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# Comprehensive analysis of fibroblast growth factor receptor expression patterns during chick forelimb development

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**ABSTRACT Specific interactions between fibroblast growth factors (Fgf1-22) and their tyrosine kinase receptors (FgfR1-4) activate different signalling pathways that are responsible for the biological processes in which Fgf signalling is implicated during embryonic development. In the chick, several Fgf ligands *(Fgf2, 4, 8, 9, 10, 12, 13* and *18 )* and the four *FgfRs (FgfR 1, 2, 3* and *4)* have been reported to be expressed in the developing limb. The precise spatial and temporal expression of these transcripts is important to guide the limb bud to develop into a wing/leg. In this paper, we present a detailed and systematic analysis of the expression patterns of *FgfR1, 2, 3* and *4* throughout chick wing development, by *in situ* hybridisation on whole mounts and sections. Moreover, we characterize for the first time the different isoforms of *FGFR1-3* by analysing their differential expression in limb ectoderm and mesodermal tissues, using RT-PCR and *in situ* hybridisation on sections. Finally, isoform-specific sequences for *FgfR1IIIb, FgfR1IIIc, FgfR3IIIb* and *FgfR3IIIc* were determined and deposited in GenBank with the following accession numbers: GU053725, GU065444, GU053726, GU065445, respectively.**

KEY WORDS: *Fgf, FgfRs, limb, chick*

The developing chick limb grows out as a protrusion of mesenchymal cells from the lateral plate mesoderm and adjacent somites. Cells from the lateral plate mesoderm form cartilage and connective tissues, such as muscle sheaths, tendons and ligaments. Meanwhile, myogenic precursor cells delaminate from the lateral part of the somites, migrate to the limb bud, colonise the dorsal and ventral limb regions and activate the myogenic program to differentiate into multinucleated myotubes, thus generating the definitive limb skeletal muscles (Christ and Brand-Saberi, 2002; Duprez, 2002; Buckingham *et al.* 2003). The mesenchyme of the limb bud is enveloped by an ectodermal jacket, whose distal tip forms a specialised epithelial structure, the apical ectodermal ridge (AER) (Todt and Fallon, 1984). Mesenchymal cells directly under the AER remain undifferentiated and populate the so-called undifferentiated zone (Tabin and Wolpert, 2007). Condensation of the cartilage elements proceeds in a proximal to distal direction based on cells' temporal and positional values, which could be provided by the reported limb molecular clock (Pascoal *et al.* 2007a; reviewed in Pascoal and Palmeirim, 2007; Tabin and Wolpert, 2007).

During development, changes occur along the three limb bud axes - anterior-posterior (AP), dorsal-ventral (DV) and proximal-distal (PD) - conducted by three distinct signalling centres, the zone of polarizing activity (ZPA), the dorsal (non-ridge) ectoderm and the AER, respectively (reviewed in Towers and Tickle, 2009). ZPA activity is mediated by the diffusible molecule Sonic hedgehog (Shh) (reviewed in Towers and Tickle, 2009). The DV axis is specified by the signalling molecule Wnt-7a and the transcription factor Lmx1b, which are expressed in the dorsal limb ectoderm and dorsal mesenchyme, respectively (Parr and McMahon, 1995; Chen *et al.* 1998). *Wnt-7a* expression is repressed in the ventral ectoderm by Engrailed1, a target of Bmp signalling (Loomis *et al.* 1996). The AER drives PD limb outgrowth (Saunders, 1948) and its activity is mediated by several fibroblast growth factor (Fgf) family members (Niswander *et al.* 1993; Fallon *et al.* 1994), whose

*Abbreviations used in this paper:* AER, apical ectodermal ridge; Fgf, fibroblast growth factor; FgfR, Fgf receptor; ZPA, zone of polarizing activity.

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sequential contribution to PD limb growth over time has been recently reported (Mariani *et al.* 2008). Fgfs comprise several structurally related polypeptides, many of which have been described to be expressed in the developing chick limb (Fallon *et al.* 1994; Savage and Fallon, 1995; Crossley *et al.* 1996; Ohuchi *et al.* 1997; Munoz-Sanjuan *et al.* 1999; Ohuchi *et al.* 2000; Havens *et al.* 2006). The expression of *Fgf4, 8* and *9* is restricted to the AER, *Fgf10, 12, 13* and *18* are expressed in the limb mesenchyme and *Fgf2* is expressed both in the ectoderm, including the AER, and in the mesenchyme. Fgf4 is also expressed in muscle from E6 (Edom-Vovard *et al.* 2001a) and Fgf8 in tendons from E8 (Edom-Vovard *et al.* 2001b). Fgf4 and 8, however, are indispensable for limb development, since targeted deletion of *Fgf4* and *Fgf 8* in the AER generated limbless mice (Sun *et al.* 2002; Boulet *et al.*, 2004). AER-derived Fgfs signal to the underlying mesenchyme, which is evidenced by the distal to proximal gradient of the Fgf8 effector *Mkp3* (Pascoal *et al.* 2007b), maintaining the subjacent mesoderm in an undifferentiated, proliferative state (Tabin and Wolpert, 2007).

Fgf signalling is implicated in many biological processes during development, such as differentiation, proliferation (Prykhozhij and Neumann, 2008), survival, migration, adhesion, apoptosis and chemotaxis (reviewed in Bottcher and Niehrs, 2005). All Fgfs mediate their cellular responses by binding to and activating appropriate Fgf receptors (FgfRs), which belong to a family of cell surface receptor tyrosine kinases. FgfRs contain an extracellular Fgf binding domain composed of three immunoglobulin-like domains, a transmembrane domain and an intracellular tyrosine kinase domain. There are four known FgfRs and among these, FgfR1-3 can generate alternatively spliced isoforms (reviewed in Eswarakumar *et al.* 2005) that display different ligand specificity (Ornitz *et al.* 1996; Zhang *et al.* 2006). Ligand binding in the presence of heparan sulfate proteoglycans leads to receptor dimerisation and activation of downstream intracellular signalling cascades, such as Ras-MAP kinase, PI3 kinase/Akt and PLC gamma/PKC (reviewed in Dailey *et al.* 2005). When mutated or misexpressed, Fgfs and their receptors cause morphogenic disorders affecting limb formation and can

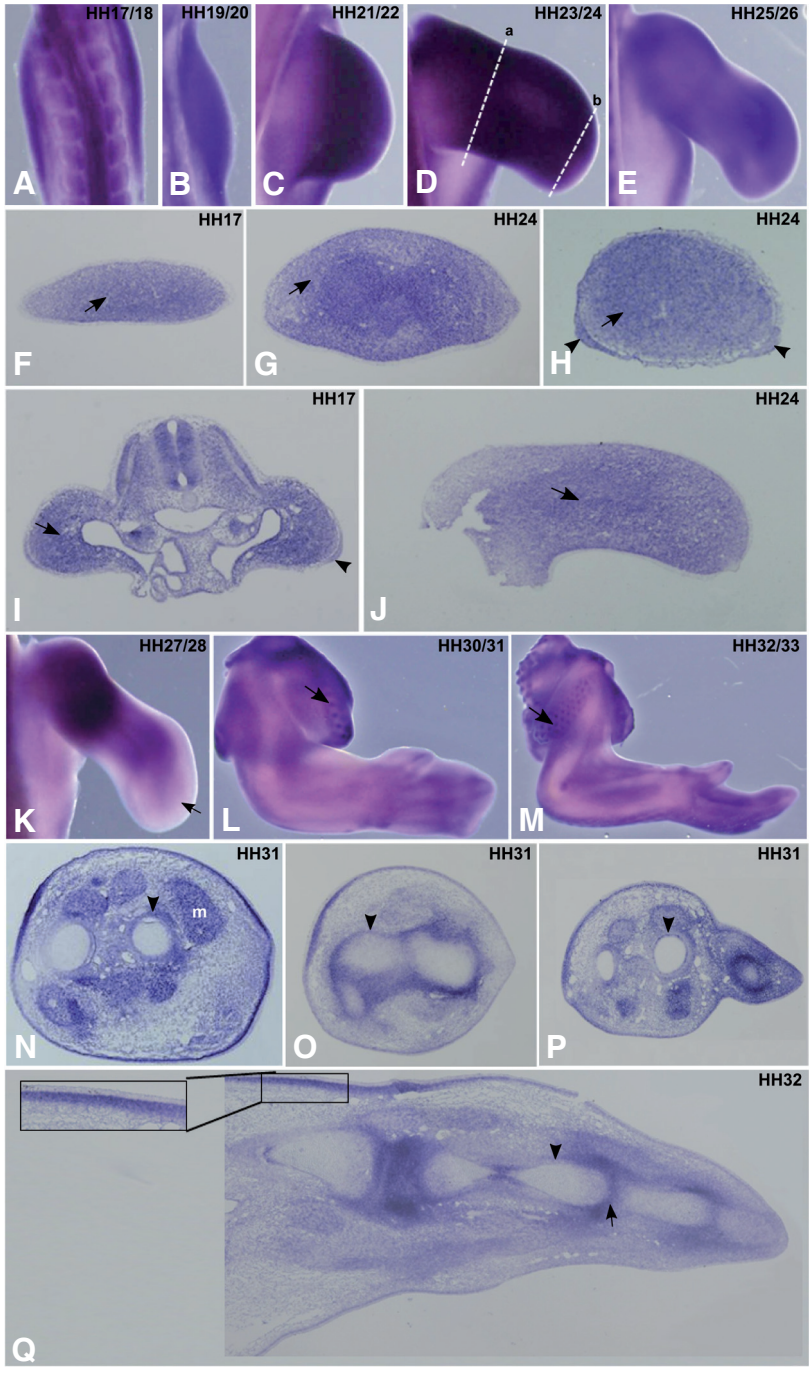
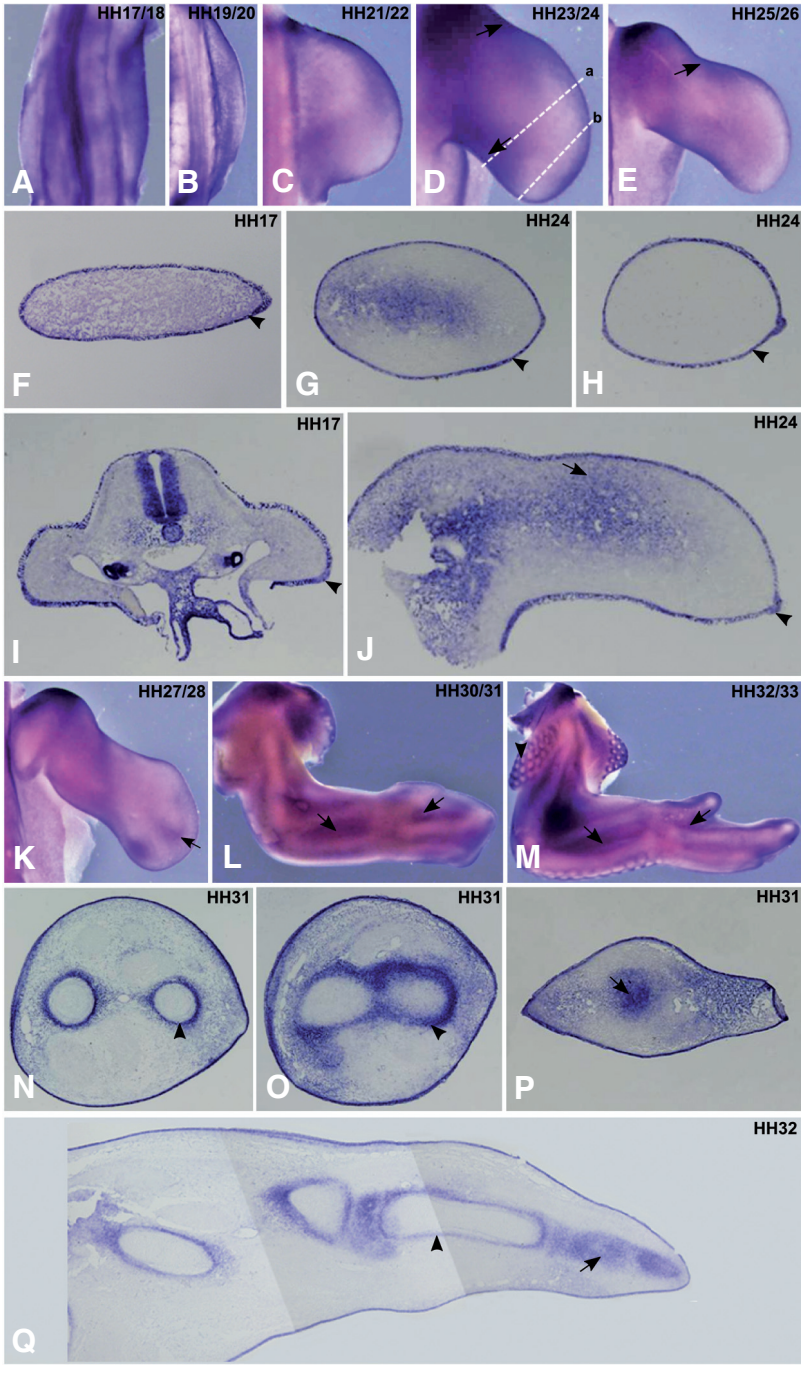



**Fig. 1. *FgfR1* expression pattern during chick forelimb development.** *Chick wings at HH17-33 stages were processed for* in situ *hybridisation in whole-mounts* **(A-E, K-M)** *and in transversal/longitudinal sections* **(F-J, N-Q)** *using a probe for non isoform-specific chick* FgfR1. **(A-E)** In situ *hybridisation in whole-mount limbs for* FgfR1 *showed a generalized expression in limb mesenchyme.* **(F-H)** *Transverse sections of limb buds at HH17 and HH24 (G: proximal, H: distal, represented in* **(D)** *as (a) and (b), respectively) show* FgfR1 *expression in the limb mesenchyme (arrows), and in the ectoderm of the distal limb (H, arrowheads).* **(I,J)** *Longitudinal limb sections at HH17 and HH24 show* FgfR1 *expression in the limb mesenchyme (arrows), and in the AER of HH17 limb bud (I, arrowhead).* **(K)** In situ *hybridisation in whole-mount limbs at HH27/28 stage show decreased*n FgfR1 *expression in the distal limb mesenchyme (arrow).* **(L,M)** FgfR1 *expression is observed in developing feather buds of the body wall (arrows).* **(N-P)** *Transverse sections of forelimb at stage HH31 show* FgfR1 *expression in perichondrium (arrowheads) and in muscles (m).* **(Q)** In situ *hybridisation in longitudinal limb sections at HH32 shows that* FgfR1 *transcripts are observed in the perichondrium (arrowhead) and in the future interphalangic joints (arrow). The inset in* **(Q)** *shows an upregulation of* FgfR1 *expression in the mesenchyme close to the proximal ectoderm.* **(A-E, K-M)** *are dorsal views of whole-mount limbs.* **(F-H, N-P)** *Transverse sections are oriented such that left – posterior, right – anterior, top – dorsal and bottom – ventral.* **(I,J,Q)** *longitudinal limb sections are positioned such that left – proximal and right – distal. Embryonic stages of the limbs are mentioned in the upper right corner of the pictures.*

also lead to cancer (reviewed in Wilkie *et al.* 2002; Eswarakumar *et al.* 2005).

The expression pattern of *FgfR1-4* on sections during chick limb development has been previously reported to some extent (Marcelle *et al.* 1995; Szebenyi *et al.* 1995; Eloy-Trinquet *et al.* 2009). Nevertheless, as our knowledge on the importance of Fgf signalling in limb development increased considerably over the last decade, a more detailed description of the spatial and temporal distribution of all *FgfRs* became compulsory. Moreover, very little is known about FGFR isoform-specific expression during limb development. Although there are studies available for the expression pattern of *FgfR2IIIb* and *FgfR2IIIc* isoforms (Lizarraga *et al.* 1999; Havens *et al.* 2006), knowledge on the expression of the *FgfR1* and *FgfR3* isoforms is lacking, in both chick and mouse. We hereby report comprehensive expression analyses of *FgfR1-4* and IIIb and IIIc isoforms of *FgfR1-3* during chick forelimb bud development, from stage HH17 to HH33 (Hamburger and Hamilton, 1951), using RT-PCR and *in situ* hybridisation on whole mount and sections.




## Results

This work presents a comprehensive analysis of the expression patterns of *FgfR1-4* during chick forelimb development in stages HH17 through HH33 by *in situ* hybridization. For each receptor, a non isoform-specific probe was used allowing an overall visualization of expression spatial distribution. Subsequently, we analysed the expression of the IIIb and IIIc isoforms of *FgfR1-3* by RT-PCR and *in situ* hybridisation on sections.

### FgfR1 *expression*

Between stages HH17 and HH26, *FgfR1* is expressed along the entire PD axis of the chick limb bud (Fig. 1A-E). Transverse and longitudinal section analyses show that *FgfR1* transcripts are present mainly in the mesenchyme (Fig. 1 F-J, arrows) and are absent from the enveloping ectoderm in the proximal part of the limb. In the distal limb, however, *FgfR1* is also observed in sections in the distal ectoderm, including the AER (Fig. 1 H,I, arrowhead). At stage HH26, when cartilage differentiation is in progress, *FgfR1* transcripts are detected throughout the limb, although in a slightly graded proximal to distal distribution (Fig. 1E, data not

**Fig. 2. *FgfR2* expression pattern during chick forelimb development.** *Chick wings at HH17-33 stages were processed for* in situ *hybridisation in whole-mounts* **(A-E, K-M)** *and transverse/longitudinal sections* **(F-J, N-Q)** *using a probe for non isoform specific chick* FgfR2. **(A-E)** In situ *hybridisation in whole mount limbs using the* FgfR2 *probe showed its presence in the limb ectoderm, including the AER.* **(F-H)** *Transverse limb sections (G: proximal, H: distal, represented in* **(D)** *as (a) and (b), respectively) confirm the ectodermal expression of* FgfR2 *(arrowheads), although a faint proximal/posterior mesenchymal expression could also be observed at HH24 (G, arrow).* **(I,J)** *Longitudinal limb sections at HH17 and HH24 show* FgfR2 *expression in the ectoderm (arrowheads) including the AER and also in the mesenchyme (arrow).* **(K-M)** In situ *hybridisation in whole-mount limbs show* FgfR2 *expression in between the zeugopod skeletal elements and in the interdigital zones (arrows) and around the feather buds in the body wall (M, arrowhead).* **(N-P)** *Transverse sections of forelimb at stage HH31 show* FgfR2n*expression in perichondrium (arrowheads) and in the entire distal cartilage element (arrow).* **(Q)** In situ *hybridisation in longitudinal limb sections at HH32 show* FgfR2 *expression in distal cartilage elements (arrow) and in perichondrium in more proximal cartilage elements (arrowhead).* **(A-E, K-M)** *are dorsal views of whole-mount limbs.* **(F-H, N-P)** *transverse sections are oriented such that left – posterior, right – anterior, top – dorsal and bottom – ventral.* **(I,J,Q)** *longitudinal limb sections are positioned such that left – proximal and right – distal. Embryonic stages of the limbs are mentioned in the upper right corner of the pictures.*

shown). Moreover, longitudinal sections revealed stronger expression in the perichondrium compared to the mesenchymal tissue (data not shown). At HH27, *FgfR1* expression decreases prominently in the distal limb mesenchyme and becomes restricted to more proximal regions (Fig. 1K, arrow). *In situ* hybridisation of stage HH31 or HH32 forelimbs show that *FgfR1* is observed in the perichondrium and in the prospective interphalangic joints (Fig. 1 N-Q, arrowheads and 1Q, arrow, respectively). In addition, *FgfR1* expression is also observed in muscles (Fig. 1 N,m), tendons (Fig. 1N) and in feather buds of the body wall (Fig. 1 L,M arrows). Interestingly, *FgfR1* is strongly expressed in a thin layer of mesenchymal cells juxtaposed the limb ectoderm, with a higher signal in the dorsal regions (Fig. 1 N-Q and 1Q, inset) which fades gradually towards the distal limb.

### FgfR2 *expression*

*FgfR2* transcripts are strongly detected in the entire ectodermal cell layer covering limb mesenchymal cells, including the AER (Fig. 2). This is clearly evidenced in the transverse and longitudinal limb sections (Fig. 2 F-J, arrowhead). Additionally, *FgfR2* transcripts can be observed in the proximal regions of stage HH23-26 limbs, including the anterior and posterior limb margins (Fig. 2 D,E,G,J, arrow). From stage HH27 onwards, *FgfR2* mRNA is observed in between the two zeugopod skeletal elements and in the interdigital domains (Fig. 2 K,L,M arrow) and surrounding feather buds of the body wall (Fig. 2M, arrowhead). Section analyses showed that *FgfR2* expression prefigures the distal developing chondrogenic elements (Fig. 2 P,Q, arrow), and was observed in the perichondrium of the more proximal cartilage elements (Fig. 2 N,O,Q, arrowhead).

### FgfR3 *expression*

*FgfR3* transcripts were first detected in limb buds at stage HH19, where they are clearly restricted to the most proximal mesoderm (Fig. 3 B-J, arrow). This expression pattern is maintained until stage HH24. However, low levels of *FgfR3* expression in the mesenchyme underlying the anterior ectoderm towards the distal part of the limb could also be observed (Fig. 3D, arrowhead). *FgfR3* mRNA was never detected neither in the ZPA nor in the AER and the underlying distal mesenchyme (Fig. 3 A-J), although it could be detected in the proximal mesoderm (Fig. 3J, arrow). From HH25 stage, *FgfR3* is strongly expressed in all the

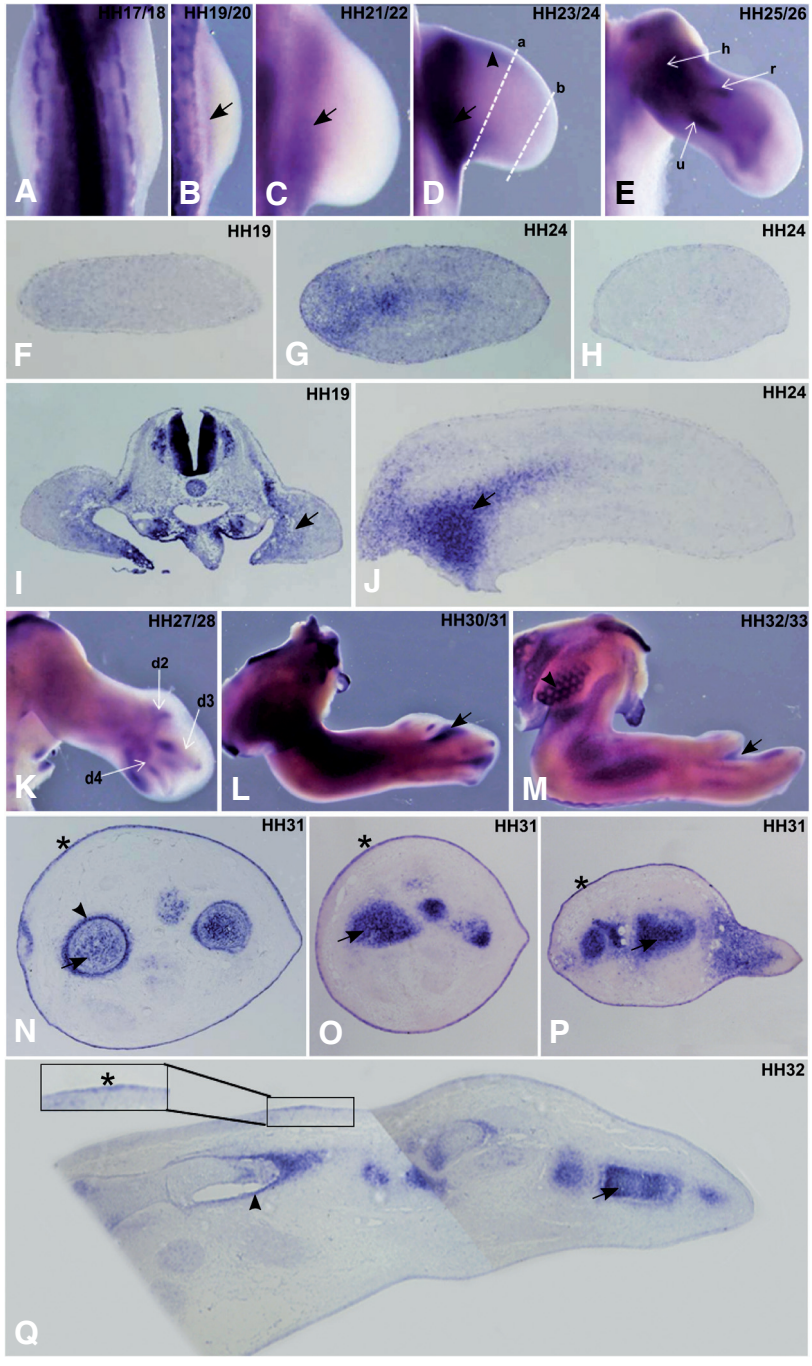



**Fig. 3. *FgfR3* expression pattern during chick forelimb development.** *Chick wings at HH17-33 stages were processed for* in situ *hybridisation in whole-mounts* **(A-E, K-M)** *and transverse/longitudinal sections* **(F-J, N-Q)** *using a probe for non isoform specific chick* FgfR3. **(A-E)** In situ *hybridisation in whole mount limbs at stages HH19-24 show* FgfR3 *expression in the more proximal part of limb buds (B-D, arrows) and in forming cartilage elements* **(E)** *of the humerus (h), radius (r) and ulna (u) at HH25/26 stages. Faint* FgfR3 *expression was also observed in the anterior mesenchyme underlying the ectoderm (D, arrowhead).* **(F-H)** *Transverse (G:proximal, H:distal, represented in D as a and b, respectively) and* **(I,J)** *longitudinal limb sections at stage HH19 and HH24 shows the specific mesenchymal expression of* FgfR3 *(arrows).* **(K-M)** In situ *hybridisation in whole-mount limbs at HH27 to HH33 shows a* FgfR3 *expression in interdigital domains (arrows), in addition to digit cartilage elements (d2, d3 and d4).* FgfR3 *expression is also observed around the feather buds in the body wall (arrowhead).* **(N-Q)** *During limb stages HH31/32* FgfR3 *is expressed in the ectoderm (asterisk & inset) and also in the cartilage elements (arrows) and perichondrium (arrowhead).* **(A-E, K-M)** *are dorsal views of whole-mount limbs.* **(F-H, N-P)** *transverse sections are oriented such that left – posterior, right – anterior, top – dorsal and bottom – ventral.* **(I,J,Q)** *longitudinal limb sections are positioned such that left – proximal and right – distal. Embryonic stages of the limbs are mentioned in the upper right corner of the pictures.*

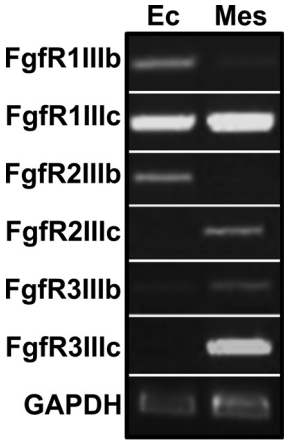



**Fig. 4. RT-PCR evaluation of the differential expression of *FgfR1-3* IIIb and IIIc isoforms in the forelimb ectoderm and mesoderm.** FgfR1-3 *isoform-specific expression was evaluated by RT-PCR using total RNA extracted from segregated ectoderm (Ec) and mesoderm (Mes) tissues from stage HH24 forelimbs.* FgfR1IIIb, FgfR1IIIc *and* FgfR3IIIb *are found in both ectoderm and mesenchyme.* FgfR2IIIb *is exclusively expressed in the ectoderm, while* FgfR2IIIc *and* FgfR3IIIc *are only expressed in the mesenchyme.*

forming cartilage elements (Fig. 3 E,K) and can also be observed in interdigital zones (Fig. 3 L,M, arrow). Sections of HH32 limbs revealed specific expression of *FgfR3* in muscles (Fig. 3N), distal cartilage elements (Fig. 3 N-Q, arrow) and in the perichondrium (Fig. 3 N,Q, arrowhead). Interestingly, *FgfR3* is also faintly expressed in the ectoderm at this stage of limb development (Fig. 3 N-Q, asterisk and inset).

### *Isoform-specific expression patterns of* FgfRs 1-3

We employed the same experimental approach as Shin *et al.* (2005) to detect the presence of the specific IIIb and IIIc isoforms of *FgfR1-3* in segregated ectoderm and mesoderm tissues of stage HH24 limb buds. RT-PCR analysis clearly revealed that *FgfR2IIIb* is exclusively expressed in the limb ectoderm, while *FgfR2IIIc* and *FgfR3IIIc* are only found in limb mesenchyme (Fig. 4). These results strongly imply that the *FgfR2* ectodermal staining observed in Fig. 2 is due to *FgfR2IIIb* isoform expression. Conversely, *FgfR2* mesodermal staining is a result of *FgfR2IIIc* expression. Since *FgfR3IIIc* can only be detected in the limb mesoderm by RT-PCR (Fig. 4), we can clearly state that at stage HH24 the faint *FgfR3* ectodermal staining observed by *in situ* hybridization (Fig. 3G) is solely due to the *FgfR3IIIb* isoform expression. However, RT-PCR also detected this splice variant in the mesenchyme (Fig. 4), so detailed *FgfR3* isoform-specific expression patterns in the limb mesenchyme are missing. The same stands for *FgfR1* isoforms, since both are detected by RT-PCR in ectoderm and mesoderm (Fig. 4).

To further elucidate the expression patterns of *FgfR1* and *FgfR3* isoforms, *in situ* hybridization probes were prepared using the amplification products obtained upon RT-PCR performed on total RNA extracted from HH24 whole chick embryos, using the primers previously described (Shin *et al.* 2005). The amplicon sequences were determined and deposited in GenBank with the following accession numbers: *FgfR1IIIb*-GU053725; *FgfR1IIIc*- GU065444; *FgfR3IIIb*- GU053726; *FgfR3IIIc*-GU065445.

*In situ* hybridization of *FgfR1IIIb* and *FgfR1IIIc* isoforms on limb sections (Fig. 5) consistently showed fainter staining compared to the non isoform-specific'*FgfR1* probe. Very subtle differences could be found for the spatial distribution of both isoforms in the limb tissues: *FgfR1IIIb* consistently marked the ectoderm stronger than the *FgfR1IIIc* isoform (Fig. 5 A,B,C,D, arrows) and, in stage HH31 limb sections, *FgfR1IIIc* presents stronger expression in the perichondrium than the other variant (Fig. 5 B,D, arrowhead).

At stage HH24, *FgfR3IIIb* is expressed throughout the entire limb and the *FgfR3IIIc* isoform is expressed in the proximal mesenchyme (Fig. 5 E,G, arrow), which is in accordance to the RT-PCR results (Fig. 4). In later developmental stages (HH30), *FgfR3IIIc* transcripts are predominantly detected in and around the cartilage, unlike the faint expression of *FgfR3IIIb* (Fig. 5

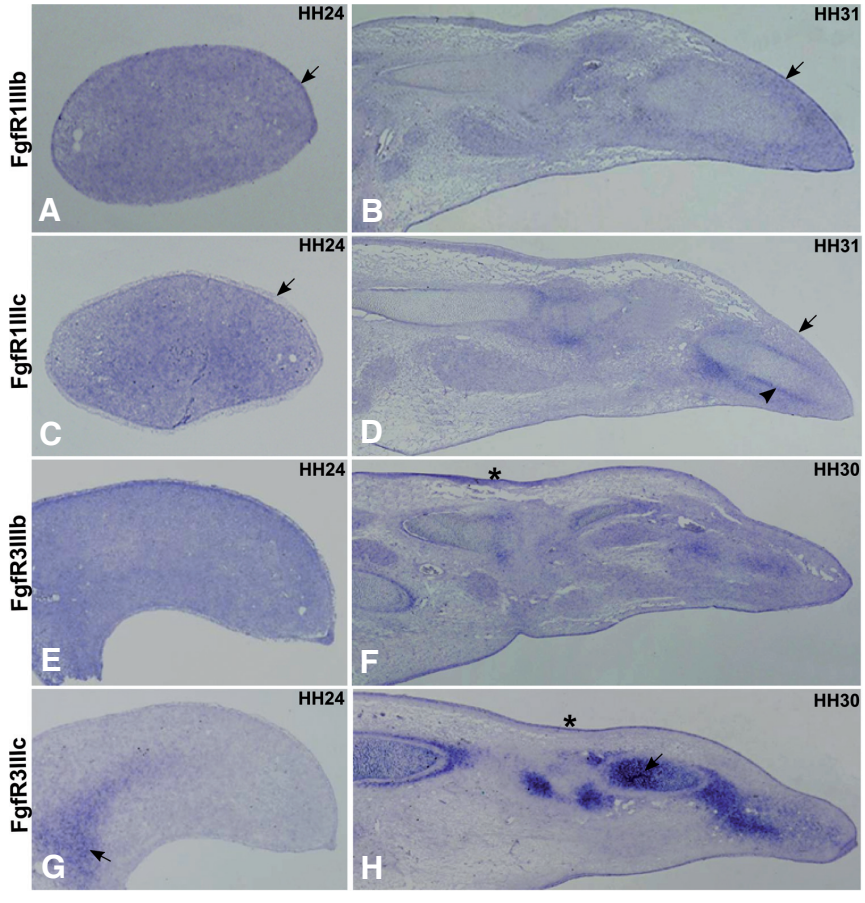



**Fig. 5. Section *in situ* hybridization analyses of *FgfR1IIIb, FgfR1IIIc, FgfR3IIIb* and *FgfR3IIIc* expression patterns.** *Chick wings at HH24 and HH30/31 stages were processed for transversal/longitudinal paraffin section* in situ *hybridisation using the isoform-specific sequences previously obtained as probes (GenBank accession numbers GU053725, GU065444, GU053726, GU065445).* FgfR1IIIb *presents stronger expression in the ectoderm than* FgfR1II*Ic; see arrows in* **(A-D)**, *while* FgfR1IIIc *transcripts could be detected in the perichondrium; see arrowhead in* **(D)** *unlike FgfR1IIIb. FgfR3IIIc is expressed in the proximal limb of stage HH24; see arrow in* **(G)**, *and in the cartilage elements at stage HH30; arrow in* **(H)**, *unlike FgfR3IIIb* **(E,F)**. *At stage HH30, both FgfR3IIIb and IIIc are expressed in the ectoderm; see asterisks in* **(F,H)**.

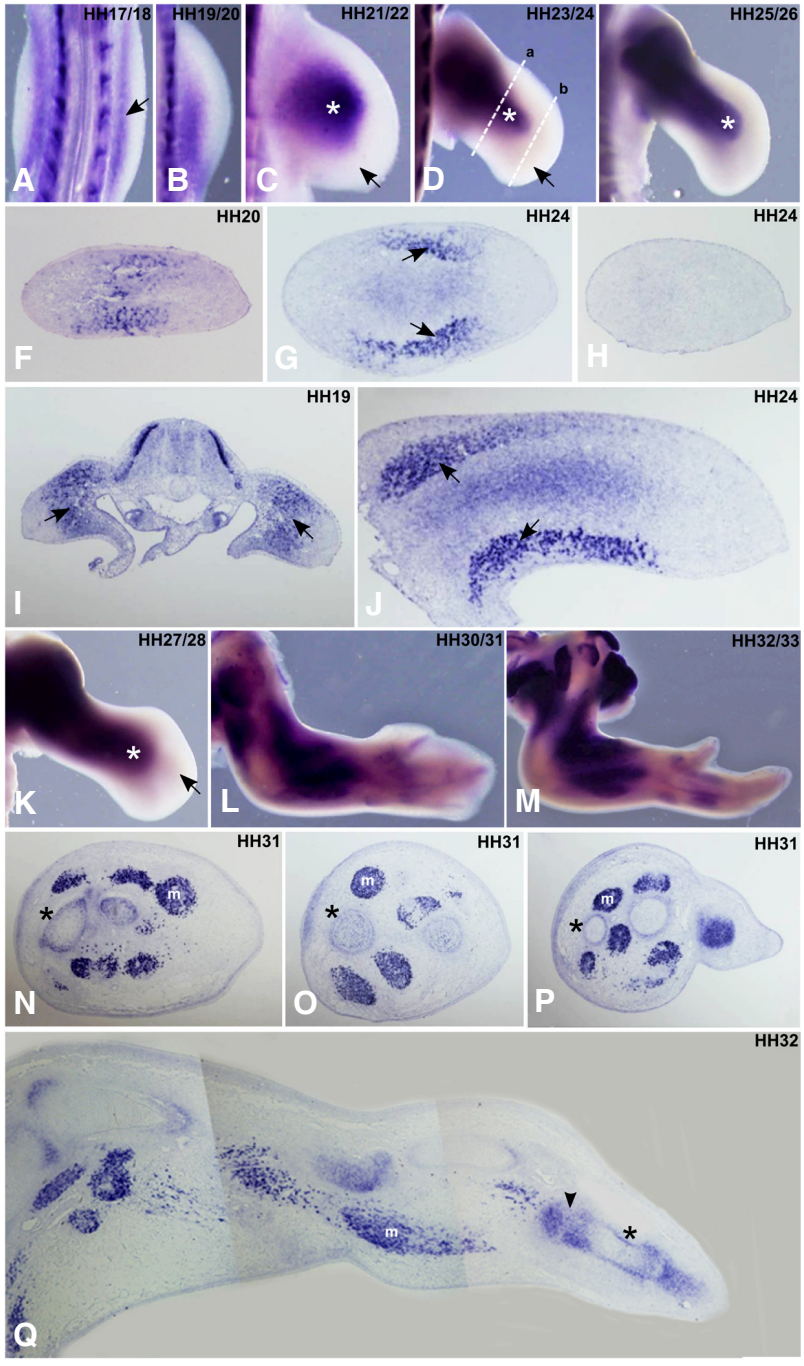



**Fig. 6. *FgfR4* expression pattern during chick forelimb development.** *Chick wings at HH17-33 stages were processed for* in situ *hybridisation in whole-mounts* **(A-E, K-M)** *and transverse/longitudinal sections* **(F-J, N-Q)** *using a probe for non isoform specific chick* FgfR4. **(A-E)** In situ *hybridisation in whole-mount limbs shows limb* FgfR4 *expression at HH17 (*A, *arrow). There is no* FgfR4 *expression in the AER and ZPA (*C,D, *arrows), while* FgfR4 *expression is observed in muscle masses (asterisks; also in* K*).* **(F-H)** *Transverse sections of HH20 and HH24 limbs (G: proximal, H: distal, represented in* (D) *as (a) and (b), respectively) reveal* FgfR4 *expression in the dorsal and ventral muscle masses (G, arrows).* **(I,J)** *Longitudinal sections of HH19 and HH24 limbs indicate* FgfR4 *expression in muscle precursors (I, arrows) and then in dorsal and ventral muscle masses (J, arrows).* **(K-M)** In situ *hybridisation experiments in whole mount limbs at HH27 to HH33 show an absence of* FgfR4 *expression in distal limb mesenchyme (K, arrow). In limb stage HH31 FgfR3 is expressed in the perichondrium (N-P, asterisk) and in the muscle tissue (N-P, m).* **(Q)** In situ *hybridisation in longitudinal limb sections at HH32 shows* FgfR4 *expression in individualised muscles (m), in the perichondrium of distal cartilage elements (asterisk) and interphalangic joints (arrowhead).* **(A-E, K-M)** *are dorsal views of whole-mount limbs.* **(F-H, N-P)** *Transverse sections are oriented such that left, posterior; right, anterior; top, dorsal; bottom, ventral.* **(I,J,Q)** *longitudinal limb sections are positioned such that left is proximal and right is distal. Embryonic stages of the limbs are mentioned in the upper right corner of the pictures.*

F,H, arrow). Moreover, both isoforms can be observed in the ectoderm at this stage (Fig. 5 F,H, asterisk).

### FgfR4 *expression*

Analysis of *FgfR4* expression patterns showed that this receptor starts being expressed in the most proximal part of limb buds at stage HH17 (Fig. 6A, arrow). From stage HH21 to HH28 *FgfR4* is persistently expressed in a central mesenchymal domain, which extends distally as the limb develops (Fig. 6 C-E,K asterisk). Transverse and longitudinal section *in situ* hybridization analyses showed that this expression is most predominant in the dorsal and ventral muscle masses (Fig. 6 G,J arrow, Marcelle *et al.* 1995). There is no *FgfR4* expression in the AER, the underlying distal mesenchyme and in the ZPA (Fig. 6 C-E). From stage HH29 on, *FgfR4* expression is also located in limb cartilage element domains (Fig. 6 L-Q), as previously described (Marcelle *et al.* 1995). Analysis of longitudinal and cross sections at stage HH31/32 revealed that this gene is weakly expressed in the perichondrium and interphalangic joints (Fig. 6 N-Q asterisk and arrowhead, respectively), in addition to its stronger expression in individualised muscles (Fig. 6 N-Q, m).

## Discussion

Fgf signalling plays an important role in many aspects of limb development, such as initiation, outgrowth and patterning (reviewed in Martin, 1998; Xu *et al.* 1999; Yu and Ornitz, 2008). In this study we performed a careful examination of the spatio-temporal expression patterns of *FgfR1-4* genes and IIIb and IIIc isoforms of *FgfR1-3* during chick wing development.

### FgfR *expression in the AER and in the underlying undifferentiated mesenchyme*

The AER constitutes the signalling centre responsible for limb outgrowth along the PD axis. Fgfs produced by the AER signal to the underlying mesenchyme or to the neighbouring ectoderm, which express Fgf receptors and various components of the Fgf signalling pathway. Our results show that during early limb patterning events (HH17-20), *FGFR1* and *FGFR2* are expressed in the developing chick forelimb buds. *FgfR1* is the main Fgf receptor to be expressed in the undifferentiated mesenchymal cells

located in the distal part of the limb. *FgfR2* expression is also detected in the mesenchyme but not in the distal limb region. Interestingly, the distal expression of *FgfR1* is progressively lost at stages HH26-28, which correspond to the stages when distal autopod skeletal elements are being laid down. FgfR1 involvement in growth and patterning of vertebrate limbs was first evidenced by the targeted mutation of *FgfR1*, which affected autopod patterning in mice (Partanen *et al.* 1998). More recently, conditional deletion of *FgfR1* gene in mouse embryos suggested a three phase function for FgfR1: elongation of PD axis at early stages, mesenchymal cell survival at middle stages and patterning autopod at later stages (Verheyden *et al.* 2005). Supporting its role in later stages, previous studies show that mutant mouse embryos carrying a targeted deletion of the *FgfR1*$\alpha$ (*IIIc*) isoform exhibited distal truncation of limb buds which were shorter in PD axis and wider in AP axis (reviewed in Xu *et al.* 1999).

Our whole-mount analyses show that *FgfR2* is the only *FgfR* to be robustly expressed in the entire ectoderm, including the AER, throughout all stages of development. This expression pattern supports the idea that chick *FgfR2* is mediating the Fgf signal in AER formation, ectodermal cell movements towards and into the limb as well as in certain aspects of dorsoventral ectodermal polarity establishment, as already described in mice (Gorivodsky and Lonai, 2003). *FgfR2* expressed in the AER has been reported to be essential for the maintenance of this structure (Lu *et al*. 2008). These authors also reported the loss of autopod in mouse forelimb upon conditional removal of *FgfR2* from the AER bringing forth the involvement of FgfR2 in distal limb patterning. Among the two FgfR2 isoforms, FgfR2IIIb variant is implicated in mesenchymal-epithelial signalling loop in limb bud initiation (De Moerlooze *et al*. 2000), whereas the FgfR2IIIc form is associated to limb skeletal bone formation (Eswarakumar *et al.* 2002). Recently, conditional inactivation of mouse *FgfR2* from the AER and both *FgfR1* and *FgfR2* from the mesenchyme suggest that AER-Fgfs function not only as survival factors but also as regulators of mesenchymal proliferation and chondrogenic differentiation (Yu and Ornitz, 2008). Overall, *FgfR1* and *FgfR2* are involved in limb initiation and patterning.

*FgfR3* and *FgfR4* are not expressed in the AER or in the underlying undifferentiated zone throughout all chick limb developmental stages.

### *Fgf signaling in the ZPA*

The ZPA is another important limb signalling centre, which produces Shh and organizes the limb along the AP axis. It has been shown that there is a positive feed-back loop between *Shh* expressed in the ZPA and *Fgf4* expressed in the AER, which is important for limb development (reviewed in Towers and Tickle, 2009). Our whole-mount *in situ* hybridisation analyses show that *FgfR1* is the unique *FgfR* continuously expressed in the ZPA from stage HH17 to 26, suggesting that FgfR1 is involved in this regulatory loop. Accordingly, conditional inactivation of *FgfR1* in posterior limb caused reduction in *Shh* RNA levels and consequently affected digit identity (Verheyden *et al.* 2005).

### *FgfRs and cartilage development*

Our whole-mount and section analyses showed that all four *FgfRs* are expressed during chondrogenesis in the chick wing. *FgfR1* is strongly expressed in perichondrium and in forming joints. *FgfR1* expression in cartilage elements displays no variation along the PD axis of the limb. In contrast, *FgfR2, 3* and *4* expression in cartilage elements varies along the PD axis, being expressed in the more distal cartilage elements and becoming restricted to the perichondrium as development proceeds. Mutations in *FgfR1, 2* or *3* genes lead to human limb congenital disorders (Wilkie *et al.* 2002; Coumoul and Deng, 2003). In addition *FgfR1* and *FgfR3* have been described to take part in bone fracture repair (Nakajima *et al.* 2001; Nakajima *et al.* 2003). The involvement of *FgfR3* in chondrogenesis and its mutation in inherited defective growth of human long bones syndrome was reported earlier (Colvin *et al.* 1996; Deng *et al.* 1996; Delezoide *et al.* 1998). Recently, the involvement of Fgf signalling through FgfR3 in the commitment of pre-chondrogenic mesenchymal cells to chondrogenesis and to cartilage production was documented (Davidson *et al.* 2005). Activating *FgfR3* in mice can mimic human dwarfism (Li *et al.* 1999) and lack of *FgfR3* causes skeletal overgrowth (Colvin *et al.* 1996; Deng *et al.* 1996), indicating that FgfR3 acts as a negative regulator of bone development. The downstream pathways responsible for the negative regulation are the MAP kinase pathway that inhibits chondrocyte differentiation and the Stat1 pathway that inhibits chondrocyte proliferation (Murakami *et al.* 2004).

Our observation of *FgfR2* expression surrounding cartilage elements during limb development is in agreement with the suggestion that *FgfR2* activation elicits a lateral inhibition of chondrogenesis that limits the expansion of developing skeletal elements (Moftah *et al.* 2002). Finally, although mouse homozygous for targeted *FgfR4* mutation was normal, double homozygous disruption for both *FgfR3* and *4* showed defects in long bone growth indicating that *FgfR4* could also be a positive regulator of long bone growth (Weinstein *et al.* 1998; Lazarus *et al.* 2007).

### *FgfRs and muscle formation*

Precursors of limb muscles orginate from the somites, migrate to the limb and undergo differentiation. In all chick limb bud stages analysed, *FgfR4* expression is observed in migrating myogenic cells, in dorsal and ventral muscle masses and then in individualised muscles, consistent with its involvement in limb myogenesis (Marcelle *et al.* 1995; Marics *et al.* 2002). FgfR4 signalling has been shown to participate in terminal skeletal muscle differentiation in the embryo and during muscle regeneration process in the adult (Marics *et al.* 2002; Yu *et al.* 2004; Zhao *et al.* 2006). However, *FgfR4* is not the only *FgfR* expressed in muscles but also *FgfR1* expression is observed (Fig. 1N), as previously described (Edom-Vovard *et al.* 2001a, b, Eloy-Trinquet *et al.* 2009). Interestingly, down-regulation of FgfR1 signalling has also been correlated with terminal myogenic differentiation (Grothe *et al.* 1996; Itoh *et al.* 1996). So, two FgfRs are associated with muscle formation, FgfR1 and 4. However, their precise role in limb myogenesis is still unclear.

### *FgfRs and feather development*

We would also like to point out the expression of *FgfRs* in the context of feather development. Our findings that all *FgfRs* except *FgfR4* are expressed either in (*FgfR1*) or around (*FgfR2* and *3)* presumptive feather buds correlates with previously reported data (Noji *et al.* 1993). Recently the importance of FgfR1 and FgfR2 in feather development was demonstrated using dominant

negative forms (Mandler and Neubuser, 2004).

### *Conclusions*

The results obtained throughout our work are overall in accordance to what has been previously described (Szebenyi *et al.* 1995). We additionally report *FgfR1* expression in the distal ectoderm, including the AER and *FgfR3* in the ectoderm of late limb developmental stages (HH31). The expression patterns of the different *FgfRs* are consistent with their involvement in different steps of limb development, such as early limb bud formation, outgrowth and patterning. *FgfR* expression is also linked with the differentiation process of various cell types including, cartilage, muscle and feathers. Finally, we have analysed, for the first time, the expression of the different isoforms of *FgfR1* and *FgfR3* (*FgfR1IIIb, FgfR1IIIc, FgfR3IIIb* and *FgfR3IIIc*). The results obtained highlight the importance of the epithelial-mesenchymal tissue interactions operating during limb development. The relative affinity of each FgfR isoform to the different limb Fgfs has been previously described (Ornitz *et al*. 1996; Zhang *et al*. 2006), and our FgfR expression profiles evidence a trend in that the ectodermal-expressed isoforms, such as FgfR2IIIb, respond to mesenchyme-produced Fgfs (Fgf10), while FgfR isoforms found in the mesoderm preferentially react to ectodermal-derived Fgfs. This is particularly evident for FgfR2IIIc and FgfR3IIIc which recognize Fgf2,4,8,9 and Fgf2,8,9, respectively.

## Materials and Methods

### *Eggs and embryos*

Fertilised chick (*Gallus gallus*) eggs obtained from commercial sources were incubated at 38°C in a 49% humidified atmosphere and staged according to the Hamburger and Hamilton (HH) classification (Hamburger and Hamilton, 1951).

### In situ *hybridisation probes*

Non isoform-specific chick FgfR1-4 probes were kindly provided by Dr. Guojun Sheng (Nakazawa *et al*. 2006). *In situ* hybridisation probes for FgfR1-3 IIIb and IIIc isoforms were generated by amplifying portions of these genes by reverse transcription and polymerase chain reactions (RT-PCR) using the isoform-specific primers previously described in Sinh *et al.* (2005). The DNA fragments generated were cloned into the pCR®II-TOPO® vector (Invitrogen, USA) and plasmid DNA was isolated. The constructs were confirmed upon sequencing. Digoxigenin-labelled RNA probes were synthesized using linearised plasmids, according to standard procedures.

### In situ *hybridisation of whole-mount embryos and tissue sections*

Embryos were fixed overnight at 4°C in a solution of 4% formaldehyde with 2 mM EGTA in PBS at pH 7.5, rinsed in PBT (PBS, 0.1% Tween 20), dehydrated in methanol and stored at –20°C. Whole mount *in situ* hybridisation was performed as previously described (Henrique *et al.* 1995).

Paraffin sections were prepared as follows: stage HH32 chick wings were collected in PBS and fixed at 4ºC over night in a solution of 60% ethanol, 30% formaldehyde and 10% acetic acid. The following day, limbs were dehydrated in series of ethanol with a final step of xylene. Finally, they were incubated in paraffin at 70ºC for 30 min, placed in the desired orientation and left to solidify. Longitudinal limb sections of 10 μm were made using Microm HM325 on SuperFrost Plus (Menzel-Glaser) slides and allowed to dry at 37ºC overnight. *In situ* hybridisation on paraffin sections was performed as described previously (Tozer *et al.* 2007).

### *RNA extraction from limb tissues and RT-PCR reactions*

Stage HH24 chick fore-limbs were submitted to pancretin-mediated digestion until the ectoderm detached freely from the mesoderm. The reaction was stopped by the addition of goat serum solution (Invitrogen). The ectoderm was isolated from the mesoderm and both tissues were collected separately for RNA isolation using the RNeasy Mini Kit Protect (Qiagen, Germany). Total mRNA quantification was done by spectrophotometry (NanoDrop Technologies, Inc., USA). Total RNA was digested with DNAse RNAse-Free (Promega, USA) according to manufacturer's instructions. Purified RNA was reverse transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen), and a PCR reaction was performed employing the FgfR isoform-specific primer pairs described by Shin *et al.* (2005).

### *Imaging*

Embryos processed for *in situ* hybridisation were photographed in PBT/0.1% azide, using an Olympus DP71 digital camera coupled to an Olympus SZX16 stereomicroscope equipped with Cell^B program. Vibratome and paraffin limb sections were photographed using an Olympus DP70 camera coupled to an Olympus BX61 microscope.

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**Competition for ligands between FGFR1 and FGFR4 regulates Xenopus neural development**
Masahiro Yamagishi and Harumasa Okamoto
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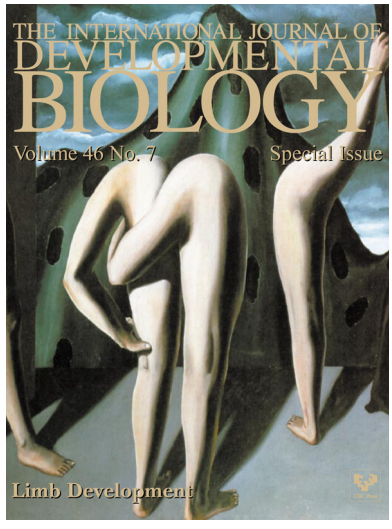


**5 yr ISI Impact Factor (2009) = 3.253**