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Induction of inner ear fate by FGF3

Victor Vendrell[1], Estela Carnicero[1], Fernando Giraldez[1], Maria Teresa Alonso[1] and Thomas Schimmang[1,2,*]

[1]Instituto de Biología y Genética Molecular, Universidad de Valladolid y Consejo Superior de Investigaciones Cientificas, Departamento de Bioquímica, Biología Molecular y Fisiología, Facultad de Medicina, E-47005 Valladolid, Spain
[2]Center for Molecular Neurobiology Hamburg, University of Hamburg, Falkenried 94, D-20251 Hamburg, Germany
*Author for correspondence (e-mail: schimman@epos.zmnh.uni-hamburg.de)

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SUMMARY

Loss-of-function experiments in avians and mammals have provided conflicting results on the capacity of fibroblast growth factor 3 (FGF3) to act as a secreted growth factor responsible for induction and morphogenesis of the vertebrate inner ear. Using a novel technique for gene transfer into chicken embryos, we have readdressed the role of FGF3 during inner ear development in avians. We find that ectopic expression of FGF3 results in the formation of ectopic placodes which express otic marker genes. The ectopically induced placodes form vesicles which show the characteristic gene expression pattern of a developing inner ear. Ectopic expression of FGF3 also influences the formation of the normal orthotopic inner ear, whereas another member of the FGF family, FGF2, shows no effects on inner ear induction. These results demonstrate that a single gene can induce inner ear fate and reveal an unexpectedly widespread competence of the surface ectoderm to form sensory placodes in higher vertebrates.

Key words: Otic placode, Otic vesicle, Fibroblast growth factor, HSV-1, Ectopic expression, Chicken

INTRODUCTION

The head ectoderm of vertebrates develops three pairs of sensory placodes, nose, lens and inner ear from anterior to posterior along the embryonic axis. Induction of the inner ear placode takes place at the 3- to 6-somite stage depending on the animal species, when an otic placode is formed in an area of surface ectoderm next to the developing hindbrain (Fritzsch et al., 1998; Torres and Giraldez, 1998). This placode invaginates to form the otic vesicle, an ellipsoid-shaped structure with a pseudostratified epithelium, which after a period of proliferative growth and a complex series of morphogenetic events will give rise to the mature inner ear.

Members of the fibroblast growth factor (FGF) family have been shown to function as developmental key regulators during pattern formation and organogenesis in vertebrates (Yamaguchi and Rossant, 1995; Martin, 1998; Hogan, 1999). Due to its expression in the otic vesicle or the neighbouring hindbrain during induction of the otic placode in mouse (Wilkinson et al., 1988, Mahmood et al., 1996), chicken (Mahmood et al., 1995) and *Xenopus* embryos (Tannahill et al.,1992; Lombardo et al., 1998), FGF3 has been proposed as the inducer of the inner ear (Fritzsch et al., 1998; Torres and Giraldez, 1998). Loss-of-function experiments, using antisense oligonucleotides or antibodies in chicken embryos provided the first functional evidence that FGF3 is required for inner ear development in avians (Represa et al., 1991). However, mice that carry a mutant FGF3 allele only showed defects during differentiation of the inner ear (Mansour et al., 1993). FGF2 has recently emerged as an alternative inducer for the inner ear since implanted beads coated with FGF2 were shown to produce vesicles with some otic characteristics in *Xenopus* embryos (Lombardo and Slack, 1998). Moreover, similar experiments using FGF3 beads indicated that FGF2 may function with a higher efficiency than FGF3 in this species (Lombardo et al., 1998). Finally, in explants of chicken embryos FGF2 had been shown to rescue induction of the otic vesicle, whose formation had been blocked by removal of the neighbouring hindbrain (Represa et al., 1991).

To further clarify the role of FGF3 and FGF2 during inner ear development in avians, we chose a gain-of-function approach using viral vectors to ectopically express these growth factors in chicken embryos. We used amplicon vectors derived from Herpes simplex virus type 1 (HSV-1), which have previously been shown efficiently to infect a wide range of cell types (Fink et al., 1996). Ectopic FGF3 expression obtained by such an amplicon vector leads to the formation of ectopic inner ears in a large area in the early embryo, most likely corresponding to a domain with multiplacodal competence. Additionally, morphogenesis of the normal inner ear was modified by exogenous FGF3. Although we observed expression of FGF2 during normal inner ear development, ectopic expression of this FGF via an amplicon vector produced no ectopic inner ears in infected embryos. These results strongly suggest that FGF3 is a key inducer of avian inner ear development and also plays a crucial role during the subsequent steps of inner ear differentiation.

MATERIALS AND METHODS

Amplicon production

Construction, packaging and titering of the pHSV*lac* amplicon vector using the helpervirus 5dl1.2 (McCarthy et al., 1989) has been

described previously (Lim et al., 1997). For construction of pHSV*fgf3* and pHSV*fgf2*, fragments containing the cDNAs encoding for mouse FGF3 (Dixon et al., 1989) or chicken FGF2 (Dono and Zeller, 1994) were inserted into the vector pHSV*pUC* (Geller et al., 1993) and their expression, controlled by the viral IE4/5 promoter, was confirmed after transfection of cell lines by immunofluorescence or by Western blots using specific antibodies for murine FGF3 (Dixon et al., 1989; Kiefer et al., 1993a) or chicken FGF2 (Dono and Zeller, 1994). The titer of packaged pHSV*fgf3* and pHSV*fgf2* as determined by immunocytochemistry was 9.1×10^6 ivu/ml and 2.4×10^6 ivu/ml, respectively. The 5dl1.2 helper virus titer was 2×10^7 pfu/ml. For immunochemistry, infected cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Fixed cells were washed with TBS (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and incubated with antibodies raised against the carboxyterminal peptide of murine FGF3 (diluted 1:4000; Dixon et al., 1989) or an antibody raised against chicken FGF2 (diluted 1:500; Dono and Zeller, 1994) in TST (1% fetal calf serum, 0.1 % Triton X-100 in TBS) overnight at 4°C. After 2 washes in TBS, samples were incubated with goat anti-rabbit/alkaline phosphatase conjugate (Sigma) diluted in TST for 1-4 hours at room temperature. Alkaline phosphatase was visualized using Nitro Blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to the manufacturers' instructions (Sigma).

Virus injection

Fertilized eggs (strain Broiler) were purchased from a local farm and stored at 17°C before incubation at 37°C with high humidity. Embryos were injected after 26-38 hours of incubation corresponding to embryos with 4-12 somites (stages HH 8-11). For virus injection, a 1/10 volume of filter-sterilized 0.25% Fast green tracking dye was added. The pipette was lowered with a micromanipulator above the region of the rhombencephalon and approximately 2-3 μl were placed as an overlay on top of the embryo using a pico-injector (PLI-100, Medical Systems Corp.). Under these conditions, formation of the inner ear in embryos injected with control amplicon vectors was unaffected (see Table 1). The only abnormality observed in pHSV*control*- and pHSV*fgf*-injected specimen was that embryos analysed 8 hours after infection were somewhat more fragile, which resulted in opening of the neural tube during staining procedures (see Fig. 1B,C). Embryos analysed 40-46 hours after infection showed no defects in closure of the neural tube. Injection of higher amounts of virus lead to high mortality rates (normal survival rate was 71% for pHSV*fgf3*) or severe physical abnormalities. No infection of embryos could be obtained when injections were performed before onset of formation of the fifth somite.

Analysis of RNA and protein expression in embryos

For whole-mount analysis of RNA expression in situ embryos were processed and hybridised at 65°C as described (Nieto et al., 1995). The corresponding RNA probes and their expression patterns have been described (Nieto et al., 1992; Vogel et al., 1995; Oh et al., 1996; Myat et al., 1996; Bovolenta et al., 1998; Giraldez, 1998; Herbrand et al., 1998; Kamachi et al., 1998). Expression of pHSV*fgf3* was detected with antisense RNA derived from the mouse cDNA (Dixon et al., 1989), which did not cross-hybridise with the endogenous chicken *fgf3* mRNA. Whole-mount staining of embryos with Pax-2 (Püschel et al., 1992), HNK-1 (Becton-Dickinson, San Jose, CA), TuJ1 (Babco, Berkeley, CA) and HSV (Dako, Glostrup, Denmark) antibodies (diluted 1:200) was performed as described (Riese et al., 1995). For histological examination, embryos were postfixed in 4% paraformaldehyde, embedded in gelatin and sectioned at 70 μm on a vibratome (TPI). For detection of endogenous FGF2 expression, paraffin sections of chicken embryos were processed with antibodies raised against chicken FGF2 (diluted 1:200; Dono and Zeller, 1994) and secondary antibodies coupled to alkaline phosphatase using standard protocols. Photographs were taken or images were captured with a DC100 camera using QWin (Leica) on a Labophot-2 microscope (Nikon).

RESULTS

HSV-1 based amplicon vectors efficiently infect chicken embryos

To address further the role of FGF3 during inner ear development in avians, we used an amplicon vector derived from HSV-1 (Fink et al., 1996) to ectopically express this growth factor in chicken embryos (Fig. 1A). To test the feasibility of using this system during chicken embryonic development, we infected various embryonic stages with a prototype amplicon vector expressing the *lac*Z gene under the control of a strong viral promoter (see Materials and Methods). We found efficient and widespread expression especially in the neural tube and surface ectoderm 8 hours after infection of the embryo from the 5-somite stage onwards. β-galactosidase was first detected 4 hours after injection of the virus and widespread infection could be observed after 8 hours (Fig. 1B; see Materials and Methods).

Ectopic expression of FGF3 induces the formation of otic placodes in a large area of the embryonic surface ectoderm

We next applied an amplicon vector expressing murine FGF3 (Fig. 1A), whose expression was confirmed in cell lines and in vivo (Fig. 1C; see Materials and Methods). To examine effects of ectopic FGF3 expression, we analysed embryos between 46 and 76 hours after infection. In approximately 50% of the

Table 1. Quantification of phenotypes of embryos injected with amplicon vectors

Vector	Stage	Embryos with ectopic placodes/vesicles	Average number of ectopic placodes/vesicles (per embryo)	Embryos with oversized endogenous inner ear	Embryos with elongated E.D.
pHSV*control*	8 (*n*=46)	0	–	0	0
	9 (*n*=39)	0	–	0	0
	10 (*n*=39)	0	–	0	0
pHSV*fgf3*	8 (*n*=92)	49 (53.2%)	1.80	44 (47.8%)	7 (7.6%)
	9 (*n*=79)	39 (49.3%)	1.68	36 (45.5%)	7 (8.8%)
	10 (*n*=28)	13 (46%)	1.76	9 (32.1%)	5 (17.8%)
pHSV*fgf2*	8 (*n*=24)	0	–	0	0
	9 (*n*=22)	0	–	0	0
	10 (*n*=6)	0	–	0	0

Embryos of the indicated stages (nomenclature of Hamburger and Hamilton; Bellairs and Osmond, 1998) were injected with amplicon vectors and the appearance and number of ectopic placodes or vesicles were analysed. Morphological alterations of the endogenous inner ear, like an increased size or elongation of the endolymphatic duct (E.D.) were also monitored. Control amplicon vectors included pHSV*lacZ* (*n*=70), pHSV*trkA* (*n*=39) and pHSV*bdnf* (*n*=15).

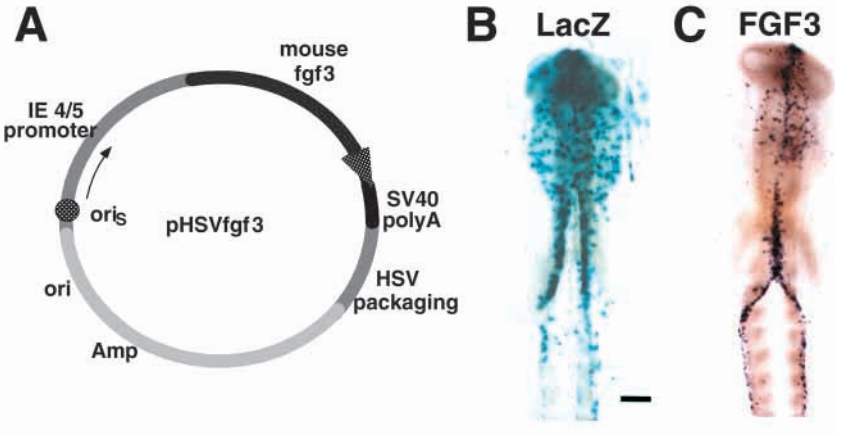

Fig. 1. Infection of chicken embryos with Herpes simplex type 1 amplicon vectors. (A) Structure of the pHSV*fgf3* amplicon consisting of the transcription unit composed of the IE 4/5 promoter, the murine *fgf3* cDNA and the SV40 polyadenylation site, the HSV-1 origin of replication (ori_S) and the HSV-1 packaging site, and sequences required for propagation in *E.coli* (ori and Amp). (B,C) Detection of β-galactosidase and murine *fgf3* RNA by whole-mount staining of chicken embryos (stage 10 after Hamburger and Hamilton, HH; Bellairs and Osmond, 1998), 8 hours after infection with pHSV*lac* and pHSV*fgf3*, respectively. The RNA probe only detects pHSV*fgf3*-derived expression. Note the efficient infection of the neural tube and surface ectoderm. Scale bar in B corresponds to 200 μm in B,C.

embryos (see Table 1), we observed the formation of morphologically distinct circular shaped structures that showed expression of *c-notch* (Fig. 2A), which is normally expressed in various cranial placodes, such as the lens, nose, otic or epibranchal placodes (Myat et al., 1996). The placodal nature of these structures was furthermore confirmed by sections, which revealed areas of thickened and invaginating surface ectoderm resembling the morphology of a forming placode (Fig. 2D). As FGF3 is implicated in induction of the otic placode, we next analysed the expression of genes expressed in early otic placodes, including *cPax*-2, HNK-1, *cek-8*, *lmx-1*, *cNkx5-1* and TuJ1 (Nieto et al., 1992; Rinkwitz-Brandt et al., 1996; Meyer zum Gottesberge and Mai, 1997; Giraldez, 1998; Herbrand et al., 1998; Molea et al., 1999). All these markers stained ectopic placodes (Fig. 2B,C,F and data not shown) which were localised over a very large area anteriorly and posteriorly of the position of the normal otic placode and vesicle (Fig. 5B), which form laterally to rhombomeres 5 and 6 of the hindbrain. Anteriorly, ectopic placodes were observed in an area of the surface ectoderm posterior to the developing eye, and lateral to the anterior hindbrain (Fig. 2A-C). Posteriorly, we found placodes laterally to the posterior hindbrain and the neural tube down to the level of the sixth somite (Fig. 2C,F). To determine the competence of more posterior areas, we performed injections targeting the viral infection to the trunk of the embryo (*n*=11). Although we found efficient infection of the neural tube and surface ectoderm, no ectopic placodes were observed in this area (data not shown). Sectioning of embryos revealed that the ectopic placodes (*n*=60) induced in the anterior part of the embryo corresponded to pseudostratified thickened epithelium staining positively for otic placode markers (Fig. 2E and data not shown). Analysis of ectopic expression of FGF3 showed that, in all cases analysed (*n*=15), placodes had formed and started to invaginate underneath cells expressing FGF3, which were localised in the surface ectoderm (Fig. 2E). A careful analysis of serial sections showed that virally infected cells of the surface ectoderm tended to round up and exclude themselves from the ectodermal layer (Fig. 2E). In contrast, although we observed ectopic expression of FGF3 in the neural tube, we found no evidence for this expression causing formation of ectopic placodes (data not shown). Cells expressing FGF3 appear thus to secrete the growth factor and act in a paracrine manner to induce the formation of ectopic placodes in the surface ectoderm. This paracrine mode of action of FGF3 has been previously described in transgenic mice, which ectopically express FGF3 in the developing lens (Robinson et al., 1998).

Ectopic otic placodes undergo normal inner ear development

The otic placode invaginates to form the otic cup and then closes to form the otic vesicle. During this process, the inner ear shows a specific expression pattern of regionalised marker

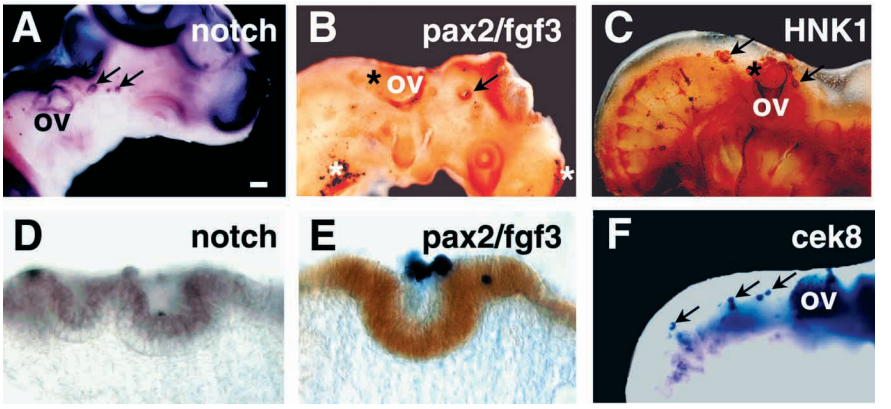

Fig. 2. Infection with pHSV*fgf3* induces formation of ectopic otic placodes. Chicken embryos infected with pHSV*fgf3* were stained as whole-mounts with the indicated RNA probes or antibodies. (A,B,C,F) Note the presence of stained rounded structures (indicated by arrows) on the head and trunk of the embryos, anteriorly and posteriorly of the normal otic vesicle (ov). The white asterisks in B indicate focal areas highly infected with pHSV*fgf3*. To improve penetration of the antibody part of the head of the embryo in B has been removed. The black asterisks in B and C indicates the poorly defined posterior border of the normal otic vesicle. (D,E) Vibratome sections of embryos show that the ectopic structures correspond to thickened and invaginating epithelial placodes, which have formed underneath blue-stained cells expressing *fgf3* mRNA in E. Scale bar in A corresponds to 200 μm in A,B,C,F and 20 μm in D,E.

genes, which reflects (a) morphological compartmentalisation in, for example, cochlear and vestibular parts or (b) differentiation of distinct cellular phenotypes, as, for example, sensory or neuronal cells (Fekete, 1996; Torres and Giraldez, 1998). We were interested to define whether the ectopic placodes would undergo the necessary steps for formation and regional specification of a normal otic vesicle or whether their ectopic localisation may lead to the acquisition of a different fate, e.g. by expressing genes specific for other sensory organs. In embryos ectopically expressing FGF3, we observed the formation of otic cups and vesicles, which in some cases reached the size of a normal otic vesicle (compare Fig. 3A and 3E). Targeted inactivation of *Pax-2* and the homeobox gene *Nkx5.1* have shown the essential roles of these genes for formation of the cochlear and vestibular regions of the otic vesicle, respectively (Torres et al., 1996; Hadrys et al., 1998; Wang et al., 1998). Their localised function is reflected by expression in distinct compartments of the otic vesicle, with *Pax-2* labelling regions closer to the neural tube and *Nkx5.1* detected in proximity to the surface ectoderm (Nornes et al., 1990; Rinkwitz-Brandt et al., 1996; Herbrand et al., 1998). Both genes were found to be expressed in their correct expression domains in ectopically forming vesicles (Fig. 3A,B). Likewise, genes expressed in the dorsal part of the otic cup and vesicle, including *cek-8* or *lmx-1* (Rinkwitz-Brandt et al., 1996; Giraldez, 1998), were detected in the dorsal part of the otic epithelium (Fig. 3C and data not shown). In contrast, expression of homeobox genes, such as *Pax-6* or *Six3*, which are marker genes for the developing lens and nose (Goulding et al., 1993; Grindley et al., 1995; Bovolenta et al., 1998; Kamachi et al., 1998) was absent in ectopic vesicles (Fig. 3I,J,M,N). Another characteristic feature of the otic vesicle is its capacity to form its proper neurones, which constitute the first cell lineage to differentiate while delaminating from the ventral area of the otic cup and vesicle (Torres and Giraldez, 1998). Staining with a β-tubulin antibody, which marks early differentiating neurones (Adam et al., 1998; Molea et al., 1999), revealed the presence of a neurogenic area in ectopic vesicles (Fig. 3K,O). The formation and differentiation of sensory areas in the developing inner ear are characterised by the expression of members of the BMP family of TGFβ-like proteins (Oh et al., 1996; Wu and Oh, 1996). The characteristic pattern of BMP7 expression flanking the neurogenic region could also be confirmed in ectopic otic vesicles (Fig. 3D). These data demonstrate that the ectopic placodes found in embryos infected with a virus expressing FGF3 undergo the program of gene expression and cell-fate specification that is characteristic for the development of an inner ear.

Ectopic FGF3 expression affects morphogenesis of the endogenous inner ear

Formation and morphogenesis of the normal otic vesicle were found to be altered by ectopic expression of FGF3. Whereas orthotopic otic vesicles in embryos injected with control amplicon vectors had usually closed by embryonic day 3, a large fraction of pHSV*fgf3*-infected embryos failed to complete closure of the vesicle (Table 1; Fig. 3F-H). This defect was apparently caused by the formation of an oversized otic placode and otic cup (especially in its posterior part, see Fig. 2B,C), which otherwise maintained the correct expression of regionally expressed marker genes (Fig. 3F-H). Additionally, in those cases where closure of the otic vesicle was achieved, we often observed that morphogenesis of the endolymphatic duct, which forms at the dorsal part of the otic vesicle was abnormal (Table 1). Expression of marker genes for the endolymphatic duct, such as HNK-1, *cPax-2* or *lmx-1* (Meyer zum Gottesberge and Mai, 1997; Giraldez, 1998; Herbrand et al., 1998), revealed an unusual lengthening of this structure (Fig. 3L,P and data not shown). Analysis of ectopically expressed FGF3 revealed a correlation between the observed phenotypes and the presence of FGF3 in the epithelia of the developing inner ear in infected embryos (data not shown).

Ectopic expression of FGF2 in chicken embryos

The fact that ectopically expressed FGF3 results in the formation of ectopic otic vesicles strongly supports the proposed role for FGF3 as an otic inducer in several species (Wilkinson et al., 1988; Represa et al., 1991; Tannahill et al., 1992; Fritzsch et al., 1998). This role of FGF3 in higher vertebrates has been questioned by loss-of-function experiments in mice, since FGF3 mutant animals showed normal induction of the inner ear (Mansour et al., 1993). These contradicting results may be explained by considering recent results obtained in *Xenopus* embryos, in which ectopic expression of both FGF3 and FGF2 induced ectopic vesicles with some otic characteristics (Lombardo and Slack, 1998; Lombardo et al., 1998). To analyse the potential involvement of FGF2 during inner ear development in avians, we examined its expression using specific antibodies for chicken FGF2 (Dono and Zeller, 1994). We observed weak expression of FGF2 in the dorsal part of the otic placode and the lumenal part of the neighbouring hindbrain, which increased during formation of the otic vesicle which also showed expression in its lumenal part (Fig. 4A,B). To address the functional role of FGF2 during chicken inner ear development, we ectopically expressed this growth factor using HSV-1 amplicon vectors. Functional activity of FGF2 produced by pHSV*fgf2* (see Materials and Methods) was confirmed by assaying its proliferative effect on various cell lines and the formation of ectopic limb bud tissue (Riley et al., 1993; Cohn et al., 1995) in infected embryos (data not shown). Although, pHSV*fgf2*-infected embryos (Fig. 4C) displayed a similar amount of HSV-1-derived expression compared to embryos injected with amplicons expressing FGF3, no ectopic otic placodes or oversized otic vesicles were observed upon ectopic expression of FGF2 (Table 1 and compare Fig. 3F-H and Fig. 4D).

DISCUSSION

In the present study, we asked whether FGF3 is able to act as an otic inducer in avians. We have demonstrated that misexpression of FGF3 leads to the formation of ectopic inner ears in chicken embryos. Moreover, FGF3 also controls size and morphogenesis of the inner ear. We also document that FGF2, which has been implicated in otic induction in *Xenopus*, appears to lack this capacity in chicken embryos. Finally, our results demonstrate a widespread competence of the avian surface ectoderm to respond to otic induction. Combined, these data show that FGF3 is sufficient to confer otic fate in a higher vertebrate and is likely to be the natural inducer of the inner ear in vivo.

HSV-1 based gene transfer into avian embryos

To our knowledge, this study presents the first example of the use of HSV-1-based vectors in the context of developmental biology. We would therefore like to emphasize the potential usefulness of the HSV-1-based amplicon system for gain-of-function studies of growth factors in developing avian embryos. Compared with other viral expression systems, we have observed a very early onset of high expression levels in infected embryos. β-galactosidase was first detected 4 hours after infection and widespread expression could be observed after 8 hours. Onset of protein expression in chicken embryos after infection with retrovirus (Homburger and Fekete, 1996) and adenovirus (Yamagata et al., 1994; Fisher et al., 1997) has not been detected before 9 and 12 hours, respectively. Additionally, the amplicon system allows a high level of expression of the transferred gene since, due to the packaging capacity of the virus, more than 20 copies of the pHSV*fgf3* vector are introduced into the target cell upon infection (Kwong and Frenkel, 1984). The amplicon system therefore appears advantageous for gain-of-function approaches for which a secreted factor is required within a short window of time and at a high local concentration.

Formation of otic vesicles in ectopic locations

Our analysis of ectopically induced vesicles has shown that they regionally express marker genes for the inner ear in their correct domains and are therefore otic in character (Fig. 3). In contrast, expression analysis of *cPax2* and *cNkx5-1* in otic vesicles transplanted to anterior locations have recently been shown to differ in their maintenance of expression patterns in ectopic locations (Herbrand et al., 1998). Whereas *cNkx5-1* showed its normal pattern in rostral locations to the otic vesicle, ectopic otocysts did not restore normal regionalisation of *cPax2* transcripts, but rather showed a random distribution or no expression at all. Presumably, rather than lacking environmental cues in ectopic locations, and in contrast to ectopically induced vesicles, transplanted vesicles appear to lose some internal components of their gene expression program during the transfer to the ectopic location. Ectopically expressed FGF3 therefore appears to induce a placode and to activate the intrinsic gene expression program that is necessary for the correct development of an inner ear. However, ectopic vesicles did not develop far enough to study further morphogenetic events like formation of the endolymphatic duct or sensory epithelia. As has been observed for transplanted otic vesicles (Herbrand et al., 1998), incubation of embryos for longer time periods only revealed the presence of ectopic vesicles that remained undifferentiated and lost expression of otic markers.

In agreement with experiments performed using transplanted otic vesicles (before stage 10), we never found ectopic formation of otic vesicles in wing buds infected with pHSV*fgf3* (Herbrand et al., 1998). These data clearly show that the wing bud surface ectoderm is not responsive to otic induction and also agrees with earlier reports demonstrating that morphogenesis and gene expression of otic vesicles (after stage 10) transplanted into wing buds is disturbed (Swanson et al., 1990; Herbrand et al., 1998).

Roles of FGF3 and FGF2 during vertebrate inner ear development

FGF3 apparently functions during two key steps of inner ear development in avians, namely induction of the otic placode and morphogenesis of the otic vesicle. The overgrowth of the endolymphatic duct observed in our experiments fits accurately with the major defect of inner ear development in FGF3 mutant mice, the failure to form an endolymphatic duct (Mansour et al., 1993). Unlike in mice, FGF3 has so far not been detected in the avian otic vesicle (Mahmood et al., 1995), suggesting at first sight that morphogenetic control of the otic vesicle by FGF3 appears to function differently in avians and mammals. However, expression analysis of FGF3 in *kreisler* and *Hoxa-1* mutant mice, which also lack an endolymphatic duct, has shown that early FGF3 expression in the otic vesicle is maintained, albeit absent or strongly reduced in the neighbouring hindbrain (Carpenter et al., 1993; Frohman et al., 1993; Mark et al., 1993; McKay et al., 1996). Therefore, as in mice, FGF3 is likely to control formation of the endolymphatic duct in avians via the neighbouring hindbrain. Expression of FGF3 is also absent in the hindbrain of retinoic acid-deficient quail embryos, reduced in the hindbrain of mouse mutants for retinaldehyde dehydrogenase-2 and not detected in otic vesicles of *Eya1*-deficient mice at embryonic day 10.5 (Maden et al., 1996; Niederreither et al., 1999; Xu et al., 1999). Under these conditions, otic vesicles fail to close or are arrested at a reduced size. Similarly, hypomorphic mouse mutants for the FGF receptor 2 (FGFR2), to which FGF3 binds with high affinity (Mathieu et al., 1995a), develop only rudimentary otic vesicles (Xu et al., 1998). These results and the increased proportions of otic vesicles observed in pHSV*fgf3*-infected chicken embryos in the present study indicate that FGF3 may also control the size of the developing inner ear. We have so far not obtained any indication for increased cell proliferation in oversized vesicles compared to normal otic vesicles (data not shown). Thus, the most likely cause for this abnormal phenotype is that FGF3 induces a larger than normal area of surface ectoderm to form placodal tissue. In vivo, FGF3 expression has been detected in the surface ectoderm of the preotic territory next to the hindbrain in chicken and mice (Mahmood et al., 1995, 1996; McKay et al., 1996). This, and the fact that ectopically expressed FGF3 results in the formation of ectopic otic placodes, fits nicely with the proposed role for FGF3 to act as a local inducer of the inner ear in the surface ectoderm next to the hindbrain (Represa et al., 1991; Fritzsch et al., 1998).

Our present data on FGF3 demonstrate that, in analogy to *Pax-6* or *Six3*, which induce ectopic lens fate (Oliver et al., 1996; Chow et al., 1999), a single gene can induce inner ear fate in vertebrates. The inductive role of FGF3 in vertebrates has been questioned by results obtained in transgenic mice (Mansour et al., 1993). In this case, when FGF3 was mutated by the insertion of a neomycin resistance gene in exon 1b by homologous recombination, induction of the otic placodes was found to be normal (Mansour et al., 1993). As discussed earlier, considering the pattern of FGF3 expression during inner ear and/or hindbrain development, the phenotypic consequences in these mutant mice are rather mild (Mahmood et al., 1995). The interpretation of the phenotype of these mice is somehow complicated by the fact that, instead of the expected 25% of homozygous mutants only 11% were recovered postnatally (Mansour et al., 1993). Therefore, the existence of homozygous mutants with a more severe inner ear phenotype has not been ruled out. Additionally, the inner ear phenotype

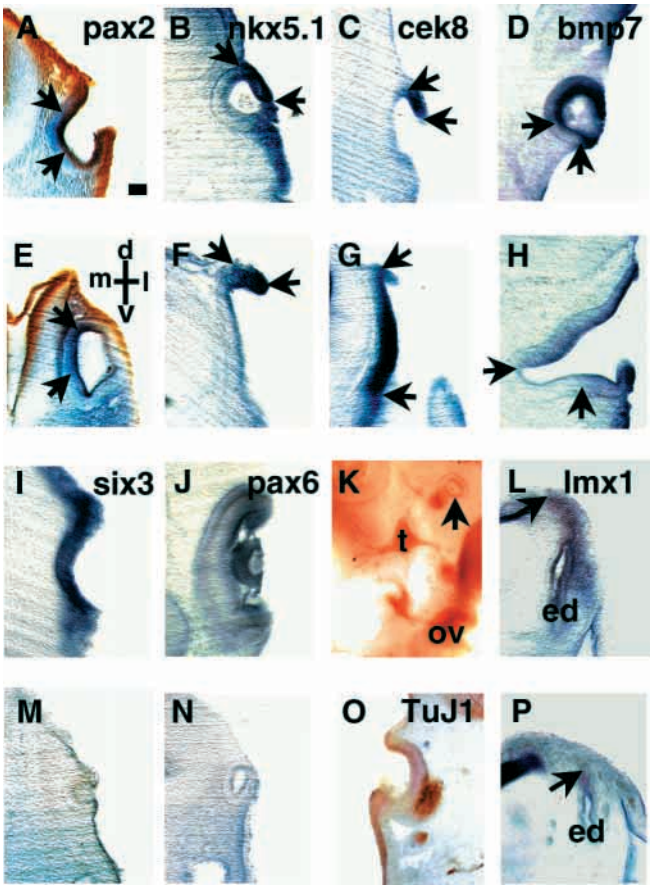

Fig. 3. Regionalisation and differentiation of the developing inner ear in pHSV*fgf3*-infected embryos. Chicken embryos were processed as whole-mounts with the indicated RNA probes or antibodies and sectioned on a vibratome. (A-H) Sections through ectopically (A-D) and normally positioned (E-H) otic cups and vesicles reveal the expression domains of otic marker genes (indicated by arrows). The orientation of the dorsoventral and mediolateral axes is shown in B. The brown background stain in A and E has been produced by a peroxidase reaction after incubation with an antibody. Note the oversized dimensions of the otic cups in F-H compared with a normally developed otic vesicle in E. (I,J,M,N) Genes expressed in the developing nose (I) and lens (J) are not expressed in ectopic inner ears (M,N). (K,O) Ectopic otic cups (arrow) form neurones (brown antibody stain) at the ventral part of the otic epithelium. The positions of the trigeminal ganglion (t) and normal otic vesicle (ov) are indicated. (L,P) The length of the endolymphatic duct (ed) in otic vesicles infected with pHSV*fgf3* (L) is increased dorsally (arrow) compared with uninfected control embryos (P). Scale bar in A corresponds to 50 μm in A-J and L-P and 150 μm in K.

of the surviving homozygotes showed variation in both penetrance and expressivity. These observations could be explained by segregation of modifying genes in a non-uniform genetic background, leaky expression of the mutant allele and/or the existence of parallel signalling pathways. Analysis of homozygous mutant embryos for FGF3 has shown the presence of a transcript comprising 60% of the 3′ portion of the cDNA at 20- to 30-fold reduced levels compared to wild-type embryos. Therefore, the possibility that the mutant FGF3 allele might represent a severe hypomorph rather than a null allele has not been completely excluded. Consequently, mice

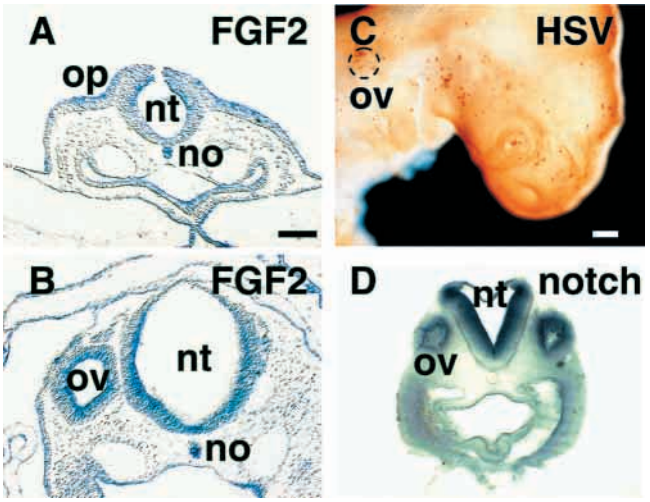

Fig. 4. Endogenous and ectopic expression of FGF2 in chicken embryos. Antibodies and RNA probes used are indicated. (A,B) Localisation of FGF2 protein in the developing inner ear and neighbouring hindbrain at stage HH11 and HH15 of embryonic development [after Hamburger and Hamilton (Bellairs and Osmond, 1998)]. FGF2 shows weak expression in the dorsal part of the otic placode (op), and then localizes to the lumenal part of the otic vesicle (ov). Expression of FGF2 is also observed in the lumenal part of the neural tube (nt) and in the notochord (no). (C) Whole-mount of an embryo (HH18) infected with pHSV*fgf2* and stained with an antibody detecting HSV-1 particles. The position of the normal otic vesicle (ov) is indicated. (D) Sections show that the otic vesicle is not oversized in pHSV*fgf2*-infected embryos stained with a *c-notch* RNA probe. Scale bar in A corresponds to 50 μm in A,B,D and 200 μm in C.

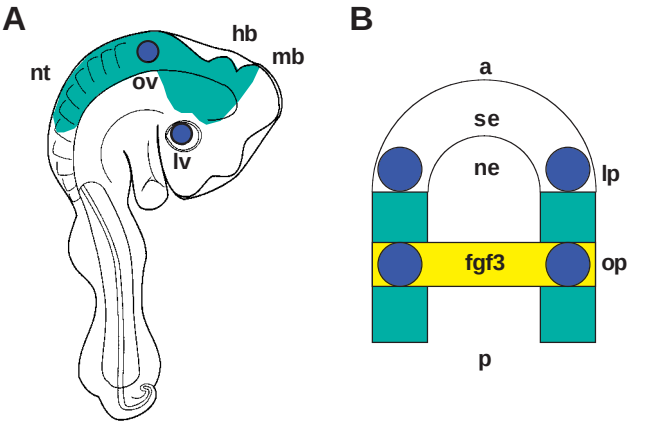

Fig. 5. Placodal competence and inner ear induction in vertebrates. (A) Ectopic expression of FGF3 reveals the presence of a large area (green; also depicted in B) anterior and posterior to the otic vesicle (ov) with the competence to respond to an induction signal. The lens vesicle (lv), midbrain (mb), hindbrain (hb) and neural tube (nt) are also indicated. (B) At the early neurula stage, a continuous stripe of surface ectoderm (se) with the capacity to form placodes surrounds the neural ectoderm (ne). The localised presence of FGF3 (yellow) along the anterior (a)-posterior (p) axis results in the induction of the otic placode (op) at its correct position and controls its size and morphogenesis of the otic vesicle. The position of the lens placode (lp) is indicated. Embryos are not drawn to scale and are modified from Torres and Giraldez (1998) and Bellairs and Osmond (1998).

that entirely lack transcripts encoding for the *fgf3* cDNA might develop a more severe phenotype affecting induction of the inner ear. Alternatively, the controversial results from gain-of-function and loss-of-function analysis of FGF3 may be explained by experiments performed in *Xenopus* embryos. In this species, FGF2 protein has been detected in the otic vesicle of the prelarval stage *Xenopus* embryo (Song and Slack, 1994). Additionally, implantation of beads coated with FGF3, but also FGF2, resulted in the formation of ectopic vesicles with some otic characteristics (Lombardo and Slack, 1998; Lombardo et al., 1998). In this context, it is worthwhile mentioning that implantation of beads coated with FGF2 induce expression of FGF3 in the dental mesenchyme of mice (Bei and Maas, 1998). It will be interesting to analyse if such an induction also takes place in the context of the FGF2-coated beads implanted in *Xenopus* embryos. However, in the present situation, the capacity of FGF3 to induce an inner ear in *Xenopus* embryos is not unique, which raises the obvious question of which FGF is the natural inducer in vivo. The fact that several members of the FGF gene family can act as inducers when expressed ectopically in avian embryos has also been observed in neural tube or limb development (Cohn et al., 1995; Alvarez et al., 1998). Moreover, as recently reported for the determination of left-right asymmetry, a single FGF can have different functions in chicken or mouse embryos (Meyers and Martin, 1999). We have addressed the potential role of FGF2 during avian inner ear induction by studying its localisation during this process. The observed expression in the otic placode and vesicle indeed suggested a direct involvement of this FGF during inner ear development. Additionally, its localisation in the hindbrain, which has been identified as an inducing tissue for the inner ear (reviewed in Torres and Giraldez, 1998), further underlined its potential role as an otic inducer. However, in contrast to FGF3 (Wilkinson et al., 1998; Tannahill et al., 1992; Mahmood et al., 1995, 1996; Lombardo et al., 1998), we found no evidence for a localised expression of FGF2 next to the inner ear within the hindbrain (data not shown). To examine the capacity of FGF2 to act as an inducer in avians, we ectopically expressed FGF2 under identical conditions and at time points when we observed the inductive capacity of FGF3. Under these experimental conditions, we found no evidence for an involvement of FGF2 during inner ear induction. In light of the fact that many members of the FGF family are functionally interchangeable (see above), this result is rather unexpected. However, at present, we cannot exclude that, under different experimental conditions, FGF2 or other FGFs may prove to act as an inducer of the inner ear in avians. Alternatively, our results may suggest that, compared to *Xenopus*, the chicken embryo shows a more restricted competence during otic induction, since it only responds to FGF3. A reasonable explanation might be that FGF3 may have become more specialised in higher vertebrates whereas, in *Xenopus*, each FGF may fulfil several functions (Kiefer et al., 1993b). A unique role for FGF2 during the induction of the inner ear in mice has also been excluded by the absence of such a phenotype in FGF2 knockout mice (Dono et al., 1998; Ortega et al., 1998). Nevertheless, the redundant functions of FGFs may explain the absence of a phenotype during otic induction in FGF3 mutant mice. Expression analysis focusing on a potential upregulation of other FGF members in these mice and production of mice mutant for combinations of FGF members may provide a more definitive answer to this issue. Alternatively, gain-of-function experiments in mice by ectopically expressing FGF3 or other FGF members should further resolve the identity of the inducer of the inner ear in mammals. Although the involvement and potential interactions with other FGF members during otic induction remains to be clarified, the expression pattern of FGF3 during inner ear development (Wilkinson et al., 1988; Tannahill et al., 1992; Mahmood et al., 1995, 1996) and our present results make this FGF the prime candidate for the natural inducer of the inner ear in lower and higher vertebrates.

Placodal competence and specification of inner ear fate by FGF3

Our experiments have also revealed that a large area of surface ectoderm is competent to form otic placodes in avian embryos (Fig. 5A). Recently, all ectodermal placodes were suggested to be derived from a common anlage (Baker et al., 1999) and a model was formulated that proposes the formation of an ectodermal stripe with multiplacodal competence in the early embryo (Torres and Giraldez, 1998), which is characterised by the expression of transcription factors such as the homeobox genes *dlx-3* or *cSix4* (Akimenko et al., 1994; Esteve and Bovolenta, 1999). Interestingly, for *dlx-3*, an essential role in the creation of such a competent stripe has been suggested by analysis of the zebrafish mutant *swirl/bmp2b*, which lacks *dlx-3* expression and does not form otic and olfactory placodes (Nguyen et al., 1998). On the other hand, ectopic expression of the murine *Six3* gene in fish embryos results in the formation of ectopic lenses in the area of the otic placode (Oliver et al., 1996). In this case, overexpression of *Six3* in the uncommitted head ectoderm has been proposed to change the bias of the otic placode towards the lens pathway by inducing a secreted factor (Oliver and Gruss, 1997). The competent zone, which forms otic placodes in response to ectopic FGF3 in avian embryos, is most likely a part of the multiplacodal ectoderm stripe, in which the usually restricted expression of this growth factor specifies the correct position of the otic placode along the embryonic axis (Fig. 5B). Competence of the surface ectoderm to induce placodes or vesicles was gradually diminished until the 12-somite stage. As suggested earlier (Gallagher et al., 1996; Torres and Giraldez, 1998), these results show an extended capacity of the competent surface ectoderm to respond to inducing signals far beyond the normal time point of induction.

Studies in several species have identified IIIb isoforms of FGF receptor 1 (FGFR1) and/or FGFR2 as the high-affinity receptors for FGF3 (Mathieu et al., 1995a,b; Kiefer et al., 1996; Ornitz et al., 1996). Although no binding studies of chicken FGF3 on FGF receptors have so far been reported, the widespread presence of FGFR1 and/or FGFR2 in the chicken surface ectoderm from the 7-somite stage onwards (Wilke et al., 1997) is likely to mediate the responses to endogenously and ectopically expressed FGF3 within the area with otic competence. It is tempting to speculate that the large area with otic competence in avians may reflect in part the much broader competence of aquatic animals to form lateral line placodes along their body axis, which give rise to sensory organs structurally and evolutionary related to the sensory patches within the inner ear (Coombs et al., 1989; Fritzsch et al., 1998). In this context, it has been suggested that lateral line placodes

are like otic placodes, which develop into otic vesicles since they lack the access to factors based on spatiotemporal differences in their location and release (Fritzsch et al., 1998). Our present study suggests that FGF3 might be the factor responsible for the different development of these two related types of placodes.

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REFERENCES

Adam, J., Myat, A., Le Roux, I., Eddison, M., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1998). Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development. *Development* **125,** 4645-4654.

Akimenko, M., Ekker, M.-A., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: Part of a homeobox gene code for the head. *J. Neurosci.* **14,** 3475-3486.

Alvarez, I. S., Araujo, M. and Nieto, M. A. (1998). Neural induction in whole chick embryo cultures by FGF. *Dev. Biol.* **199,** 42-54.

Baker, C. V., Stark, M. R., Marcelle, C. and Bronner-Fraser, M. (1999). Competence, specification and induction of Pax-3 in the trigeminal placode. *Development* **126**, 147-156.

Bellairs, R. and Osmond, M. (1998). *The Atlas of Chick Development*. London: Academic Press.

Bei, M. and Maas, R. (1998). FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. *Development* **125**, 4325-4333.

Bovolenta, P., Mallamaci, A., Puelles, L. and Boncinelli, E. (1998). Expression pattern of *cSix3*, a member of the Six/sine oculis family of transcription factors. *Mech. Dev.* **70,** 201-203.

Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi., M. R. (1993). Loss of *Hox-A1 (Hox-1.6)* function results in the reorganization of the murine hindbrain. *Development* **118,** 1063-1075.

Chow, R. L., Altmann, C. R., Lang, R. A. and Hemmati-Brivanlou, A. (1999). Pax6 induces ectopic eyes in a vertebrate. *Development* **126**, 4213-4222.

Cohn, M. J., Izpisua-Belmonte, J.-C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80,** 729-746.

Coombs, S., Görner, P. and Münz, H. (1989). *The Mechanosensory Lateral Line: Neurobiology and Evolution.* New York: Springer Verlag.

Dixon, M., Deed, R., Acland, P., Moore, R., Whyte, A., Peters, G. and Dickson, C. (1989). Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. *Mol. Cell. Biol.* **9,** 4896-4902.

Dono, R. and Zeller, R. (1994). Cell-type-specific nuclear translocation of fibroblast growth factor-2 isoforms during chicken kidney and limb morphogenesis. *Dev. Biol.* **163,** 316-330.

Dono, R., Texido, G., Dussel, R., Ehmke, H. and Zeller R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *EMBO J.* **17**, 4213-4225.

Esteve, P. and Bovolenta, P. (1999). *cSix4*, a member of the *six* gene family of transcription factors, is expressed during placode and somite development. *Mech. Dev.* **85**, 161-165.

Fekete, D. M. (1996). Cell fate specification in the inner ear. *Curr. Opin. Neurobiol.* **6,** 533-541.

Fink, D. J., DeLuca, N. A., Goins, W. F. and Glorioso, J. C. (1996). Gene transfer to neurones using herpes simplex virus-based vectors. *Annu. Rev. Neurosci.* **19,** 265-287.

Fisher, S. A., Siwik, E., Branellec, D., Walsh, K. and Watanabe, M. (1997). Forced expression of the homeodomain protein Gax inhibits cardiomyocyte proliferation and perturbs heart morphogenesis. *Development* **124,** 4405-4413.

Fritzsch, B., Barald, K. F. and Lomax, M. I. (1998). Early embryology of the vertebrate ear. In *Development of the Auditory System* (eds. E. W. Rubel, A. N. Popper and R. R. Fay) pp. 80-145. New York: Springer Verlag.

Frohman, M. A., Martin, G. R., Cordes, S. P., Halamek, L. P. and Barsh, G. S. (1993). Altered rhombomere-specific gene expression and hyoid bone differentiation in the mouse segmentation mutant, kreisler (kr). *Development* **117,** 925-936.

Gallagher, B. C., Jonathan, J. H. and Grainger, R. M. (1996). Inductive processes leading to inner ear formation during *Xenopus* development. *Dev. Biol.* **175,** 95-107 (1996).

Geller, A. I., During, M. J., Haycock, A., Freese, A. and Neve, R. (1993). Long-term increases in neurotransmitter release from neuronal cells expressing a constitutively active adenylate cyclase from a herpes simplex virus type 1 vector. *Proc. Natl. Acad. Sci. USA* **90,** 7603-7607.

Giraldez, F. (1998). Regionalized organizing activity of the neural tube revealed by the regulation of lmx1 in the otic vesicle. *Dev. Biol.* **203,** 189-200.

Goulding, M. D., Lumsden, A. and Gruss, P. (1993). Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. *Development* **117**, 1001-1016.

Grindley, J. C., Davidson, D. R. and Hill, R. E. (1995). The role of Pax-6 in eye and nasal development. *Development* **121**, 1433-1442.

Hadrys, T., Braun, T., Rinkwitz-Brandt, S., Arnold, H.-H. and Bober, E. (1998). Nkx5-1 controls semicircular canal formation in the mouse inner ear. *Development* **125,** 33-39.

Herbrand, H., Guthrie, S., Hadrys, T., Hoffmann, S., Arnold, H.-H., Rinkwitz-Brandt, S. and Bober, E. (1998). Two regulatory genes, *cNkx5-1* and *cPax2*, show different responses to local signals during otic placode and vesicle formation in the chick embryo. *Development* **125,** 645-654.

Hogan, B. L. M. (1999). Morphogenesis. *Cell* **96**, 225-233.

Homburger, S. H. and Fekete, D. M. (1996). High efficiency gene transfer into the embryonic chicken CNS using B-subgroup retroviruses. *Dev. Dyn.* **206,** 112-120.

Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R. and Kondoh, H. (1998). Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. *Development* **125,** 2521-2532.

Kiefer, P., Peters, G. and Dickson, C. (1993a). Retention of fibroblast growth factor 3 in the Golgi complex may regulate its export from cells. *Mol. Cell. Biol.* **13**, 5781-5793.

Kiefer, P., Mathieu, M., Close, M. J., Peters, G. and Dickson, C. (1993b). FGF3 from *Xenopus laevis*. *EMBO J.* **12**, 4159-4168.

Kwong, A. D. and Frenkel, N. (1984). Herpes simplex virus amplicon: Effect of size on replication of constructed defective genomes containing eucaryotic DNA sequences. *J. Virol.* **51**, 595-603.

Lim, F., Hartley, D., Starr, P., Song, S., Yu, L., Wang, Y. and Geller, A. I. (1997). Use of defective herpes-derived plasmid vectors. *Meth. Mol. Biol.* **62,** 223-232.

Lombardo, A. and Slack, J. M. W. (1998). Postgastrulation effects of fibroblast growth factor on *Xenopus* development. *Dev. Dyn.* **212,** 75-85.

Lombardo, A., Isaacs, H. V. and Slack, J. M. W. (1998). Expression and functions of FGF-3 in *Xenopus* development. *Int. J. Dev. Biol.* **42**, 1101-1107.

Maden, M., Gale, E., Kostetskii, I. and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6**, 417-426.

Mahmood, R., Kiefer, P., Gunthrie, S., Dickson, C. and Mason, I. (1995). Multiple roles for FGF-3 during cranial neural development in the chicken. *Development* **121,** 1399-1410.

Mahmood, R., Mason, I. and Morriss-Kay, G. M. (1996). Expression of *Fgf-3* in relation to hindbrain segmentation, otic pit position and pharyngeal arch morphology in normal and retinoic acid-exposed mouse embryos. *Anat. Embryol.* **194,** 13-22.

Mansour, S. L., Goddard, J. M. and Cappechi, M. R. (1993). Mice homozygous for a targeted disruption of the proto-oncogene *int-2* have developmental defects in the tail and inner ear. *Development* **117,** 13-28.

Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Olivo, J.-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119,** 319-338.

Martin, G. R. (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes Dev*. **12**, 1571-1586.

Mathieu, M., Chatelain, E., Ornitz, D., Bresnick, J., Mason, I., Kiefer, P. and Dickson, C. J. (1995a). Receptor binding and mitogenic properties of mouse fibroblast growth factor 3. Modulation of response by heparin. *J. Biol. Chem.* **13**, 24197-24203.

Mathieu, M., Kiefer, P., Mason, I. and Dickson C. (1995b). Fibroblast

growth factor (FGF) 3 from *Xenopus laevis* (XFGF3) binds with high affinity to FGF receptor 2. *J.Biol.Chem*. **270**, 6779-6787.

McCarthy, A. M., McMahan, L. and Schaffer, P. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. *J. Virol.* **63,** 18-27.

McKay, I. J., Lewis, J. and Lumsden, A. (1996). The role of FGF-3 in early inner ear development: An analysis in normal and *kreisler* mutant mice. *Dev. Biol*. **174,** 370-378.

Meyer zum Gottesberge, A. M. and Mai, J. K. (1997). Pre- and postnatal expression of glycoconjugates (3-fucosyl-N-acetyllactosamine and HNK-1 epitopes) in the mouse inner ear. *Int. J. Dev. Neurosci*. **15,** 645-656.

Meyers, E. N. and Martin, G. R. (1999). Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. *Science* **285**, 403-406.

Molea, D., Stone, J. S. and Rubel, E. W. (1999). Class III β-tubulin expression in sensory and non-sensory regions of the developing avian inner ear. *J. Comp. Neurol.* **406,** 183-198.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of *Serrate* and its relationship with *Notch* and *Delta* homologues during central neurogenesis. *Dev. Biol.* **174,** 233-247.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M. and Mullins, M. C. (1998). Ventral and lateral region of the zebrafish gastrula, including the neural crest progenitors, are established by a *bmp2b/swirl* pathway of genes. *Dev. Biol.* **199,** 93-110.

Niederreither, K., Subbarayan, V., Dollé, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.

Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116,** 1137-1150.

Nieto, M. A., Patel, K. and Wilkinson, D. G. (1995). In situ hybridisation analysis of chick embryos in whole mount and tissue sections. *Meth. Cell Biol.* **51,** 219-234.

Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U. and Gruss, P. (1990). Spatially and temporally restricted expression of *Pax2* during murine neurogenesis. *Development* **109**, 797-809.

Oh, S. H., Johnson, R. and Wu, D. K. (1996). Differential expression of bone morphogenetic proteins in the developing vestibular and auditory sensory organs. *J. Neurosci.* **16,** 6463-6475.

Oliver, G., Loosli, F., Köster, R., Wittbrodt, J. and Gruss, P. (1996). Ectopic lens induction in fish in response to the murine homeobox gene *Six3*. *Mech. Dev.* **60**, 233-239.

Oliver, G. and Gruss, P. (1997). Current views on eye development. *Trends Neurosci.* **20**, 415-421.

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G. and Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.* **271**, 15292-15297.

Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M. and Basilico, C. (1998). Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc. Natl. Acad. Sci. USA* **95,** 5672-5677.

Püschel, A., Westerfield, M. and Dressler, G. R. Comparative analysis of Pax-2 protein distributions during neurulation in mice and zebrafish.(1992). *Mech. Dev.* **38**, 197-208.

Represa, J., León, Y., Miner, C. and Giraldez, F. (1991). The *int-2* proto-oncogene is responsible for induction of the inner ear. *Nature* **353,** 561-563.

Riese, J., Zeller, R. and Dono, R. (1995). Nucleocytoplasmic translocation and secretion of fibroblast growth factor-2 during avian gastrulation. *Mech. Dev.* **49,** 13-22.

Riley, B. B., Savage, M. P., Simandl, B. K., Olwin, B. B. and Fallon, J. F. (1993). Retroviral expression of FGF-2 (bFGF) affects patterning in chick limb bud. *Development* **118**, 95-104.

Rinkwitz-Brandt, S., Arnold, H.-H. and Bober, E. (1996). Regionalized expression of *Nkx5-1, Nkx5-2, Pax2* and *sek* genes during mouse inner ear development. *Hearing Res.* **99,** 129-138.

Robinson, M. L., Ohtaka-Maruyama, C., Chan, C.-C., Jamieson, S., Dickson, C., Overbeek, P. A. and Chepelinsky, A. B. (1998). Disregulation of ocular morphogenesis by lens-specific expression of FGF-3/int-2 in transgenic mice. *Dev. Biol.* **198**, 13-31.

Song, J. and Slack, J. M. W. (1994). Spatial and temporal expression of basic fibroblast growth factor (FGF-2) mRNA and protein in early *Xenopus* development. *Mech. Dev.* **48**, 141-151.

Swanson, G. J., Howard, M. and Lewis, J. (1990). Epithelial autonomy in the development of the inner ear of a bird embryo. *Dev. Biol*. **137**, 243-257.

Tannahill, D., Isaacs, H. V., Close, M. J., Peters, G. and Slack, J. M. W. (1992). Developmental expression of the Xenopus *int-2* (fgf-3) gene: activation by mesodermal and neural induction. *Development* **115,** 695-702.

Torres, M. and Giraldez, F. (1998). The development of the vertebrate inner ear. *Mech. Dev.* **71,** 5-21.

Torres, M., Gómez-Pardo, E. and Gruss, P. (1996). *Pax2* contributes to inner ear patterning and optic nerve trajectory. *Development* **122,** 3381-3391.

Vogel, A., Rodriguez, C., Warnken, W. and Izpisua-Belmonte, J. C. (1995). Dorsal cell fate specified by chick *Lmx1* during vertebrate limb development. *Nature* **378,** 716-720.

Wang, W., Van de Water, T. and Lufkin, T. (1998). Inner ear and maternal reproductive defects in mice lacking the *Hmx3* homeobox gene. *Development* **125,** 621-634.

Wilke T. A., Gubbels, S., Schwartz, J. and Richman, J. M. (1997). Expression of fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3) in the developing head and face**.** *Dev. Dyn.* **210**, 41-52.

Wilkinson, D. G., Peters, G., Dickson, C. and McMahon, A. P. (1988). Expression of the FGF-related proto-oncogene *int-2* during gastrulation and neurulation in the mouse. *EMBO J.* **7,** 691-695.

Wu, D. K. and Oh, S. H. (1996). Sensory organ generation in the chick inner ear. *J. Neurosci.* **16**, 6454-6462.

Xu, X., Weinstein, M., Li, C., Naski, M., Cohen, R. I., Ornitz, D. M., Leder, P. and Deng C. (1998). Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* **125**, 753-765.

Xu, P.-X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). *Eya1*-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet.* **23**, 113-117.

Yamagata, M., Jaye, D. L. and Sanes, J. R. (1994). Gene transfer to avian embryos with a recombinant adenovirus. *Dev. Biol*. **166**, 355-359.

Yamaguchi, T. P. and Rossant, J. (1995). Fibroblast growth factors in mammalian development. *Curr. Opin. Gen. Dev.* **5,** 485-491.