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Developmental Expression Pattern

The expression of *Flrt3* during chick limb development

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ABSTRACT The *Flrt3* (*F*ibronectin-*L*eucine-*R*ich *T*ransmembrane protein) gene encodes a fibronectin and leucine-rich repeat transmembrane protein whose expression is controlled by fibroblast growth factors (FGFs). FLRT3 has been implicated in neurite outgrowth after nerve damage, as a positive regulator of FGF signalling and in homotypic cell adhesion. Here we describe *Flrt3* expression during chick embryonic limb development using whole-mount *in situ* hybridization. We found very dynamic expression during apical ridge formation and limb bud outgrowth. Initially *Flrt3* is expressed in the apical ectodermal ridge and underlying mesenchyme, but then becomes restricted to the dorsal and ventral sides of the apical ridge as a twin stripe. At later stages, abundant expression is seen in the hindlimb and in both the pectoral and pelvic girdle-forming regions. FLRT3 may have a crucial role in regulating cellular adhesion between the epithelial apical ridge and the underlying mesenchyme and in establishing the dorso-ventral position of the ridge.

KEY WORDS: *FLRT3, apical ectodermal ridge, FGF, limb bud*

The novel *FLRT* (*f* ibronectin-/ eucine-*r* ich *t* ransmembrane protein) human gene family encodes type-I transmembrane cell surface proteins (Lacy *et al.*, 1999). The extracellular region of FLRT contains a conserved fibronectin type-III domain and multiple leucine-rich repeats (Lacy *et al.*, 1999). The focus of many recent studies has been on the third member of the family, FLRT3. The *Xenopus* orthologue of FLRT3 has been identified and when injected into animal caps induced the mesodermal marker *Xbra*, suggesting that XFLRT3 acts to enhance Fibroblast growth factor (FGF) signalling (Bottcher *et al.*, 2004). XFLRT3 directly associated with FGFR1 and FGFR4a in co-immunoprecipitation experiments and deletion of the fibronectin domain reduced this interaction (Bottcher *et al.*, 2004). Induction of microcephaly, ectopic tail-like structures and phosphorylation of ERK MAPK was mediated by the intracellular carboxy-terminal region (Bottcher *et al.*, 2004). More recently, FLRT3 has been implicated during neurite outgrowth following injury and the leucine-rich repeats of FLRT3 have been proposed to function during homotypic cell adhesion (Tsuji *et al.*, 2004, Karaulanov *et al.*, 2006).

The chick limb has been used for many years as an ideal tool to study how FGFs and other signalling pathways relay information between epithelial and mesenchymal tissues (Tickle, 2003). The apical ectodermal ridge (AER) is a specialized pseudostratified epithelium at the dorso-ventral boundary of the distal limb bud. It is a source of many FGFs, including FGF-8, which signal to the underlying mesenchyme and are required for limb bud growth (Niswander *et al.*, 1994, Sun *et al.*, 2002). *XFLRT3* coincided with sites of *fgf-8* expression in *Xenopus*, including the mid-brain hindbrain boundary (MHB) and the tail bud and was regulated by FGF signalling. *XFLRT3* transcripts were increased in animal caps after *fgf-8* mRNA injection and were absent in ventral marginal zone cells expressing a dominant-negative FGFR1 (Bottcher *et al.*, 2004). We therefore cloned the chick orthologue of *FLRT3* (*cFlrt3)* and present an account of its expression specifically during embryonic limb development.

cFlrt3 is expressed in the forelimb ventral mesoderm as the AER is forming from stage HH16-17, but its expression is delayed in the hindlimb (Fig. 1A). Between HH18 and early stage HH19, *cFlrt3* is expressed as a single stripe throughout the AER and in the mesenchyme directly beneath it (Fig. 1B, C). From HH20 onwards until late stage HH26, *cFlrt3* transcripts are restricted to the AER at the interface between apical ridge and limb mesenchyme (Fig. 1D, E, F, G, H, Fig. 2B, C, D, E and data not shown). This expression is along the AER, but absent from the most posterior ridge ectoderm (Fig. 1E). Face on, it appears as a twin-stripe expressed either side of the border between the ventral and dorsal limb ectoderm (Fig. 1G). At limb

Abbreviations used in this paper: AER, apical ectodermal ridge; ERK MAPK, extracellular regulated kinase mitogen-activated protein kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; FLRT, fibronectin-leucine-rich transmembrane protein; MHB, mid-brain hindbrain boundary.

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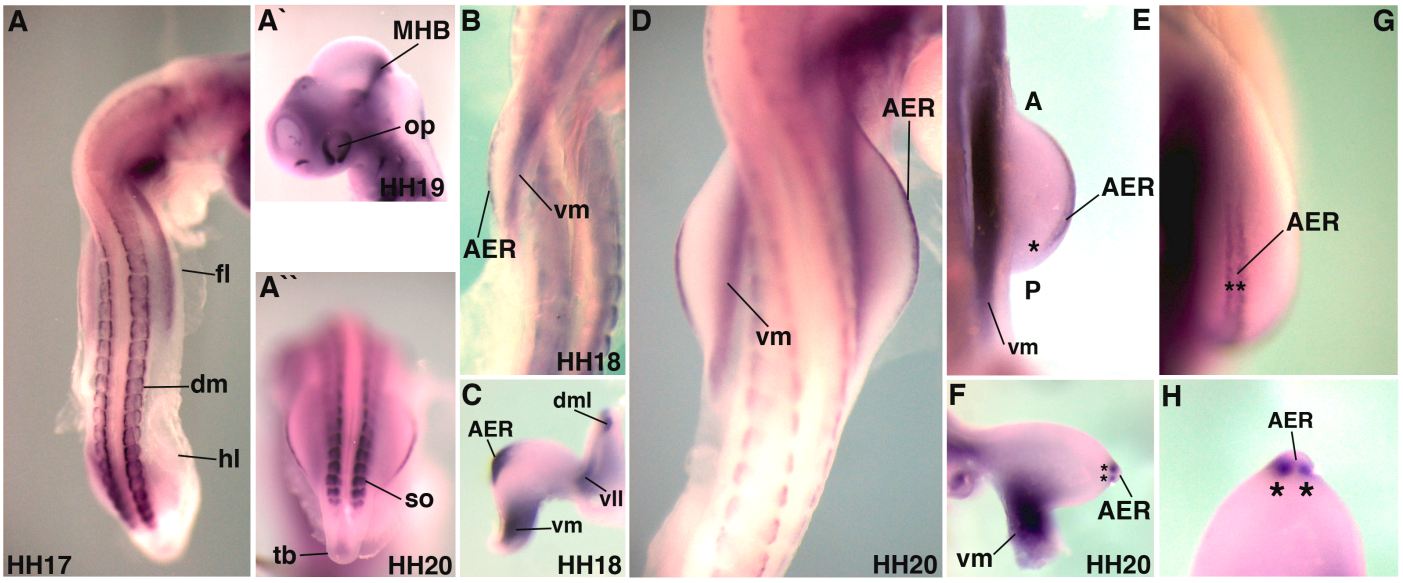

Fig. 1. Expression of *cFlrt3* during early limb development. *Whole-mount* in situ *hybridization showing expression of chick* Flrt3 *during limb formation.* ***(A)*** *Stage HH17 embryo showing initial* cFlrt3 *expression in the ventral mesoderm of the developing forelimb bud. Strong expression is also seen in the dermomyotome of somites.* ***(A')*** *Expression of* cFlrt3 *in the optic vesicle and MHB in a stage HH19 head.* ***(A'')*** *Transcripts are present in early epithelial somites and weakly in the tailbud at HH20.* ***(B)*** *Ventral view of a stage HH18 forelimb bud. Expression persists in the ventral mesoderm and emerges in the newly forming apical ectodermal ridge as a single stripe.* ***(C)*** *Transverse section of HH18 forelimb bud. Expression can be seen in the ventral mesoderm of the flank and in the apical ridge and subjacent mesenchyme. The section also shows clear* cFlrt3 *expression in the dorso-medial and ventro-lateral lips of the dermomyotome.* ***(D)*** *Dorsal view of forelimb buds at stage HH20.* ***(E)*** *Ventral view of HH20 forelimb bud showing* cFlrt3 *expression along the ridge until the posterior ridge ectoderm, marked by an asterisk. Expression in the ventral mesoderm of the flank can also be seen, which extends just beyond the position of the limb bud.* ***(F)*** *Transverse section of a HH20 limb. Asterisks mark the twin expression lines of* cFlrt3 *in the AER that run alongside the subjacent mesenchyme. This pattern persists throughout limb development until late stage HH26. From the flank,* cFlrt3 *expression slightly enters the limb mesenchyme at the base of the bud.* ***(G)*** *Face on view of a whole-mount stage HH20 limb bud clearly showing the tramline-like expression either side of the dorso-ventral border of the apical ridge. Marked by asterisks.* ***(H)*** *A transverse section showing a closer view of* cFlrt3 *expression. It is at the epithelial-mesenchymal boundary in the AER. Two asterisks mark the twin-stripe expression. A; anterior. AER; apical ectodermal ridge. dm; dermomyotome. dml; dorso-medial lip. fl; forelimb bud. hl; hindlimb bud. MHB; mid-brain hindbrain boundary. op; optic vesicle. P; posterior. so; somite. tb; tailbud. vll; ventro-lateral lip. vm; ventral mesoderm.*

axial positions, *cFlrt3* is abundantly expressed in the ventral mesoderm of the flank (Fig. 1A, B, D, E, Fig. 2A, E). *cFlrt3* expression extending from the flank slightly enters the limb bud mesenchyme itself (Fig. 1F, Fig. 2E).

At HH24-25, *cFlrt3* expression is very pronounced within pelvic and pectoral-forming regions of the embryo at the base of the limb bud (Fig. 2A, B). At these stages, the hindlimb contains an additional domain of expression stronger than that seen in the forelimb (Fig. 2A, B, C, D). This expression could be within muscle progenitors that have migrated from the dermomyotome ventro-lateral lip into the hindlimb from adjacent somites (Fig. 1A, C) (Dietrich, 1999).

Upon closer inspection, a new domain of *cFlrt3* expression in the distal limb was noticed. While expression can be clearly seen at the epithelial-mesenchymal boundary in the AER, there exists an additional isolated splinter of expression further away from the AER in both fore- and hind-limb buds (Fig. 2C, D). This expression is mesenchymal and seems to be under the avascular zone between the blood vessel network and the rim of the limb bud (Mohammed, 1986 and M. G. Davey, personal communication).

These distal expression patterns cease and are absent at HH28 (Fig. 2F). From stages HH27-28, *cFlrt3* expression is associated with some muscle primordia in both limbs and patches of expression are in the digital plate (Fig. 2F and data not shown).

Like *XFLRT3*, the chick *cFlrt3* orthologue is expressed at sites of FGF-8 signalling in the embryo, such as the MHB, optic vesicle, weakly in the tail bud and like *XFLRT2*, in somites (Fig. 1A, A', A'', C) (Bottcher *et al.*, 2004, Smith *et al.*, 2005). In the limb bud we found prominent *cFlrt3* expression in the AER, which is critical for limb formation. However the target of FGF-8 signalling is the limb bud mesenchyme (Tickle and Munsterberg, 2001). An FGF that signals to overlying ectodermal cells is FGF-10 (Isaac *et al.*, 2000) and could be responsible for *cFlrt3* expression in the ridge. FGF-10 is expressed before limb initiation and continues until stage HH28 (Ohuchi *et al.*, 1997), similarly to *cFlrt3* (Fig. 2F). FGF-10 is critical for AER formation (Yonei-Tamura *et al.*, 1999) and in maintaining *fgf-8* expression during limb bud outgrowth (Xu *et al.*, 1998). As both *fgf-8* and *cFlrt3* are similarly expressed along the anterior-posterior axis of the AER (Fig. 1E) and XFLRT3 was shown to positively modulate FGF activity (Bottcher *et al.*, 2004), cFLRT3 could therefore reinforce FGF-10 activity within the AER to drive the FGF-10/FGF-8 feedback loop (Xu *et al.*, 1998). It would be interesting to determine whether *fgf-8* expression is

maintained after knock-down of *cFlrt3* by RNAi.

The *cFlrt3* expression pattern is reminiscent of the chick *cux-1* gene, a transcription factor that mediates Notch signalling during dorso-ventral limb patterning. Similar to *cFlrt3* (Fig. 1G, H), *cux-1* is expressed as a twin-stripe in the AER (Tavares *et al.*, 2000). Canonical Wnt-3a signalling via β-catenin is important in AER formation (Kengaku *et al.*, 1998) and induces *cux-1* expression (Tavares *et al.*, 2000). Therefore FLRT3 could be downstream of signalling pathways implicated in determining the dorso-ventral position and formation of the AER (Tickle and Munsterberg, 2001). In addition we find it particularly striking, considering the role of FLRT3 in cell adhesion (Karaulanov *et al.*, 2006), that *cFlrt3* is expressed at the interface between AER and the mesenchyme it adheres to (Fig. 1H).

cFlrt3 is expressed in many other tissues during organogenesis that has yet to be explored. FGF signalling is a key player during limb and organ development and while negative regulators of FGF signalling have been intensely studied, it is equally crucial that we begin to study the role that positive FGF regulators have in these tissues (Thisse and Thisse, 2005).

Experimental Procedures

Production of the cFlrt3 *probe*

The chick *Flrt3* orthologue was discovered from a search in the ENSEMBL *Gallus gallus* genome browser: Identification ENSGALG00000008716. PCR was performed on stage HH16 whole-embryo cDNA to generate two probes that hybridized to different regions of the *cFlrt3* gene. Primers used were
1) For: 5'- AGATGAATTCCCCACTAACCTC -3' and
Rev: 5'- ACGTGCGGCCGCATCAGCCCACGTACGTTCAC -3'.
2) For: 5'- ACGTGAATTCATGACATGAGCACCATCCAG -3'
Rev: 5'- ACGTGCGGCCGCGGAATACCACTGTCTCTGTAG -3'.
Each PCR product was cloned into pBluescript (KS) using

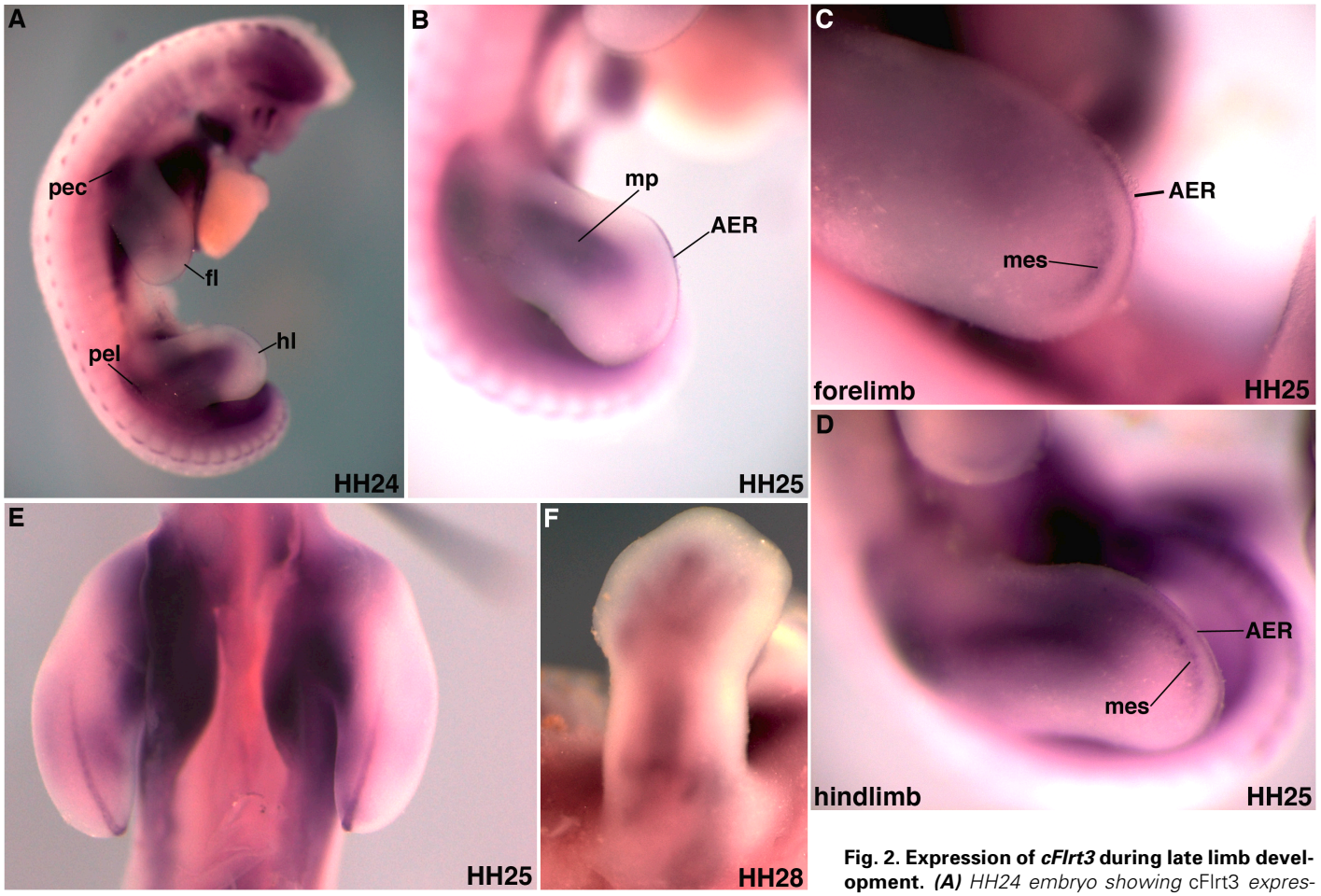

Fig. 2. Expression of *cFlrt3* during late limb development. ***(A)*** *HH24 embryo showing* cFlrt3 *expression in various tissues. There is expression in both the pectoral and pelvic-forming regions of the limb buds and a robust band of expression in the hindlimb, which is weaker in the forelimb.* ***(B)*** *Closer view of a stage HH25 hindlimb showing the band of expression possibly in muscle progenitors.* ***(C)*** *Close view of a stage HH25 forelimb bud showing the borderline ectodermal expression of* cFlrt3 *and a new proximal domain of mesenchymal expression.* ***(D)*** *Close view of a stage HH25 hindlimb, showing the new expression domain in the distal limb.* ***(E)*** *Ventral view of stage HH25 forelimb buds. The heart has been removed to reveal more clearly the abundant expression in the ventral mesoderm of the flank region.* ***(F)*** *At stage HH28 there is no ectodermal expression of* cFlrt3. *Instead expression is associated with some muscle masses in the shank and digital plate. mes; mesenchyme. mp; muscle progenitors. pec; pectoral girdle region. pel; pelvic girdle region.*

EcoR1 and Not1 sites. *In situ* hybridizations were performed with both probes and each gave consistent patterns of expression.

In situ *hybridization*

Embryos were incubated at 37°C until stages HH16 to HH28 and analyzed by whole-mount *in situ* hybridization as described previously (Smith *et al.*, 2005). However, for stages HH24-26 and HH27-28, embryos were incubated with 10 μg/ml and 15 μg/ml respectively of Proteinase K at room temperature for 20 min. Embryos were then post-fixed in 4% PFA for a further 20 min.

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