Published in final edited form as:
Dev Dyn. 2011 June ; 240(6): 1537–1547. doi:10.1002/dvdy.22616.

Detection of Isoform-Specific FGF Receptors by Whole Mount in Situ Hybridization in Early Chick Embryos

Junko Nishita[1,2], **Sho Ohta**[1], **Steven B. Bleyl**[3], and **Gary C. Schoenwolf**[1,3]

[1] Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, Utah, USA

[3] Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, Utah, USA

Abstract

We have developed 'b' and 'c' isoform-specific chicken FGF receptor 1–3 probes for in situ hybridization. We rigorously demonstrate the specificity of these probes by using both dot blot hybridization and whole mount in situ hybridization during neurulation and early postneurulation stages, and we compare expression patterns of each of the three isoform-specific probes to one another and to generic probes to each of the three (non-isoform-specific) FGF receptors. We show that the expression pattern of each receptor is represented by the collective expression of each of its two isoforms, with the expression of each FGF receptor being most similar to that of its 'c' isoform at two of the three stages studied, and that tissue and stage differences exist in the patterns of expression of the six isoforms. We demonstrate the usefulness of these probes for defining the differential tissue expression of FGF receptor 1–3 isoforms.

Keywords

FGFs; Isoforms; FGF Receptors; In Situ Hybridization; Dot Blots

INTRODUCTION

Whole mount in situ hybridization has been widely used to investigate patterns of gene expression during embryogenesis (e.g., see the online chick expression database GEISHA: http://geisha.arizona.edu/geisha; also see links to expression databases of other species at: http://geisha.arizona.edu/geisha/links.jsp?category=5; Bell et al., 2004; Darnell et al., 2007). Particularly useful for understanding signaling during development are comprehensive expression studies comparing transcripts of multiple members of a family of ligands across several stages or different species, as well as the comparison of patterns of expression of transcripts of both ligands and their receptors or other downstream or related components. Owing to the typically high conservation of nucleotide sequence between family members, synthesizing specific probes for in situ hybridization can be challenging. This is especially true for isoforms of a gene.

The fibroblast growth factor (FGF) family plays critical roles in development by regulating cell proliferation, differentiation, and cell migration, and FGF signaling directs the development of a number of organ rudiments (Goldfarb, 1996; Ornitz and Itoh, 2001;

Author for correspondence: Gary C. Schoenwolf, Ph.D., Department of Neurobiology and Anatomy, University of Utah School of Medicine, 30 N. 1900 E., Room 2R066, Salt Lake City, Utah, 84132-3401, Schoenwolf@neuro.utah.edu, Phone: 801-581-6453, Fax: 801-581-8852.
[2]Current address: Junko Nishita, National Center for Child Health and Development, Tokyo, Japan

Böttcher and Niehrs, 2005; Itoh and Ornitz, 2010). The FGF family consists of 22 ligands in mammals and 4 receptors (R1-4), with receptors 1–3 each having two isoforms, termed 'b' and 'c' isoforms (Ornitz and Itoh, 2001). Although studies of the expression of transcripts for many of the FGF ligands have been conducted during early chick development (e.g., Karabagli et al., 2002a, b; Paxton et al., 2010), as well as those for FGF receptors 1–3 (Walshe and Mason, 2000; Lunn et al., 2007), expression of transcripts for isoform-specific FGF receptors have not been described. Thus, progress in understanding potential roles for these isoforms in FGF signaling during early chick development has been hindered.

We have developed isoform-specific chick FGF receptor probes to detect FGF receptors 1–3 by whole mount in situ hybridization. We describe their patterns of expression in chick embryos during neurulation and early postneurulation stages, and we demonstrate that at these stages 'b' and 'c' isoforms have different patterns of expression that change over time, suggesting potentially unique roles for individual isoforms during early development.

RESULTS AND DISCUSSION

FGF receptor isoforms are structurally similar

FGF receptors (FGFRs) contain several common structural features including a hydrophobic leader sequence, three immunoglobulin–like (Ig) domains, an acidic box, a cell adhesion molecule homology domain, a transmembrane domain, and a divided tyrosine kinase domain (Fig. 1A). FGFRs normally exist as inactive monomers, and upon ligand binding, they become dimerized and activated (Spivak-Kroizman et al., 1994). Activated FGFRs serve as binding sites for proteins with SH2 domains (Mohammadi et al., 1991). The recruitment of SH2 domain proteins results in phosphorylation and activation of downstream signaling intermediates that together trigger a network of complex signal transduction cascades (Eswarakumar et al., 2005).

An intriguing feature of FGFRs is that a variety of isoforms are generated by alternative splicing of *FGFR* transcripts. Three exons encode Ig domains IIIa, b, and c of FGFRs (Givol and Yayon, 1992; Yayon et al., 1992) with two undergoing alternative splicing, such that the Ig domain III of FGFRs consists of either IgIIIa/IgIIIb or IgIIIa/IgIIIc, which constitutes the 'b' and 'c' isoforms of FGFRs, respectively (Fig. 1B). Alternative splicing in Ig domain III affects ligand-binding specificity (Miki et al., 1992; Yayon et al., 1992). For example, FGFR1b shows an affinity for binding with FGF3 and FGF10, but not with FGF4 and FGF6, whereas FGFR1c shows an affinity for binding with FGF4 and FGF6, but not with FGF3 and FGF10. Furthermore, although FGFR2b binds with FGF3 and FGF10, but not with FGF4 and FGF6, the FGFR2c isoform binds with FGF4 and FGF6, but not with FGF3 and FGF10 (Eswarakumar et al., 2005). Comparison of the nucleotide sequence of the exons encoding IIIb/c (Fig. 1C, D) showed that *FGFR1b* and *FGFR2b* have a higher sequence identity (Fig. 1C: 76%) than each has with its 'c' isoform (Fig. 1C: 49% and 51%, respectively), and *FGFR1c* and *FGFR2c* have a higher sequence identity (Fig. 1C: 82%) than each has with its 'b' isoform, consistent with the preferred combinations of receptor and ligand binding described above. Among the 'b' isoform of the FGF receptors, *FGFR1b* and *FGFR2b* showed the highest sequence identity (Fig. 1C: 76%; pink box), and among the 'c' isoform of the receptor, all three receptors share more than 75% sequence identity (Fig. 1C, blue boxes, 82%, 76%, 76%).

Nucleotide alignment within the alternative spliced region for several vertebrates is included in Supplemental Figure 1. The figure demonstrates a high level of nucleotide identity in this region among species.

Isoform-specific probes hybridize with high specificity in dot blots

As just discussed, analysis of nucleotide sequence revealed a high degree of identity among some of the FGF receptor isoforms. It has been reported that if a non-target sequence has >70–80% identity to the target sequence, the probe generated against the target sequence might hybridize indiscriminately to the non-target sequence (Evertsz et al., 2001; Xu et al., 2001). To test the specificity of our riboprobes, we performed dot blot hybridization.

Dot blot membranes containing DNA templates of all six of the ‘b’ and ‘c’ isoforms of FGF receptors 1–3 were hybridized with each of our candidate isoform-specific riboprobes. All of our candidate isoform-specific riboprobes hybridized strongly with the appropriate template (Fig. 2), demonstrating their specificity. However, at the highest probe concentrations some non-specific hybridization occurred. For example, although our *FGFR1b* isoform riboprobe strongly recognized the *FGFR1b* target sequence, it also hybridized weakly to the *FGFR1c* template at the highest probe concentrations (Fig. 2A, left panel), despite having less than 50% sequence identity (Fig. 1C). The hybridization affinity of our *FGFR1b* probe to *FGFR1b* template was about 5-fold greater than that of our *FGFR1b* isoform probe to *FGFR1c* template (Fig. 2A, right panel). Similarly, our other isoform probes, particularly our *FGFR1c* (Fig. 2B, left panel), *FGFR2c* (Fig. 2D, left panel), and *FGFR3b* (Fig. 2E, left panel) probes, weakly hybridized with blots other than those that contained target sequence; again, hybridization was significantly greater with the target sequence (3–10 fold; Figs. 2B, D, and E, right panels). Interestingly, in only one of these three cases did cross-hybridization occur between probe and template having greater than 70% sequence identity (82%: *FGFR1c* probe to *FGFR2c* template; Fig. 1C). Thus, the strong hybridization of our riboprobes to their target DNA and their minimal and significantly weaker (i.e., only at the highest probe concentrations) hybridization to non-target DNA suggested that our probes were isoform-specific and likely to provide reliable results for whole mount in situ hybridization.

Overview of the tissue specificity of ‘b’ and ‘c’ isoforms of FGF receptors

It is generally believed that in developing organs, ‘b’ isoforms of FGF receptors are expressed within epithelial tissues, whereas ‘c’ isoforms are expressed within mesenchymal tissues (e.g., reviewed by Ornitz et al., 1996; Ornitz and Itoh, 2001). This tissue relationship seems to apply specifically for *FGFR2* isoforms, but not necessarily for other *FGFRs* (e.g., *FGFR3* isoforms; Scotet and Houssaint, 1995). As detailed below for early stages of chick development, this tissue restriction in isoform expression did not exist. For example, in HH stage 9 embryos, both the ‘b’ and ‘c’ isoforms of *FGFR1*, *2*, and *3* were expressed in the neural tube—an epithelial structure (Figs. 3B, C; 3E, F; 3H, I). However, expression of the ‘b’ isoform was consistently weaker and more spatially restricted than that of the ‘c’ isoform. Similarly, at HH stage 13, both the ‘b’ and ‘c’ isoforms of FGFR1, 2, and 3 were expressed in the neural tube, with the expression of the ‘b’ isoform being consistently weaker and more spatially restricted than that of the ‘c’ isoform (Figs. 4B, C; 4E, F; 4H, I). At both HH stage 9 and 13, isoform expression (as well as generic *FGFR* expression), was largely restricted to epithelial tissue, rather than mesenchyme, including areas of epithelialized mesoderm such as the developing presomitic mesoderm (somites) or lateral plate mesoderm (e.g., HH stage 9: Figs. 3f.2′, i.2″; HH stage 13: Figs. 4C.2‴, 4i.2″, 4i2″). Interestingly, only the ‘c’ isoform was expressed in epithelialized mesoderm, revealing at least some tissue specificity at these early stages.

In later stages of chick development, a higher level of tissue specificity exists. For example, in HH stage 24 chick wing buds, as assessed with RT-PCR, *FGFR2b* is expressed only in the ectoderm, whereas *FGFR2c* and *FGFR3c* are expressed only in the mesenchyme. In contrast, *FGFR1b*, *FGFR1c*, and *FGFR3c* are expressed in both ectoderm and mesenchyme

(Sheeba et al., 2010). Moreover, in the developing chick mandible (HH stages 22–30), as assessed with section in situ hybridization, *FGFR2b* is expressed mainly in the epithelium, whereas *FGFR2c* is expressed in the mesenchyme, including Meckel's cartilage (Havens et al., 2006). Furthermore, in chick proventriculus and gizzard (6.5 days of incubation), as assessed with RT-PCR, *FGFR1b* and *FGFR2b* are expressed in the epithelium, whereas *FGFR2c* and *FGFR3c* are mainly expressed in the mesenchyme; *FGFR1c* is expressed at high levels in both the epithelium and mesenchyme (Shin et al., 2005).

Table 3 summarizes the detailed patterns of expression of generic and 'b' and 'c' isoform of FGF receptors at HH stages 7, 9, and 13. A more detailed description of these patterns at each HH stage studied is given below.

In situ hybridization of *FGFR* isoform riboprobes in HH stage 9 chick embryos reveals unique patterns of expression of various probes

To begin to ask whether our isoform-specific *FGFR* riboprobes had unique patterns of expression in developing embryos, we performed whole mount in situ hybridization on HH stage 9 chick embryos. The generic *FGFR1* riboprobe (like the generic *FGFR2* and *FGFR3* riboprobes) was generated against sequence outside of the alternatively spliced region to recognize both isoforms. Generic *FGFR1* was expressed throughout the newly formed neural tube along its entire craniocaudal extent, and extended into the primitive streak (Fig. 3A). The expression of the *FGFR1b* isoform was markedly different than that of generic *FGFR1*, being restricted to a short craniocaudal span of the hindbrain (Fig. 3B). In contrast, expression of the *FGFR1c* isoform essentially mirrored that of generic *FGFR1* (Fig. 3C), clearly showing that most of the expression pattern observed with the generic *FGFR1* probe was due to the expression of the *FGFR1c* isoform, and that the *FGFR1b* and *FGFR1c* expression patterns were additive, collectively accounting for the generic *FGFR1* expression pattern.

Generic *FGFR2*, like generic *FGFR1*, was also expressed throughout most of the craniocaudal extent of the neural tube, beginning cranially in the caudal half of the forebrain and stopping caudally before extending into the primitive streak. In addition, generic *FGFR2* was expressed in the head mesenchyme flanking the developing brain, and in the caudal part of the straight heart tube (sinoatrial region at the anterior intestinal portal). Expression of the *FGFR2b* isoform was restricted to the brain (beginning cranially in the caudal half of the forebrain) and cranial spinal cord (Fig. 3E), whereas expression of the *FGFR2c* isoform mirrored that of generic *FGFR2*, occurring throughout the craniocaudal extent of the neural tube (with cranial and caudal borders essentially the same as those of generic *FGFR2*) and in the head mesenchyme and caudal heart tube (Fig. 3F). This again shows (as for *FGFR1*) that most of the expression pattern observed with the generic *FGFR2* probe was due to the expression of the *FGFR2c* isoform, and that the *FGFR2b* and *FGFR2c* expressions patterns were additive.

Generic *FGFR3* was expressed throughout most of the craniocaudal extent of the neural tube, from the caudal forebrain to the spinal cord up to the level of the last formed somite (Fig. 3G). In addition, generic *FGFR3* was expressed in the last 6 or so formed somites, in the flanking nephrotome (intermediate mesoderm), and in two wings of lateral plate mesoderm that extended cranially from the most cranially expressing somite pair. Expression of the *FGFR3b* isoform was restricted to the brain and spinal cord, with a gap in expression at the hindbrain level, and in the caudal 2–3 somite pairs (Fig. 3H). Expression of the *FGFR3c* isoform (Fig. 3I) closely paralleled that of generic *FGFR3*, again showing (as for *FGFR1* and *FGFR2*) that most of the expression pattern observed with the generic *FGFR3* probe was due to the expression of the *FGFR3c* isoform, and that the *FGFR3b* and *FGFR3c* expression patterns were additive.

Next, we compared in detail the gene expression patterns of probes that showed high sequence identity. Although the *FGFR1b* and *FGFR2b* isoforms had 76% sequence identity, their patterns of expression were very different. *FGFR1b* expression was restricted to the hindbrain (Fig. 3b.1), whereas *FGFR2b* was expressed throughout the brain, except in the cranial forebrain (Fig. 3e.1). Comparison of sections at the midbrain and hindbrain levels showed that *FGFR1b* was uniquely expressed in the extraembryonic endoderm at the midbrain level, and not in the neuroectoderm (Fig. 3b.1′), whereas *FGFR2b* was expressed in the neuroectoderm of the midbrain, and not in the endoderm (Fig. 3e.1′). Furthermore, *FGFR1b* was expressed strongly in the ventral hindbrain neuroectoderm, and weakly (arrow) in the ventral midline endoderm of the foregut (Fig. 3b.1″), whereas *FGFR2b* was expressed weakly in the hindbrain neuroectoderm, and strongly in the ventral midline endoderm of the foregut (Fig. 3e.1″).

FGFR1c and *FGFR2c*, the isoforms with the highest sequence identity (82%), also had significantly different expression patterns. Although both transcripts were broadly expressed in the brain (Figs. 3c.1, 3f.1), *FGFR1c* expression extended to the cranial end of the forebrain neuroectoderm, but not into the lateral extremes of the optic vesicles (Fig. 3c.1), whereas *FGFR2c* expression extended only to the caudal half of the forebrain, but also laterally into the optic vesicles (Fig. 3f.1). In contrast to *FGFR1c*, *FGFR2c* was also expressed in the head mesenchyme (Fig. 3f.1′), caudal heart tube, and the endoderm of the underlying anterior intestinal portal (Fig. 3f.1″). Finally, both probes were expressed in the ventral midline endoderm of the foregut (thyroid rudiment; Figs. 3c.1″ and f.1″).

Differences also existed in the expression patterns of *FGFR1c* and *FGFR3c* (76% sequence identity) and *FGFR2c* and *FGFR3c* (76% sequence identity). The most notable difference between the expression patterns of *FGFR1c* and *FGFR3c* was that *FGFR1c* was expressed only in the neuroectoderm of the spinal cord, whereas *FGFR3c* was expressed both in the spinal cord and the flanking mesoderm (cf. Figs. 3c.2, 3c.2′, and 3c.2″ with Figs. 3i.2, 3i.2′, and 3i.2″). Similarly, although both *FGFR2c* and *FGFR3c* were expressed in the neuroectoderm of the spinal cord, expression of *FGFR3c* stopped at the level of the last formed somites, whereas expression of *FGFR2c* extended to the level of the primitive streak. Moreover, FGFR3c was expressed within three subdivisions of trunk mesoderm—somite, lateral plate mesoderm, and nephrotome (Figs. 3f.2, f.2′, f.2″)—whereas *FGFR2c* was expressed only in the lateral plate mesoderm (Figs. 3i.2, i.2′, i.2″).

Unique patterns of *FGFR* isoform riboprobe expression are maintained in HH stage 13 chick embryos

Both generic *FGFR1* and *FGFR2* continued to be uniformly expressed throughout most of the craniocaudal extent of the neural tube in HH stage 13 chick embryos (Figs. 4, A, D). Moreover, the patterns of expression of these two receptor probes were mirrored by the expression patterns of the *FGFR1c* and *FGFR2c* probes (Figs. 4C, F), but not by that of the *FGFR1b* and *FGFR2b* probes (Figs. 4B, E), which were considerably more restricted. A truly unique feature of *FGFR1b* at this stage is that it was also expressed in the otic cups (arrows, Fig. 4B), as no other *FGFR* probe was expressed in these structures.

In the mouse embryo, the expression of two isoform-specific FGF receptors, *FgfR2c* and *FgfR3c*, have been examined by in situ hybridization. In contrast to the chick embryo, in which only *FGFR1b* is expressed, both *FgfR2c* and *FgfR3c* are expressed in the otic cups and early otic vesicles (Wright et al., 2003).

Generic *FGFR3* expression was considerably more restricted at HH stage 13 than that of the other two generic probes (cf. Figs. 4A and 4D with Fig. 4G), but again, the generic *FGFR3* pattern was largely mirrored by the expression of the *FGFR3c* probe (Fig. 4I), with the

FGFR3b probe being expressed only in the caudal hindbrain/cranial spinal cord (Fig. 4H, asterisk). Also, unlike generic *FGFR1* and *FGFR2* probes at this stage, the generic *FGFR3* and the *FGFR3c* probes were robustly expressed in lateral plate mesoderm (somatic mesoderm in particular) and nephrotome (Figs. 4G, I).

As at HH stage 9, detailed examination at HH stage 13 of the expression patterns of the isoforms with the highest sequence identify, showed unique patterns of expression. Whole mounts and sections through various levels of the brain showed that *FGFR1b* was expressed in the otic pits, whereas *FGFR2b* was expressed in the thyroid rudiment (cf. Figs. 4b.1, 4b.1′, and 4b.1″ with Figs. 4e.1, 4e.1′, and 4e.1″). Similarly, *FGFR1c* and *FGFR2c* had different expression patterns in the diencephalon and developing optic cups (cf. Figs. 4c.1 and 4c.1′ with Figs. 4f.1 and 4f.1′), and the expression patterns of the two other isoforms with high sequence identity differed at the spinal cord level, including the caudal extent of isoform expression in the neural tube and the particular mesodermal subdivision in which each isoform was expressed (cf. Figs. 4c.2 and sections with Figs. 4i.2 and sections; cf. Figs. 4f.2 and sections with Figs. 4i.2 and sections).

FGFR isoform riboprobe expression at later stages of chick development

The expression of isoform-specific FGF receptors has been examined in chick by in situ hybridization at later stages. This includes *FGFR2b* and *FGFR2c* in the developing chick mandible (Havens et al., 2006) and *FGFR1b*, *FGFR1c*, *FGFR3b*, and *FGFR3c* in the developing forelimb bud (Sheeba et al., 2010).

Patterns of receptor isoform expression in neurula stage embryos (HH stage 7) are less unique than at later stages

At neurula stages, all three FGF receptors were expressed almost exclusively within the neuroectoderm, with generic *FGFR1* and *FGFR2* being expressed throughout the craniocaudal extent of the neuraxis (Figs. 5A, D), and generic *FGFR3* being restricted to the midbrain/forebrain and caudal hindbrain/cranial spinal cord levels (Fig. 5G). Our results using generic *FGFR1*, *FGFR2*, and *FGFR3* probes confirmed previous published patterns of FGF receptors at these stages (Walshe and Mason, 2000; Lunn et al., 2007). In contrast to later stages (discussed above), the patterns of expression of the generic *FGFR1* and *FGFR3* probes were mirrored by the expression of both the *FGFR1b* and *FGFR3b* probes, respectively, and that of the *FGFR1c* and *FGFR3c* probes, rather than by just by the ‘c’ isoform probes alone, although for *FGFR2*, the pattern of expression of the ‘c’ isoform (and not the ‘b’ isoform) mirrored that of the generic *FGFR2* pattern, as at later stages. Sections revealed more subtle differences (Figs. 5a-i, .1-.4). In particular, the *FGFR1b* probe was expressed in the future ectoderm of the otic placode (Fig. 5b.3), as well as more rostrally along the midbrain and forebrain, presaging its expression in the otic pits at stage 13. Moreover, some probes were expressed in extraembryonic tissues (generic *FGFR2*: arrows Figs. 5d.1-d.3; *FGFR2c*: arrows Figs. 5f.1-f.4; generic *FGFR3*: arrows Figs. 5g.1-g.3; *FGFR3c*: arrows Figs. 5i.1, i.2), somites (generic *FGFR3*: Figs. 5g.3, g.4; *FGFR3c*: Figs. 5i.3, i.4), or embryonic endoderm (and some overlying tissue) (generic *FGFR2*: arrows Figs. 5d.2, d.3; *FGFR2c*: arrows Figs. 5f.2, f.3).

Similarly, in mouse at comparable (i.e., gastrula/neurula) stages, considerable overlap occurs in the expression patterns of *Fgfr2* and its ‘b’ and ‘c’ isoforms (Orr-Urtreger al., 1991, 1993). Thus, in both mouse and chick, expression patterns of the generic and isoform-specific *Fgf* receptors at these stages are less unique.

EXPERIMENTAL PROCEDURES

Cloning of FGF receptor isoforms

Primers were designed (Table 1) using the *Gallus gallus* reference genome sequence (May 2006; WUGSC 2.1/galGal3) to amplify each of the FGF receptor isoforms by reverse transcription PCR from total RNA of whole chick embryos at HH stage 6–7 (total RNA isolation: QIAGEN RNeasy Micro kit, RT-PCR: Invitrogen SuperScript One-Step RT-PCR with Platinum *Taq*). The resulting DNA sequences, containing both isoform-specific and flanking regions of each FGF receptor, were ligated into pCRII-TOPO vector (Invitrogen) and were sequenced. New primers (Table 2) were designed based on this sequencing and were used to amplify only the smaller isoform-specific regions of each FGF receptor, which were cloned into pCRII-TOPO vectors. These plasmids were used as template DNA for both dot blot hybridization and for digoxigenin (DIG)-labeled RNA probe synthesis.

During preparation of this manuscript, a paper appeared online describing the cloning of 4 chicken FGF receptor isoforms and their expression, along with the remaining two isoforms, by in situ hybridization in the developing wing bud (Sheeba et al., 2010).

Standardized chicken gene nomenclature was used throughout the text, as described previously (Burt et al., 2009).

Dot blot hybridization

To minimize contamination of bacterial sequence derived from pCRII-TOPO vector, template DNA for dot blot hybridization was prepared by amplifying the specific region of each isoform by PCR (primers shown in Table 2). Each PCR product was purified by QIAquick Gel Extraction (QIAGEN). DIG-labeled RNA probe synthesis was done using a Digoxigenin RNA labeling kit (Roche) according to the manufacturer's instructions.

Dot blot hybridization was done using standard methods. Briefly, nitrocellulose membranes (GE Healthcare) were presoaked in sterilized water and briefly dried at room temperature. Template DNA containing the sequence of each individual FGF receptor isoform was serially diluted with sterilized water and 1μl of each diluted template was applied onto the membrane and dried at room temperature. Template DNA was denatured in a solution of 1.5M NaCl and 0.5N NaOH, neutralized in a solution of 1.5M NaCl and 1.5M Tris-HCl (pH 7.5), and then rinsed with 2xSSC. Membranes were heated at 70 for 2 hours to crosslink the spotted DNA. The membranes were then prehybridized with DIG Easy hyb solution (Roche) at 55°C for 30 min, the prehybridization solution was decanted, and the preheated DIG Easy hyb solution containing the appropriate RNA probe (at 0.5μg/ml) was added to the membrane and then hybridized at 55°C for 3 hours. After hybridization, the membranes were rinsed with a solution containing 2xSSC and 0.1% SDS at room temperature and washed with a solution containing 0.1xSSC and 0.1% SDS at 68°C. After stringency washes, the membranes were briefly washed with washing buffer containing 0.1M Maleic acid, 0.15M NaCl, and 0.3% Tween 20 (pH 7.5) at room temperature. The membranes were incubated for 30 min in blocking solution containing 1.0% Blocking reagent (Roche), and they were then incubated in blocking solution containing a 1:10000 dilution of anti-DIG-AP antibody. The membranes were washed with washing buffer and equilibrated for 15 min in detection buffer containing 0.1M Tris-HCl (pH 9.5) and 0.1M NaCl. Finally, the membranes were soaked in a 1:200 dilution of CSPD solution (Roche) and exposed to X-ray film to detect the signals.

In situ hybridization for gene expression analysis

Fertilized White Leghorn chicken eggs were purchased from Merrill's Poultry Farm (Paul, Idaho, USA) and incubated at 38.5°C until embryos reached the desired stages according to the criteria of Hamburger and Hamilton (1951; HH stages; reprinted as Hamburger and Hamilton, 1992). Embryos were fixed with 4% PFA/PBS overnight at 4 and processed for in situ hybridization and subsequent vibratome sectioning (Chapman et al., 2002). For in situ hybridization, the following modifications were made from the published protocol: hybridization was done at 65°C over night, with a probe concentration of 0.5μg/ml; the use of proteinase K was omitted; 2–30 min washes were done with Solution X; and the color reaction was allowed to occur for 7–10 days. Finally, embryos in each group of stages were hybridized in parallel under identical conditions using the three generic and six isoform-specific *FGFR* probes.

Image levels, brightness, and contrast were adjusted uniformly across each photograph using Photoshop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the essential financial support of this project from grant no. DC004185 from the NIH, and the helpful discussion of our colleagues.

References

Bell GW, Yatskievych TA, Antin PB. GEISHA, a whole-mount in situ hybridization gene expression screen in chicken embryos. Dev Dyn. 2004; 229:677–687. [PubMed: 14991723]

Böttcher RT, Niehrs C. Fibroblast growth factor signaling during early vertebrate development. Endocr Rev. 2005; 26:63–77. [PubMed: 15689573]

Burt DW, Carrë W, Fell M, Law AS, Antin PB, Maglott DR, Weber JA, Schmidt CJ, Burgess SC, McCarthy FM. The Chicken Gene Nomenclature Committee report. BMC Genomics. 2009; 10(Suppl 2):S5. [PubMed: 19607656]

Chapman SC, Schubert FR, Schoenwolf GC, Lumsden A. Analysis of spatial and temporal gene expression patterns in blastula and gastrula stage chick embryos. Dev Biol. 2002; 245:187–199. [PubMed: 11969265]

Darnell DK, Kaur S, Stanislaw S, Davey S, Konieczka JH, Yatskievych TA, Antin PB. GEISHA: an in situ hybridization gene expression resource for the chicken embryo. Cytogenet Genome Res. 2007; 117:30–35. [PubMed: 17675842]

Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 2005; 16:139–149. [PubMed: 15863030]

Evertsz EM, Au-Young J, Ruvolo MV, Lim AC, Reynolds MA. Hybridization cross-reactivity within homologous gene families on glass cDNA microarrays. BioTechniques. 2001; 31:1182, 1184, 1186. passim. [PubMed: 11730025]

Givol D, Yayon A. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. FASEB J. 1992; 6:3362–3369. [PubMed: 1464370]

Goldfarb M. Functions of fibroblast growth factors in vertebrate development. Cytokine Growth Factor Rev. 1996; 7:311–325. [PubMed: 9023055]

Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. 1951. Dev Dyn. 1992; 195:231–272. [PubMed: 1304821]

Havens BA, Rodgers B, Mina M. Tissue-specific expression of Fgfr2b and Fgfr2c isoforms, Fgf10 and Fgf9 in the developing chick mandible. Arch Oral Biol. 2006; 51:134–145. [PubMed: 16105644]

Itoh N, Ornitz DM. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J Biochem. 2010 in press.

Karabagli H, Karabagli P, Ladher RK, Schoenwolf GC. Comparison of the expression patterns of several fibroblast growth factors during chick gastrulation and neurulation. Anat Embryol. 2002a; 205:365–370. [PubMed: 12382140]

Karabagli H, Karabagli P, Ladher RK, Schoenwolf GC. Survey of fibroblast growth factor expression during chick organogenesis. Anat Rec. 2002b; 268:1–6. [PubMed: 12209559]

Lunn JS, Fishwick KJ, Halley PA, Storey KG. A spatial and temporal map of FGF/Erk1/2 activity and response repertoires in the early chick embryo. Dev Biol. 2007; 302:536–552. [PubMed: 17123506]

Miki T, Bottaro DP, Fleming TP, Smith CL, Burgess WH, Chan AM, Aaronson SA. Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. Proc Natl Acad Sci USA. 1992; 89:246–250. [PubMed: 1309608]

Mohammadi M, Honegger AM, Rotin D, Fischer R, Bellot F, Li W, Dionne CA, Jaye M, Rubinstein M, Schlessinger J. A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. Mol Cell Biol. 1991; 11:5068–5078. [PubMed: 1656221]

Ornitz DM, Itoh N. Fibroblast growth factors. Genome Biol. 2001; 2:REVIEWS3005. [PubMed: 11276432]

Orr-Urtreger A, Bedford MT, Burakova T, Arman E, Zimmer Y, Yayon A, Givol D, Lonai P. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev Biol. 1993; 158:475–486. [PubMed: 8393815]

Orr-Urtreger A, Givol D, Yayon A, Yarden Y, Lonai P. Developmental expression of two murine fibroblast growth factor receptors, flg and bek. Development. 1991; 113:1419–1434. [PubMed: 1667382]

Paxton CN, Bleyl SB, Chapman SC, Schoenwolf GC. Identification of differentially expressed genes in early inner ear development. Gene Expr Patterns. 2010; 10:31–43. [PubMed: 19913109]

Scotet E, Houssaint E. The choice between alternative IIIb and IIIc exons of the FGFR-3 gene is not strictly tissue-specific. Biochim Biophys Acta. 1995; 1264:238–242. [PubMed: 7495869]

Sheeba CJ, Andrade RP, Duprez D, Palmeirim I. Comprehensive analysis of fibroblast growth factor receptor expression patterns during chick forelimb development. Int J Dev Biol. 2010; 54:1517–1526. [PubMed: 21302260]

Shin M, Watanuki K, Yasugi S. Expression of Fgf10 and Fgf receptors during development of the embryonic chicken stomach. Gene Expr Patterns. 2005; 5:511–516. [PubMed: 15749079]

Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D, Huang J, Jaye M, Crumley G, Schlessinger J, Lax I. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. Cell. 1994; 79:1015–1024. [PubMed: 7528103]

Walshe J, Mason I. Expression of FGFR1, FGFR2 and FGFR3 during early neural development in the chick embryo. Mech Dev. 2000; 90:103–110. [PubMed: 10585567]

Wright TJ, Hatch EP, Karabagli H, Karabagli P, Schoenwolf GC, Mansour SL. Expression of mouse fibroblast growth factor and fibroblast growth factor receptor genes during early inner ear development. Dev Dyn. 2003; 228:267–272. [PubMed: 14517998]

Xu W, Bak S, Decker A, Paquette SM, Feyereisen R, Galbraith DW. Microarray-based analysis of gene expression in very large gene families: the cytochrome P450 gene superfamily of Arabidopsis thaliana. Gene. 2001; 272:61–74. [PubMed: 11470511]

Yayon A, Zimmer Y, Shen GH, Avivi A, Yarden Y, Givol D. A confined variable region confers ligand specificity on fibroblast growth factor receptors: implications for the origin of the immunoglobulin fold. EMBO J. 1992; 11:1885–1890. [PubMed: 1316275]

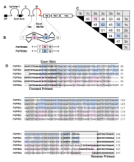

Figure 1. The FGF receptor isoforms generated by alternative splicing

A, Diagram of the primary structure of FGF receptors showing a signal peptide (SP), three immunoglobulin-like (Ig) domains (labeled as Ig-loop-I, II, III), a stretch of seven conserved acidic amino acids between IgI and II (Acid box), a single transmembrane domain (TM), and two intracellular tyrosine kinase domains (TK1, 2). **B,** Alternative splicing of FGF receptors occurs in the IgIII domain, resulting in the corresponding 'b' or 'c' FGF receptor isoforms. As an example, *FGFR2* is diagrammed to show that exons 8 and 9 are alternatively spliced to form the 'b' and 'c' isoforms. **C,** The nucleotide identity of the C-terminal half of the IgIII domain of each isoform is tabulated as percent identity, with an identity over 75% being highlighted in pink (isoform 'b') or blue (isoform 'c'). The highest identity occurs between members of isoform 'c', with the highest identity of 82% occurring between *FGFR1c* and *FGFR2c*. **D,** The nucleotide sequence alignment of the C-terminal half of the IgIII domain of each isoform. High base pair identity occurs among all six isoforms (shown shaded in gray), with further identity within 'c' (blue) and 'b' (pink) subgroups. The boxed sequences indicate the location of primers for each isoform.

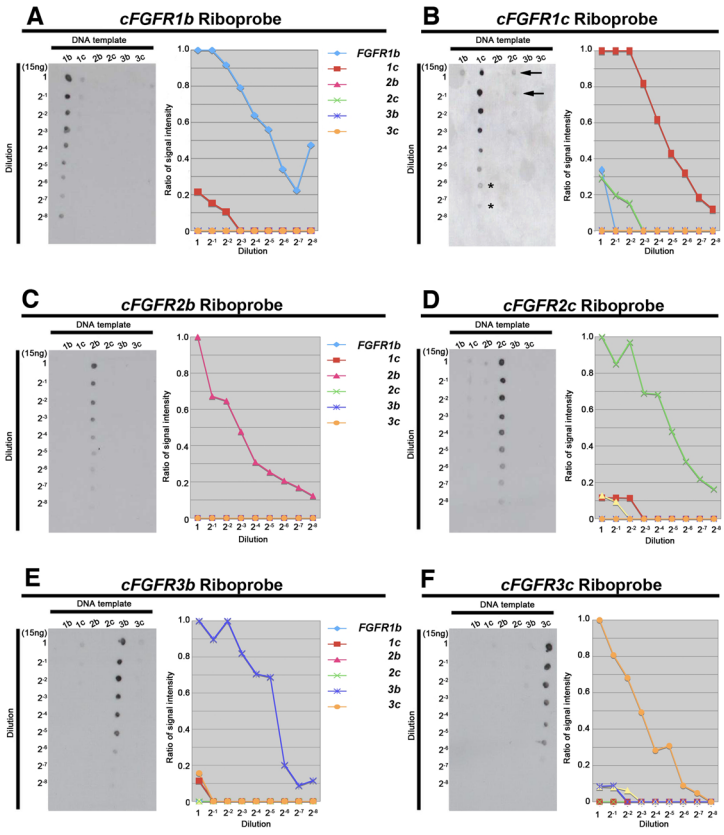

Figure 2. Dot blot hybridization demonstrates the high specificity of FGF receptor isoform riboprobes

Isoform target DNAs were diluted serially and spotted onto nitrocellulose membranes. Hybridization was performed with a single FGF receptor isoform riboprobe using the same conditions for all riboprobes. **A-F,** All probes identified their corresponding targets on dot blots (left panel in each figure) with the highest specificity. Faint cross-hybridization can be seen, for example, in the *FGFR1c* and *FGFR2c* probe/target interactions (**B,** arrows), however, the intensity of this cross-hybridization corresponds to a 1:128–256 dilution (2^{-7}, 2^{-8}; asterisks), providing evidence that riboprobes have high specificity for their targets, even in the instance of *FGFR1c* and *FGFR2c*, where base pair identity is the highest (82%). The dot blot signal was quantified with ImageJ to produce the graphs (right panel in each figure).

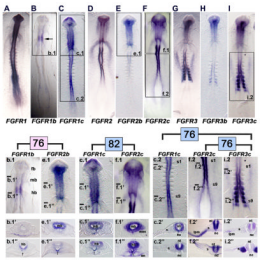

Figure 3. Whole mount in situ hybridization demonstrates the specificity of hybridization of FGF receptor isoform riboprobes in HH stage 9 embryos

A, *FGFR1*, **B,** *FGFR1b* (the boxed area is shown at a higher magnification in b.1; arrow, restricted expression of *FGFR1b* to the caudal midbrain region), and **C,** *FGFR1c* (the boxed areas are shown at a higher magnification in c.1 and c.2) show patterns of expression of FGF receptor 1. **b.1,** Head region of the *FGFR1b* labeled embryo shown in B (transverse sections at the craniocaudal levels indicated by the lines are shown in b.1′ and b.1″). **c.1,** Head region of the *FGFR1c* labeled embryo shown in C (transverse sections at the craniocaudal levels indicated by the lines are shown in c.1′ and c.1″). **c.2,** Trunk region of the *FGFR1c* labeled embryo shown in C (transverse sections at the craniocaudal levels indicated by the lines are shown in c.2′ and c.2″). fb, forebrain; mb, midbrain; hb, hindbrain; s1, somite 1; s9, somite 9; s, somite; nt, neural tube (spinal cord level); nc, notochord; arrows in b.1′ and c.1″ indicate the expression in the ventral midline foregut.

D, *FGFR2*, **E,** *FGFR2b* (the boxed area is shown at a higher magnification in e.1), and **F,** *FGFR2c* (the boxed areas are shown at a higher magnification in f.1 and f.2) show patterns of expression of FGF receptor 2. **e.1,** Head region of the *FGFR2b* labeled embryo shown in E (transverse sections at the craniocaudal levels indicated by the lines are shown in e.1′ and e.1″). **f.1,** Head region of the *FGFR2c* labeled embryo shown in F (transverse sections at the craniocaudal levels indicated by the lines are shown in f.1′ and f.1″). **f.2,** Trunk region of the *FGFR2c* labeled embryo shown in F (transverse sections at the craniocaudal levels indicated by the lines are shown in f.2′ and f.2″). mb, midbrain; hb, hindbrain; en, endoderm; mes, head mesenchyme; en, endoderm of the anterior intestinal portal; lpm, lateral plate mesoderm; s, somite; nt, neural tube; nc, notochord; arrows in e.1″ and f.1″ indicate expression in the ventral midline foregut.

G, *FGFR3*, **H,** *FGFR3b*, and **I,** *FGFR3c* (the boxed area is shown at a higher magnification in i.2) show patterns of expression of FGF receptor 3. **i.2,** Trunk region of the *FGFR3c* labeled embryo shown in I (transverse sections at the craniocaudal levels indicated by the lines are shown in i.2′ and i.2″). s1, somite 1; s9, somite 9; s, somite; lpm, lateral plate mesoderm; nt, neural tube; nc, notochord.

Enlargements and sections are arranged to facilitate comparisons of expression patterns across the receptor family membranes that share the highest identity, indicated by the pink and blue numbers (74, 81, 77, and 72), as explained in Fig. 1C. In all combinations, differences occur in the patterns of expression as detailed in the text.

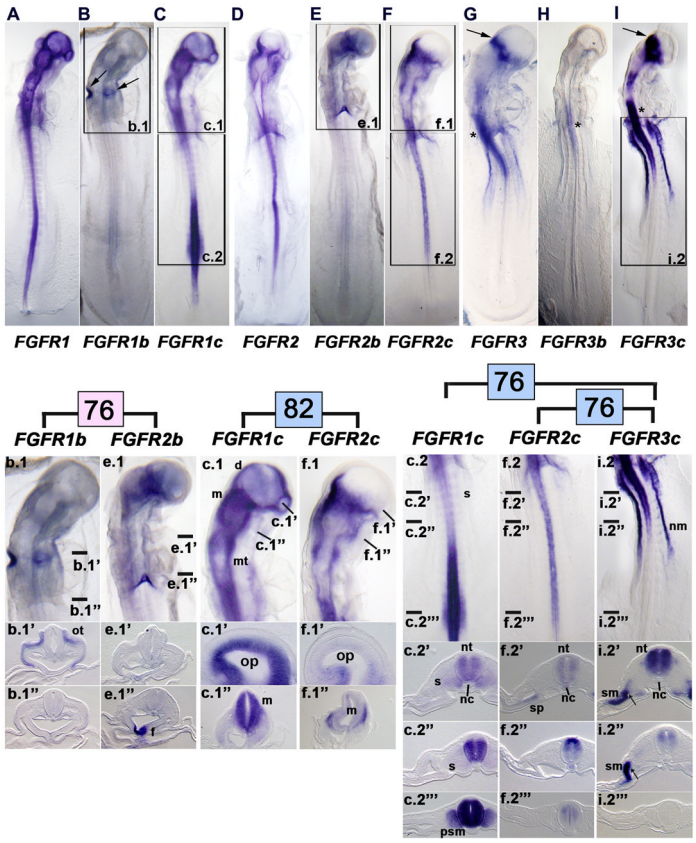

Figure 4. Whole mount in situ hybridization demonstrates the specificity of hybridization of FGF receptor isoform riboprobes in HH stage 13 embryos

A, *FGFR1*, **B,** *FGFR1b* (the boxed area is shown at a higher magnification in b.1; arrows, expression of *FGFR1b* in the otic cups), and **C,** *FGFR1c* (the boxed areas are shown at a higher magnification in c.1 and c.2). **b.1,** Head region of the *FGFR1b* labeled embryo shown in B (transverse sections at the craniocaudal levels indicated by the lines are shown in b.1′ and b.1″). **c.1,** Head region of the *FGFR1c* labeled embryo shown in C (transverse sections at the craniocaudal levels indicated by the lines are shown in c.1′ and c.1″). **c.2,** Trunk region of the *FGFR1c* labeled embryo (transverse sections at the craniocaudal levels indicated by the lines are shown in c.2′, c.2″, and c.2‴). ot, otic cup; d, diencephalon; m, mesencephalon; mt, metaencephalon; op, optic vesicle; s, somite; nt, neural tube (spinal cord level); nc, notochord; psm, presomitic mesoderm (segmental plate mesoderm).

D, *FGFR2*, **E,** *FGFR2b* (the boxed area is shown at a higher magnification in e.1), and **F,** *FGFR2c* (the boxed areas are shown at a higher magnification in f.1 and f.2). **e.1,** Head region of the *FGFR2b* embryo shown in E (transverse sections at the craniocaudal levels indicated by the lines are shown in e.1′ and e.1″). **f.1,** Head region of the *FGFR2c* embryo shown in F (transverse sections along the indicated line are shown in f.1′ and f.1″). **f.2,** Trunk region of the *FGFR2c* labeled embryo shown in F (transverse sections at the craniocaudal levels indicated by the lines are shown in f.2′, f.2″, and f.2‴). f, expressing ventral midline of foregut (thyroid rudiment); op, optic vesicle; m, mesencephalon; nt, neural tube (spinal cord level); nc, notochord; sp, medial splanchnic mesoderm.

G, *FGFR3* (arrow, strong expression at the border between the diencephalon and mesencephalon; asterisk, expression within the hindbrain/cranial spinal cord), **H,** *FGFR3b*, and **I,** *FGFR3c* (the boxed area is shown at a higher magnification in i.2′; arrow, strong expression at the border between the diencephalon and mesencephalon; asterisk, expression

within the hindbrain/cranial spinal cord). **i.2,** Trunk region of the *FGFR3c* labeled embryo (transverse sections at the craniocaudal levels indicated by the lines are shown in i.2′, i.2″, and i.2‴). nm, nephrogenic mesoderm (nephrotome or intermediate mesoderm); nt, neural tube (spinal cord level); nc, notochord; sm, somatic mesoderm; arrows in i.2′ and i.2″ indicate nephrogenic mesoderm

Enlargements and sections are arranged to facilitate comparisons of expression patterns across the receptor family membranes that share the highest identity, indicated by the pink and blue numbers (74, 81, 77, and 72), as explained in Fig. 1C. In all combinations, differences occur in the patterns of expression as detailed in the text.

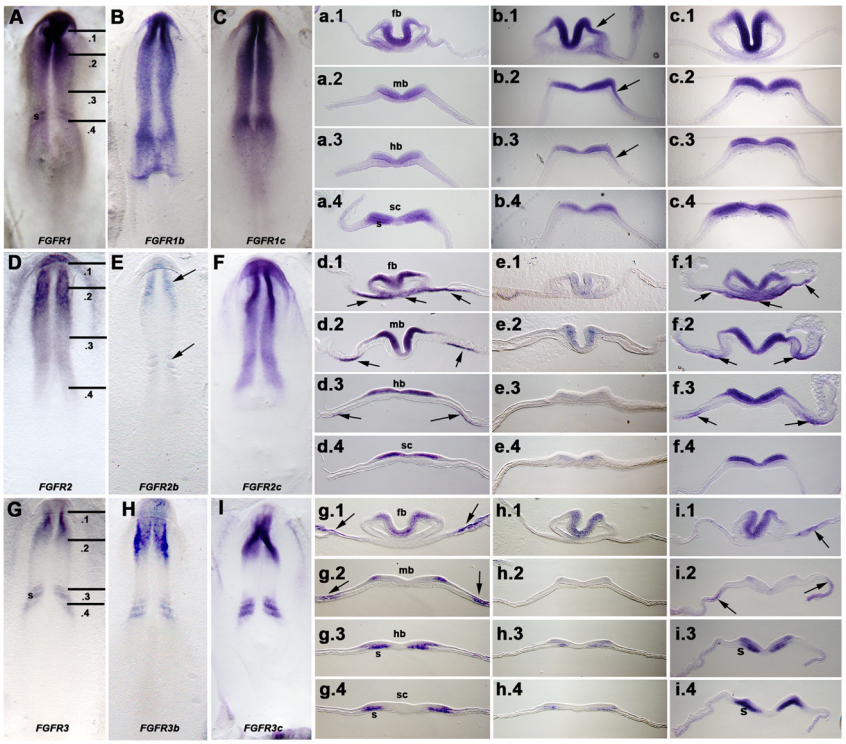

Figure 5. Whole mount in situ hybridization demonstrates similarity in the patterns of expression of FGF receptor isoforms in HH stage 7 embryos

A, *FGFR1*, **B,** *FGFR1b*, and **C,** *FGFR1c*. Lines in A indicate section planes shown in a.1-a.4, b.1-b.4, and c.1-c.4. fb, forebrain level; mb, midbrain level; hb, hindbrain level; sc, spinal cord level. Arrow in b.3 indicates expression in the ectoderm of the future otic placode, which extends cranially to flank both the midbrain (b.2, arrow) and forebrain (b.1, arrow) levels.

D, *FGFR2*, **E,** *FGFR2b* (weak expression restricted to the cranial and caudal levels of neuraxis), and **F,** *FGFR2c*. Lines in D indicate section planes shown in d.1-d.4, e.1-e.4, and f.1-f.4. fb, forebrain level; mb, midbrain level; hb, hindbrain level; sc, spinal cord level. Arrows in d.1, d.2, d.3, f1, f.2, and f.3 indicate expressing areas of endoderm (and in some regions, also overlying tissue).

G, *FGFR3*, **H,** *FGFR3b*, and **I,** *FGFR3c*. Lines in G indicate section planes shown in g.1-g.4, h.1-h.4, and i.1-i.4. fb, forebrain level; mb, midbrain level; hb, hindbrain level; sc, spinal cord level; s, somite. Arrows in g.1, g.2, i1 and i.2 indicate expressing areas of ectoderm and/or underlying lateral plate mesoderm.

Table 1

Primers for cloning of FGF receptor isoforms.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
FGFR1b	TCCCCGCCAACAAAACGGTG	TCTGGCAAAAGCTACTGAGC
FGFR1c	TCCCCGCCAACAAAACGGTG	AGTTCTCGAAGCTACTGAGC
FGFR2b	CAAACGCCTCGGCTGTAGTC	GAGTTTACAAGCTCCTGAAA
FGFR2c	CAAACGCCTCGGCTGTAGTC	AGTTCTGCCAGCTCCTGAAA
FGFR3b	CAATCAGACTGTGGTGGTCG	CAAACCAAAAGCAGAGGAGC
FGFR3c	CAATCAGACTGTGGTGGTCG	GGTGCTACCAGCAGAGGAGC

Table 2

Primers for preparation of generic and isoform-specific templates and riboprobes.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
FGFR1	ACGCAGGACGGGCCCCTCTAT	CCATCAGAGTGATGTTTGGT
FGFR1b	GATCCTGAAGCACTCGGGAA	TCTGGCAAAAGCTACTGAGC
FGFR1c	GATCCTGAAGACGGCTGGCG	AGTTCTCGAAGCTACTGAGC
FGFR2	GAGGCATGGAGTACTTGGCT	AAAGATTCTGCTTGTACATA
FGFR2b	GGTTTTAAAGCATTCGGGGA	GAGTTTACAAGCTCCTGAAA
FGFR2c	GGTTTTAAAGGCTGCCGGTG	AGTTCTGCCAGCTCCTGAAA
FGFR3	TCGGTGCTTGCACGCAGGAC	CAGAGCGATGTCTGGTCTTT
FGFR3b	GCTGAAGACGTCTTGGATCA	CAAACCAAAAGCAGAGGAGC
FGFR3c	GCTGAAGACGGCAGGTGTTA	GGTGCTACCAGCAGAGGAGC

Table 3

Summary of the tissue expression of *FGFRs* at three stages of chick development. ND, not detected.

Stage and domain of expression		*FGFR1*	*FGFR1b*	*FGFR1c*	*FGFR2*	*FGFR2b*	*FGFR2c*	*FGFR3*	*FGFR3b*	*FGFR3c*
HH stage 7, 24 hours										
neural plate	forebrain level	+++	+++	+++	+++	+	+++	++	++	++
	midbrain level	+++	+++	+++	+++	+	+++	++	++	++
	hindbrain level	++	++	++	++	+	++	++	+	++
	spinal cord level	++	++	++	++	ND	++	ND	ND	ND
endoderm		ND	ND	ND	++	ND	++	ND	ND	ND
extraembryonic tissues		ND	ND	ND	ND	ND	ND	++	ND	++
somite		++	ND	++	ND	ND	ND	+++	+	+++
preotic placode		ND	++	ND	ND	ND	ND	ND	ND	ND
HH stage 9, 29 hours										
neural tube	forebrain	+++	ND	++	+++	++	+++	+++	++	+++
	midbrain	+++	ND	++	++	++	++	++	++	++
	hindbrain	+++	++	+++	++	++	++	++	+	++
	spinal cord	+++	ND	+++	+++	ND	+++	++	+	++
head mesenchyme		ND	ND	ND	++	ND	++	ND	ND	ND
anterior intestinal portal lateral		+	ND	+	++	ND	+	ND	ND	ND
lateral plate mesoderm		ND	ND	ND	+	ND	+	++	ND	+
intermediate mesoderm		ND	ND	ND	ND	ND	ND	++	ND	++
somite		ND	ND	ND	ND	ND	ND	+++	+	+++
primitive streak		++	ND	++	ND	ND	ND	ND	ND	ND
HH stage 13, 50 hours										
neural tube	diencephalon	+++	ND	++	++	++	++	ND	ND	ND
	mesencephalon	+++	+	++	++	++	++	ND	ND	ND
	met/myelencephalon	+++	ND	+++	+++	ND	+++	+++	ND	+++
	spinal cord	+++	ND	+++	++	ND	++	+++	+	+++
head mesenchyme		ND	ND	ND	++	ND	++	ND	ND	ND
lateral plate mesoderm	somatic mesoderm	ND	ND	ND	ND	ND	ND	+++	ND	+++
	splanchnic mesoderm	ND	ND	ND	+	ND	+	ND	ND	ND
intermediate mesoderm		ND	ND	ND	ND	ND	ND	+	ND	+

Stage and domain of expression	*FGFR1*	*FGFR1b*	*FGFR1c*	*FGFR2*	*FGFR2b*	*FGFR2c*	*FGFR3*	*FGFR3b*	*FGFR3c*
foregut	ND	ND	ND	++	++	++	ND	ND	ND
somite	ND	ND	ND	ND	ND	ND	+	ND	+
presomitic mesoderm	+	ND	++	ND	ND	ND	ND	ND	ND
otic cup	ND	+++	ND	ND	ND	ND	ND	ND	ND
optic vesicle	+++	ND	+++	ND	ND	ND	ND	ND	ND

Dev Dyn. Author manuscript; available in PMC 2012 June 1.