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, colonize 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...
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 morpogenic 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 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; Szabeni 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. 1F-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. 1H, 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 shown).
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 interphalangeal joints (Fig. 1N-Q, arrowheads and 1Q, arrow, respectively). In addition, FgfR1 expression is also observed in muscles (Fig. 1N,m), tendons (Fig. 1N) and in feather buds of the body wall (Fig. 1L,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. 1N-Q and 1Q, inset) which fades gradually towards the distal limb.

**FgfR1 expression**

FgfR1 transcripts were first detected in limb buds at stage HH19, where they are clearly restricted to the most proximal mesoderm (Fig. 3B-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. 3A-J), although it could be detected in the proximal mesoderm (Fig. 3J, arrow). From HH25 stage, FgfR3 is strongly expressed in all the expressed in a thin layer of mesenchymal cells juxtaposed the limb ectoderm, with a higher signal in the dorsal regions (Fig. 1N-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. 2F-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. 2D,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. 2K,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. 2P,Q, arrow), and was observed in the perichondrium of the more proximal cartilage elements (Fig. 2N,O,Q, arrowhead).

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**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 stages HH19 and HH24 show 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.
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. 3 N), 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 IIb and IIc isoforms of FgfR1-3 in segregated ectoderm and mesoderm tissues of stage HH24 limb buds. RT-PCR analysis clearly revealed that FgfR2IIb is exclusively expressed in the limb ectoderm, while FgfR2IIc and FgfR3IIc are only found in limb mesenchyme (Fig. 4). These results strongly imply that the FgfR2 ectodermal staining observed in Fig. 2 is due to FgfR2IIb isoform expression. Conversely, FgfR2 mesodermal staining is a result of FgfR2IIc expression. Since FgfR3IIc 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 FgfR3IIb 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: FgfR1IIlb-GU053725; FgfR1IIlc-GU065444; FgfR3IIlb-GU053726; FgfR3IIlc-GU065445.

*In situ* hybridization of FgfR1IIlb and FgfR1IIlc 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: FgfR1IIlb consistently marked the ectoderm stronger than the FgfR1IIlc isoform (Fig. 5 A,B,C,D, arrows) and, in stage HH31 limb sections, FgfR1IIlc presents stronger expression in the perichondrium than the other variant (Fig. 5 B,D, arrowhead).

At stage HH24, FgfR3IIlb is expressed throughout the entire limb and the FgfR3IIlc 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), FgfR3IIlc transcripts are predominantly detected in and around the cartilage, unlike the faint expression of FgfR3IIlb (Fig. 5 G,H).
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 FgfR1α (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, FgfR2IIb variant is implicated in mesenchymal-epithelial signalling loop in limb bud initiation (De Meeuwoof 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-Fgf’s 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 feedback 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; Delezioide 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 homologous for targeted FgfR4 mutation was normal, double homologous 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 originate 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-Trinquét 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 (Szepenyei 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 also linked with the differentiation process of various cell types according to what has been previously described (Szebenyi et al. 1996; Zhang et al. 2006), and our Fgfr expression profiles evidence a trend in that the ectodermal-expressed isoforms, such as Fgfr2IIb, respond to mesenchyme-produced Fgfs (Fgf10), while Fgfr isoforms found in the mesoderm preferentially react to ectodermal-derived Fgfs. This is particularly evident for Fgfr2IIc and Fgfr3IIc which recognize Fgfs 2,4,8,9 and Fgfs 8,9, respectively.

Materials and Methods

Eggs and embryos
Fertilised chick (Gallus gallus) eggs obtained from 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 Fgfr1-3IIb and IIc isoforms were generated by amplifying portions of these genes by reverse transcription and polymerase chain reaction (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 4°C 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 70°C 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 37°C 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 pancreatin-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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References


LOOMIS, C.A., HARRIS, E., MICHAUD, J., WURST, W., HANKS, M. and JOYNER, 
MOFTAH, M.Z., DOWNIE, S.A., BRONSTEIN, N.B., MEZENTSEVA, N., PU, J., 
MURAKAMI, S., BALMES, G., MCKINNEY, S., ZIVIOL, D. and DE 
Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell 84: 911-921. 


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*Int. J. Dev. Biol.* (1996) 40: 1185-118

**Expression of FGFR-4 mRNA in developing mouse tissues**
J Korhonen, J Partanen and K Alitalo
*Int. J. Dev. Biol.* (1992) 36: 323-329

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