/-d2.3;Mox2+/-) also show a severe phenotype that results in the formation of microvesicles which 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 develop a complete otic vesicle 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.

Examination of the hindbrain of Fgf3+/-Fgf10+/- and Fgf3+/-Fgf8+/-neo/neos 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; Walseh 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; Thisset et al., 2001; Nechiporuk et al., 2007). Zebrafish mutants or morphants for one-eyed pinhead (ope) that lack mesendodermal tissue underlying the otic placode show a loss of Fgf3 and Fgf8 in their mesendodermal 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 acquisition 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 (Miliyak 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 Millyak 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 Fgf8 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 Fgf8−/− 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 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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FGFs and inner ear induction 481


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