Anat Embryol (2002) 205:365–370
DOI 10.1007/s00429-002-0264-7

ORIGINAL ARTICLE

Hakan Karabagli · Pinar Karabagli · Raj K. Ladher
Gary C. Schoenwolf

Comparison of the expression patterns of several fibroblast growth factors during chick gastrulation and neurulation

Accepted: 27 May 2002 / Published online: 4 September 2002

Abstract The fibroblast growth factor family consists of a large number of secreted polypeptide growth factors. The developmental importance of the family has been highlighted in many studies, which serve to underscore their role as local instructive signals directing diverse processes in the developing embryo. We wished to characterize and compare the expression patterns of nine members of the fibroblast growth factor family in the chick embryo. In this study, we survey the expression patterns of *fgf-2*, *-3*, *-4*, *-8*, *-10*, *-12*, *-13*, *-14* and *-18* during gastrula and neurula stages (Hamburger and Hamilton stages: 4–13). As well as providing a comparison of the expression patterns of those fibroblast growth factors already published, we provide new data on the expression patterns of some of these genes at early stages.

Keywords Chick embryos · Ectoderm · Endoderm · Hensen's node · Mesoderm · Neural plate · Neural tube · Primitive streak

Introduction

A large number of secreted growth factors comprise the fibroblast growth factor (FGF) family. Family members have diverse roles in regulating cell proliferation, migration and differentiation, and they also function as localized instructive cues that direct particular aspects of embryogenesis (Ornitz and Itoh 2001).

H. Karabagli
Department of Neurosurgery,
Haydarpasa Numune Educational Hospital, Istanbul, Turkey

P. Karabagli
Department of Pathology, SSK Goztepe Educational Hospital, Istanbul, Turkey

R.K. Ladher · G.C. Schoenwolf (✉)
Department of Neurobiology and Anatomy and Children's Health Research Center, Room 401 MREB, University of Utah,
20 North 1900 East, 84132-3401 Salt Lake City, Utah, USA
e-mail: Schoenwolf@hsc.utah.edu
Tel.: +801-581-6453, Fax: +801-581-4233

Several FGF family members are known to be expressed during early development, but a comparative study of their spatiotemporal patterns of expression during critical stages of gastrulation and neurulation is lacking. During gastrulation and neurulation, the axes of the embryo are established, germ layers are segregated and cells undergo changes in behavior resulting in the generation of morphogenetic movements. Because of the functional redundancy that occurs among FGF family members, ascertaining a precise role for individual FGFs in any of these complex processes of early development is difficult. Comparative studies of gene expression provide insight into which family members may play roles in the various developmental events occurring during early development, and they can reveal sites where multiple family members are co-expressed, suggesting functional redundancy.

Aside from the issue of redundancy, another factor, the possibility of "pseudo"-hetero-dimeric FGF molecules, makes a comparative study of the localization of FGF family members valuable. FGFs signal through cognate membrane-bound receptors, with a stoichiometric relationship of one ligand bound to one receptor, stabilized by heparin (Ornitz 2000; Pellegrini 2001). This binding causes the receptor to change conformation and allows it to be brought into close apposition with another receptor (Wiesmann and de Vos 1999). The resulting tetramer can then signal. Thus, it becomes likely that at least two FGF molecules can participate in the same signaling complex, and although the ligands are not strictly heterodimers, owing to the lack of a covalent association, they can be considered in much the same way.

In this report we characterize and compare by in situ hybridization the expression patterns of several members of the FGF family in the early chick embryo between Hamburger and Hamilton (1951) stages 4–13. The expression patterns of *fgf-2*, *-3*, *-4*, *-8*, *10*, *-12*, *-13*, *-14* and *-18* were examined in both whole mounts and sections.

Materials and methods

Chick embryos

Fertilized hen's eggs were obtained from Dunlap Hatchery, Caldwell, ID, and incubated at 38°C in humidified incubators to obtain embryos at stages 4–13 (Hamburger and Hamilton 1951). Embryos were dissected from eggs, washed in 123 mM saline and then fixed overnight in 4% paraformaldehyde, buffered with PBS.

Whole mount in situ analysis

Antisense riboprobes, labeled with digoxygenin, were transcribed from plasmids containing the relevant cDNA corresponding to a member of the FGF family. These were hybridized to fixed embryos overnight at 65°C as described previously (Ladher et al. 2000). Probes were used for the following genes (numbers in parentheses indicate the Genbank accession numbers): *fgf-2* (M95706), *-3* (Z47555), *-4* (U14654), *-8* (U41467), *-10* (D86333), *-12* (AF199602), *-13* (AF108757), *-14* (AF199606) and *-18* (AB030229).

Vibratome sectioning

Stained embryos were sectioned to aid further analysis. Embryos were embedded in a buffered solution of 30% gelatin at 60°C. Once hardened, a block of gelatin containing the embryo was excised and placed into 4% paraformaldehyde overnight at 4°C. After rinsing the block in PBS, the block was mounted onto the base plate of a Leica VT1000 vibratome, and sections were cut using a Gillette razor blade. Sections were mounted onto a glass slide, drained of excess liquid and cover slipped using Aquapolymount.

Results

Following whole mount in situ hybridization, embryos were inspected for the presence or absence of expression of a particular FGF (Table 1). Then, detailed analyses of expression patterns were undertaken (Fig. 1, Fig. 2, Fig. 3).

Table 1 The expression patterns of riboprobes for nine FGFs were examined by in situ hybridization at three groups of stages. + indicates that expression occurred at a particular group of stages; – indicates that expression was not detected. Figure numbers and panel letters illustrate results from each cell

		Stages 4–5 Fig. 1	Stages 7–9 Fig. 2	Stages 10–13 Fig. 3
Fgf-2	Panel A	+	+	+
Fgf-3	Panel B	+	+	+
Fgf-4	Panel C	+	+	+
Fgf-8	Panel D	+	+	+
Fgf-12	Panel E	+	+	+
Fgf-13	Panel F	+	+	+
Fgf-18	Panel G	+	+	+
Fgf-14	Panel H	–	+	+
Fgf-10	Panel I	–	–	+

Fgf-2

Using a construct that recognizes both *fgf-2* and the N-terminally divergent isoform *altfgf-2* (Zhu and Lough 1996), weak expression of *fgf-2* was first detected during gastrulation. At stage 4, *fgf-2* is expressed in a punctate pattern in the epiblast of and just rostral to Hensen's node (Fig. 1A). As development proceeds, expression occurs throughout the neural plate (Fig. 2A) and later, once neurulation is completed, in the rostral (forebrain/midbrain) neural tube (Fig. 3A). *Fgf-2* is also expressed in the notochord and walls of the heart tube. These results are in general agreement with previously published studies (Riese et al. 1995; Zhu and Lough 1996; Zuniga Mejia Borja et al. 1996).

Fgf-3

Expression of *fgf-3* occurs within the early head process and throughout the rostrocaudal extent of the primitive streak, with the exception of a gap just caudal to Hensen's node, which shows either weak expression or no expression (Fig. 1B; see also Mahmood et al. 1995). During neurulation, *fgf-3* continues to be expressed in the primitive streak (now throughout its rostrocaudal extent, but excluded from its ventromost portion) and in bilateral patches in the paraxial mesoderm just rostral to the first somites (Fig. 2B); slightly later, *fgf-3* is expressed in the neuroectoderm overlying the paraxial mesodermal patches. After completion of neurulation and

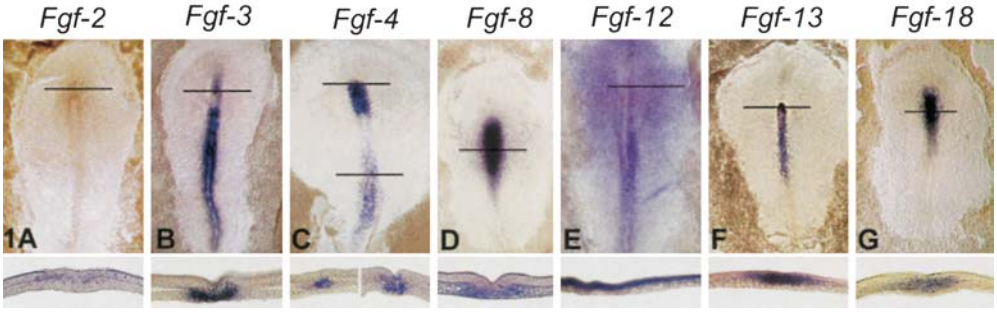

Fig. 1 Whole mounts (*top*) and transverse sections (*bottom*) after labeling by in situ hybridization using riboprobes to *fgf-2*, *-3*, *-4*, *-8*, *-12*, *-13* and *-18*. In whole mounts, rostral is at the *top* and caudal is at the *bottom* of each illustration. *Transverse lines* indicate the level at which transverse sections were taken. In **C**, the *line* near the top of the whole mount indicates the more rostral section on the left and the line near the bottom of the whole mount indicates the more caudal section on the right

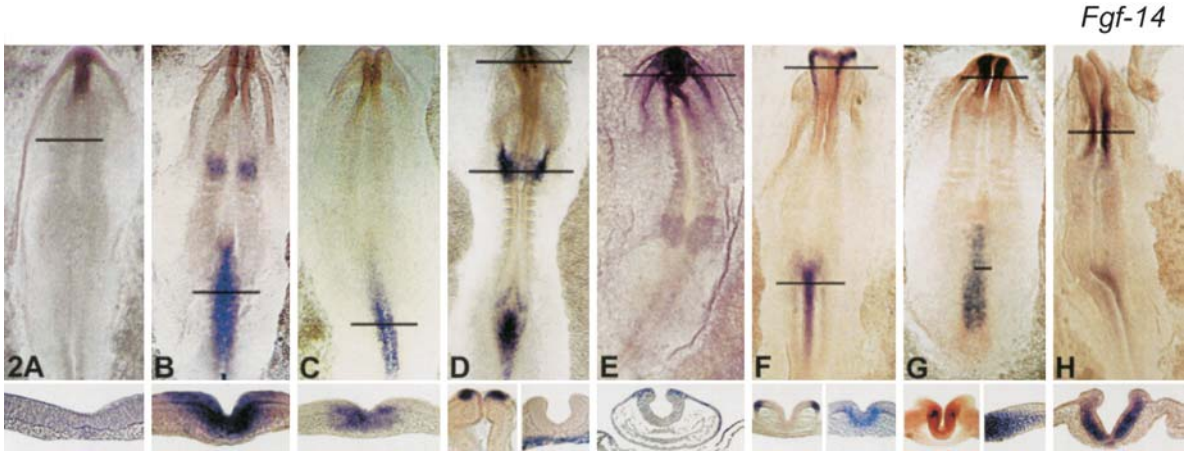

Fig. 2 Whole mounts (*top*) and transverse sections (*bottom*) after labeling by in situ hybridization using riboprobes to *fgf-2*, *-3*, *-4*, *-8*, *-12*, *-13*, *-18* and *-14*. In whole mounts, rostral is at the top and caudal is at the bottom of each illustration. *Transverse lines* indicate the level at which transverse sections were taken. In **D**, **F** and **G**, the *line* near the top of the whole mount indicates the more rostral section on the left and the *line* near the bottom of the whole mount indicates the more caudal section on the right

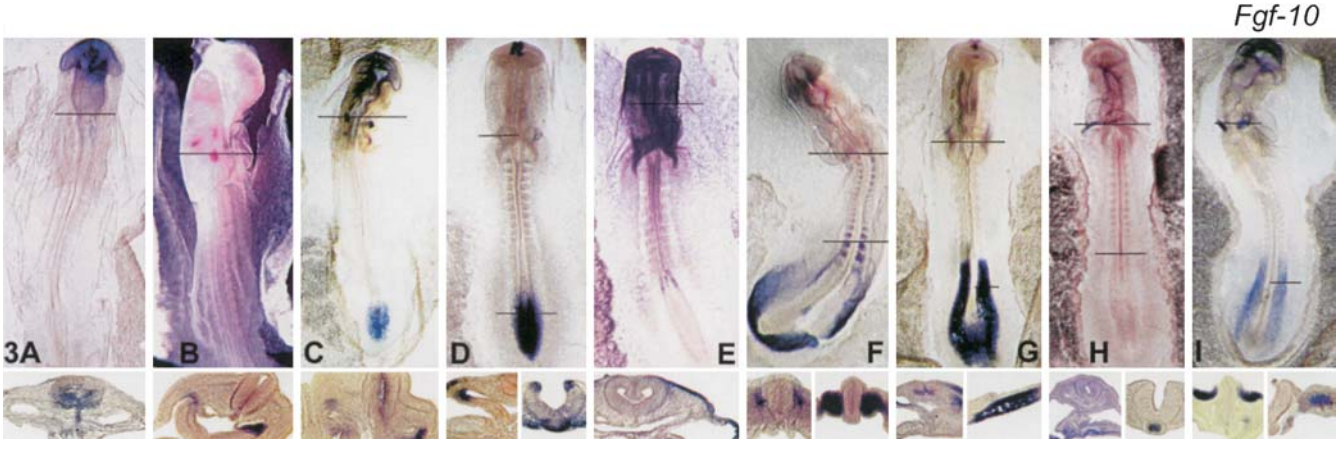

Fig. 3 Whole mounts (*top*) and transverse sections (*bottom*) after labeling by in situ hybridization using riboprobes to *fgf-2*, *-3*, *-4*, *-8*, *-12*, *-13*, *-18*, *-14* and *-10*. In whole mounts, rostral is at the top and caudal is at the bottom of each illustration. *Transverse lines* indicate the level at which transverse sections were taken. In **D**, **F**, **G**, **H** and **I**, the *line* near the top of the whole mount indicates the more rostral section on the left and the *line* near the bottom of the whole mount indicates the more caudal section on the rights

during formation of the otocyst, *fgf-3* is expressed in the ventral part of the caudal hindbrain and in the endoderm of pharyngeal pouches 1 and 3 (Fig. 3B).

Fgf-4

Expression of *fgf-4* first can be detected in the primitive streak at around stage 3 (data not shown; see also Shamim and Mason 1999). By stage 4, two distinct domains are apparent: a rostral domain encompassing Hensen's node and the early head process and a caudal domain marking cells of the caudal primitive streak (excluding its ventromost portion; Fig. 1C). By stage 7, expression occurs only in the caudal primitive streak (again, excluding its ventromost portion; Fig. 2C), which persists through stage 13 in the incipient tail bud (Fig. 3C). At stage 11 and onward, *fgf-4* is expressed in part of the pharyngeal endoderm, and by stage 13 this expression has resolved into two distinct spots marking the endoderm of pharyngeal pouches 1 and 3 (Fig. 3C). Additionally, *fgf-4* is expressed in the neuroectoderm of the forebrain through the rostral hindbrain (Fig. 3C).

Fgf-8

Expression of *fgf-8* begins early during chick embryogenesis, and the stages prior to gastrulation have already been described (Lawson et al. 2001). At stages 3–4, *fgf-8* expression occurs in the rostral half to two-thirds of the primitive streak and early ingressing mesendoderm (Fig. 1D). Expression in the streak persists through stage 10, as its rostral end begins to become incorporated into

the forming tail bud (Fig. 3D); at this stage, scattered cells of the neural plate just rostral to Hensen's node also express *fgf-8*. By stage 6 and continuing through stage 9, expression occurs in bilateral patches constituting part of the future pharyngeal endoderm caudal to the level of the cranial (anterior) intestinal portal and just rostral to the first somite (Fig. 2D). As the cranial neuropore is closing, the surrounding neural folds express *fgf-8* (Fig. 2D). At stage 11 (Fig. 3D), expression is still occurring in the neural folds at the closing cranial neuropore and in part of the pharyngeal endoderm, which has now localized to the level of pharyngeal pouches 2 and 3. Expression within the pharyngeal pouches is associated with similar patches of expression in the overlying branchial ectoderm. In other reports, the expression of *fgf-8* has been emphasized at the border between the midbrain and hindbrain regions of the neural tube from stage 8 onwards (Hidalgo-Sanchez et al. 1999a, Hidalgo-Sanchez et al. 1999b; Liu et al. 1999; Martinez et al. 1999; Ohuchi et al. 2000; Shamim et al. 1999). We chose to curtail the staining procedure shortly after staining began to prevent the other expression domains, which have not been as emphasized, from becoming over stained. Nevertheless, our results support previous studies in that we have observed that *fgf-8* is robustly expressed in the isthmus from stage 12 onwards, but at stage 9 it is considerably weaker as compared to other areas that were stained for the same length of time.

Fgf-12

Expression of *fgf-12* (Munoz-Sanjuan et al. 2000) is first detected at stage 3 in the epiblast. By stage 4, *fgf-12* is expressed in the rostral ectoderm (neural plate and epidermal ectoderm) and throughout the rostrocaudal extent of the primitive streak (Fig. 1E). As neurulation proceeds, *fgf-12* becomes largely excluded from the neural plate (except for bilateral patches flanking Hensen's node) and is expressed throughout the epidermal ectoderm (Fig. 2E). This domain moves caudally as somites form, and it is restricted to the area of the closing caudal neuropore (i.e., the rostral end of the sinus rhomboidalis) by stage 11 (Fig. 3E). Otherwise, expression of *fgf-12* is restricted to the epidermal ectoderm (Fig. 3E).

Fgf-13

Expression of *fgf-13* is detected in the stage 3 embryo in the developing primitive streak. By stage 4, expression occurs throughout the rostral two-thirds of the primitive streak (Fig. 1F), with the caudal end of the streak being devoid of transcripts. Expression within the primitive streak persists throughout neurulation (Fig. 2F), and with formation of the tail bud, expression is extinguished in this tissue (Fig. 3F). At stage 8, expression occurs throughout the neural folds, precisely at the interface between the neural and epidermal ectoderm (Fig. 2F). At stage 10, expression of *fgf-13* occurs in the walls of the rostral neural tube, somites (medial portions of rostral somites and entire somites more caudally), caudal intermediate mesoderm and caudal lateral plate (Fig. 3F; see also Munoz-Sanjuan et al. 1999). The pattern of expression of *fgf-13* in the caudal portion of the trunk is complementary to those of *fgf-18* and *fgf-10* (discussed below) in that *fgf-13* is expressed in the lateral plate mesoderm, whereas *fgf-10* is expressed in the segmental plate mesoderm.

Fgf-18

At stage 4, *fgf-18* is expressed in Hensen's node, but by stage 4+, expression extends into the notochordal precursor cells of the early head process (Fig. 1G). Subsequently, expression occurs throughout the rostrocaudal extent of the regressing primitive streak in both epiblast and ingressing mesendodermal cells (Fig. 2G). At stage 7, expression occurs in the rostral neural folds, but in contrast to the expression of *fgf-13* (see Fig. 2F), *fgf-8* is expressed more ventrally in the neuroepithelium per se, near the level of the future dorsolateral hinge points (Fig. 2G; see Colas and Schoenwolf 2001). It is worth noting that *fgf-18* and *fgf-8* are two phylogenetically closely related members of the fibroblast growth factor family (Ornitz and Itoh 2001), and by stage 10, expression of *fgf-18* within the neural folds is confined to those folds flanking the closing cranial neuropore, virtually identical to the pattern of expression of *fgf-8* at this stage (see Fig. 3D). At stage 10, several other regions of *fgf-18* expression occur (Fig. 3G; also see Ohuchi et al. 2000). The most striking is in the caudal segmental plate, but additional sites include the portion of the pharyngeal endoderm spanning pouches 2 and 3, as well as the overlying ectoderm in a pattern similar to that seen for *fgf-8* (see Fig. 3D) and the isthmus region of the neural tube (i.e., the junction between the midbrain and hindbrain).

Fgf-14

The expression of *fgf-14* is first detected at around stage 8 in the lateral walls of the forming neural tube (Fig. 2H); expression persists within the closed neural tube only within the mesencephalon level (Fig. 3H). In addition, *fgf-14* is expressed at stage 8 throughout the rostrocaudal extents of the notochord and primitive streak (Fig. 2H). At stage 12, expression occurs in a discrete domain in the ventral portion of the sinoatrial region of the heart (and underlying endoderm) and throughout the rostrocaudal extent of the trunk notochord (Fig. 3H).

Fgf-10

Expression of *fgf-10* is first detected at stage 9, weakly in the segmental plate and intermediate mesoderm, as has already been described (Ohuchi et al. 1997). By stage 10, stronger expression occurs rostrally in the otic placodes and caudally in the segmental plate mesoderm (data not shown). By stage 13 (Fig. 3I), the caudal domain of expression within the segmental plate mesoderm is more distinct, and the more rostral expression within the otic placode becomes localized to the dorso-rostral quadrant of the placode.

Discussion

We have compared the spatiotemporal patterns of expression of nine members of the fibroblast growth factor family among chick stages 4 to 13. During this period of development several key processes occur. Gastrulation results in the morphological segregation of the mesoderm, ectoderm and endoderm. During this time the neural plate is also specified and neurulation occurs, resulting in the formation of the neural tube, the rudiment of the adult central nervous system. Regional patterning also occurs, with the dorsal-ventral, rostral-caudal and left-right axes becoming specified at this time. Organ rudiments are also being formed, with the heart and somites (in addition to the neural tube) being the most obvious examples. In all of these processes, localized environmental instructions play key roles, directing and coordinating changes in cellular behavior and fate.

During gastrulation *fgf-2*, *-3*, *-4*, *-8*, *-12*, *-13* and *-18* are expressed in the midline of the embryo, all at some location within the primitive streak or in the head process. Of these, *fgf-3*, *-4* and *-12* are expressed in the caudal part of the streak. *Fgf-2*, *-3*, *-4*, *-8*, *13* and *-18* are expressed rostrally, either in Hensen's node or in the head process. It is possible that all have distinct functions during gastrulation. However, it is likely that when and where expression overlaps, individual members modify each other's effect. *Fgf-12* is expressed from stage 3 throughout the future ectoderm and remains expressed throughout the non-neural ectoderm until at least stage 13. Although functional studies have yet to be performed, it is possible that *fgf-12* acts in ectodermal competence or is involved in epidermal ectodermal differentiation because it is excluded from neural ectoderm as neurulation occurs.

Another important aspect of development at these stages is the acquisition of pattern. *Fgf-8*, *-13* and *-18* are all expressed in the anterior neural fold, a site that has been postulated to impart rostral identity on the neural plate (Crossley et al. 2001; Shimamura and Rubenstein 1997). Again, for all three to be expressed, and presumably to function, implies either a degree of redundancy or a modification of each other's action, allowing patterning of the neuroectoderm. These genes may also play a role in the mechanics of neurulation. *Fgf-8* and *-13* are both expressed in the anterior neural ridge itself, whereas *fgf-18* is expressed in the anterior dorsolateral hinge points of the neural groove; such localized growth factor function could be vital in coordinating the closure of the anterior neural plate.

Patterning of the head also commences at these stages. Recent data (Couly et al. 2002) have suggested that pharyngeal endoderm plays a role in patterning the head. *Fgf-3*, *-4*, *-8* and *-18* are all expressed in the pharyngeal endoderm and in locations that are consistent with a role in patterning the head of the embryo. Again, with respect to function, our results suggest the factors should be considered as a group that can modify each other's signaling characteristics rather than individual factors.

Acknowledgements The following generously provided us with plasmids for making riboprobes: John Lough (*fgf-2*), Ivor Mason (*fgf-3*), Gail Martin (*fgf-4* and *-8*), Sumihare Noji (*fgf-10*), Jeremy Nathan and Ignacio Munoz-Sanjuan (*fgf-12*, *-13* and *-14*) and Nobuyuki Itoh (*fgf-18*). We gratefully acknowledge the support of the Turkish Neurosurgical Society and the NIH (grant nos. NS 18112 and DC 04185).

References

Colas JF, Schoenwolf GC (2001) Towards a cellular and molecular understanding of neurulation. Dev Dyn 221: 117–145

Couly G, Creuzet S, Bennaceur S, Vincent C, Le Douarin NM (2002) Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. Development 129: 1061–1073

Crossley PH, Martinez S, Ohkubo Y, Rubenstein JL (2001) Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. Neuroscience 108: 183–206

Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J Morph 88: 49–92

Hidalgo-Sanchez M, Millet S, Simeone A, Alvarado-Mallart RM (1999a) Comparative analysis of Otx2, Gbx2, Pax2, Fgf8 and Wnt1 gene expressions during the formation of the chick midbrain/hindbrain domain. Mech Dev 81: 175–178

Hidalgo-Sanchez M, Simeone A, Alvarado-Mallart RM (1999b) Fgf8 and Gbx2 induction concomitant with Otx2 repression is correlated with midbrain-hindbrain fate of caudal prosencephalon. Development 126: 3191–3203

Ladher RK, Church VL, Allen S, Robson L, Abdelfattah A, Brown NA, Hattersley G, Rosen V, Luyten FP, Dale L, Francis-West PH (2000) Cloning and expression of the Wnt antagonists Sfrp-2 and Frzb during chick development. Dev Biol 218: 183–198

Lawson A, Colas JF, Schoenwolf GC (2001) Classification scheme for genes expressed during formation and progression of the avian primitive streak. Anat Rec 262: 221–226

Liu A, Losos K, Joyner AL (1999) FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development 126: 4827–4838

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

Martinez S, Crossley PH, Cobos I, Rubenstein JL, Martin GR (1999) FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. Development 126: 1189–1200

Munoz-Sanjuan I, Simandl BK, Fallon JF, Nathans J (1999) Expression of chicken fibroblast growth factor homologous factor (FHF)-1 and of differentially spliced isoforms of FHF-2 during development and involvement of FHF-2 in chicken limb development. Development 126: 409–421

Munoz-Sanjuan I, Fallon JF, Nathans J (2000) Expression and regulation of chicken fibroblast growth factor homologous factor (FHF)-4 at the base of the developing limbs. Mech Dev 95: 101–112

Ohuchi H, Nakagawa T, Yamamoto A, Araga A, Ohata T, Ishimaru Y, Yoshioka H, Kuwana T, Nohno T, Yamasaki M, Itoh N, Noji S (1997) The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. Development 124: 2235–2244

Ohuchi H, Kimura S, Watamoto M, Itoh N (2000) Involvement of fibroblast growth factor (FGF)18-FGF8 signaling in specification of left-right asymmetry and brain and limb development of the chick embryo. Mech Dev 95: 55–66

Ornitz DM (2000) FGFs, heparan sulfate and FGFRs: complex interactions essential for development. Bioessays 22: 108–112

Ornitz DM, Itoh N (2001). Fibroblast growth factors. Genome Biol 2: 1–12

Pellegrini L (2001) Role of heparan sulfate in fibroblast growth factor signalling: a structural view. Curr Opin Struct Biol 11: 629–634

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

Shamim H, Mason I (1999) Expression of Fgf4 during early development of the chick embryo. Mech Dev 85: 189–192

Shamim H, Mahmood R, Logan C, Doherty P, Lumsden A, Mason I (1999) Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain. Development 126: 945–959

Shimamura K, Rubenstein JL (1997) Inductive interactions direct early regionalization of the mouse forebrain. Development 124: 2709–2718

Wiesmann C, de Vos AM (1999) Putting two and two together: crystal structure of the FGF-receptor complex. Structure Fold Des 7: R251–255

Zhu X, Lough J (1996) Expression of alternatively spliced and canonical basic fibroblast growth factor mRNAs in the early embryo and developing heart. Dev Dyn 206: 139–145

Zuniga Mejia Borja A, Murphy C, Zeller R (1996) AltFGF-2, a novel ER-associated FGF-2 protein isoform: its embryonic distribution and functional analysis during neural tube development. Dev Biol 180: 680–692