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Original Article

Developmental expression of chick *Twist* and its regulation during limb patterning

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ABSTRACT We have isolated a chick *Twist* gene *(cTwist)* and examined its expression pattern during development by whole mount *in situ* hybridization. In early embryos, *cTwist* transcripts are found in the developing somites, lateral plate mesoderm, limb mesenchyme, branchial arches and head mesenchyme. At later stages, *cTwist* expression is found in the sclerotome and dermatome, limb bud mesenchyme, interdigital regions, and distal mesenchyme of the maxillary and mandibular processes. In the developing feathers, *cTwist* is expressed in the mesenchyme of the buds and becomes restricted to the proximal region of the feather filaments. Additionally, we report that the expression of *cTwist* in the limb mesenchyme is regulated by the AER, FGFs, RA and SHH. The FGFs secreted by the AER seem to have a critical role in maintaining *cTwist* expression. SHH is also able to maintain *cTwist* expression but only in the presence of the AER. Overall, our results provide new evidence that reinforce the existence of an interplay between the cTwist and FGF signalling pathways.

KEY WORDS: *Twist, chick embryo, limb development, FGF, SHH*

Introduction

Twist encodes a basic-helix-loop-helix (bHLH) transcription factor that was initially identified in *Drosophila* as one of the zygotic genes required for dorso-ventral patterning and mesoderm differentiation during embryogenesis (Thisse *et al.,* 1987; Leptin *et al.,* 1992). *Twist* homologues have been later found in other species, namely *CeTwist* in *Caenorhabditis elegans* (Harfe *et al.,* 1998), *Twist* in jellyfish (Spring *et al.,* 2000), *Twist-1* in zebrafish (Kim and Chitnis, 1999 in Genbank), *XTwi* in Xenopus (Hopwood *et al.,* 1989), *MTwist* in mouse (Wolf *et al.,* 1991), *RTwist* in rat (Bloch-Zupan *et al.,* 2001) and *TWIST* in humans (Wang *et al.,* 1997). Although a chick *Twist* homologue has been previously reported (Bushdid *et al.,* 1998; Kanegae *et al.,* 1998; Isaac *et al.,* 2000), neither a detailed analysis of its expression nor its putative function during chick development have been published.

Twist expression has been shown to correlate with mesenchymal regions which are under the influence of Fibroblast Growth Factors (FGFs) and Sonic hedgehog (SHH). A good model for studying the influence of these molecules on *cTwist* expression is the chick limb bud. During limb development, *cTwist* expression was detected in regions controlled by the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA; Bushdid *et al.,* 1998; Kanegae *et al.,* 1998; Isaac *et al.,* 2000). The AER is a specialized epithelial structure that forms at the tip of the vertebrate limb bud, secretes several FGFs (Niswander and Martin, 1992, Savage *et al.,* 1993; Heikinheimo *et al.,* 1994, Ohuchi *et al.,* 1994, Crossley and Martin, 1995; Savage and Fallon, 1995; Colvin *et al.,* 1999) and controls the proximal-distal outgrowth (Saunders, 1948). When the AER is excised, limbs appear truncated along this axis (Summerbell, 1974). Furthermore, AER function can be replaced by the action of several members of the FGF family (Niswander *et al.,* 1993; Fallon *et al.,* 1994; Crossley *et al.,* 1996; Vogel *et al.,* 1996). The ZPA is a group of cells located at the posterior region of the developing limb bud that expresses *shh* (Riddle *et al.,* 1993) and controls the anterior-posterior patterning of the vertebrate limb (Saunders and Gasseling, 1968). Application of a retinoic acid (RA) bead induces polarizing activity when implanted in the anterior part of the limb bud (Tickle *et al.,* 1982). Moreover, SHH was shown to be one of the molecules responsible for mediating the ZPA (Riddle *et al.,* 1993). In addition, the activities of these two organizing centers, the AER and the ZPA, depend on each other since there is a positive feedback loop between the FGFs expressed in the AER and SHH expressed in the ZPA. As *Fgf-4* is expressed in the posterior part

Abbreviations used in this paper: AER, apical ectodermal ridge; bHLH, basic helix-loop-helix; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; RA, retinoic acid; SHH, sonic hedgehog; ZPA, zone of polarizing activity.

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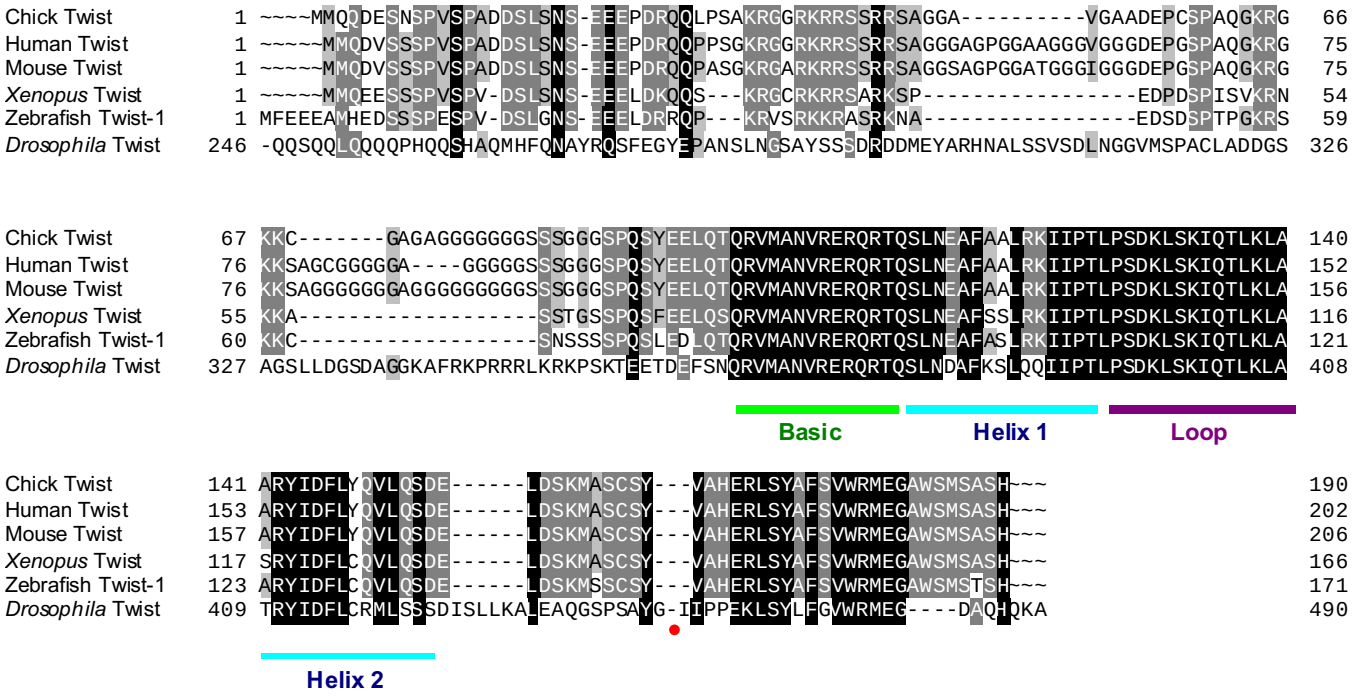

Fig. 1. Alignment of the chick Twist deduced amino acid sequence with the human, mouse, *Xenopus*, zebrafish and *Drosophila* homologues
(Drosophila sequence upstream of the bHLH domain is not included due to its high divergence). The lines under the sequences indicate the bHLH domains (basic in green, helix in blue, and loop in purple). Suppressed portion of the Drosophila sequence is indicated by a red dot.

of the AER (Niswander and Martin, 1992), it was thought to be responsible for maintaining this loop with SHH (Laufer *et al.,* 1994; Niswander *et al.,* 1994). However, recent studies made with *Fgf-4* knockout mice suggest that other FGFs may be involved in these epithelial-mesenchymal interactions (Moon *et al.,* 2000; Sun *et al.,* 2000).

In *twist* knockout mice, limb buds are reduced and the AER is absent in the forelimb buds (Chen and Behringer, 1995). In these mice, even though limbs are absent, limb budding does indeed occur possibly because of compensation by the Twist-related protein Dermo-1, which is also expressed in the limb bud mesenchyme (Li *et al.,* 1995). Heterozygous *MTwist*-null mice display craniofacial and limb malformations that resemble those of the human Saethre-Chotzen syndrome (Bourgeois *et al.,* 1998), a craniosynostosis syndrome recently associated with mutations in the *TWIST* gene (reviewed in Gripp *et al.,* 2000). *Fgf receptor* (*Fgfr*) *-1* and *-2* knockout mice also display defects in limb outgrowth (reviewed in Xu *et al.,* 1999). Moreover, mutations in human *FGFR1*, *-2* and *-3*, like those of *TWIST*, were found in patients with several craniosynostotic syndromes (reviewed in De Moerlooze and Dickson, 1997), associated mainly with broad thumbs and toes, syndactyly and brachydactyly. These observations are consistent with TWIST haploinsufficiency in Saethre-Chotzen syndrome and with a positive regulation of *Fgfr* expression by Twist. Accordingly, recent reports have shown that *Twist* expression precedes that of *Fgfr* genes in the developing mouse coronal suture (Johnson *et al.,* 2000), and FGFR2 protein expression is altered in *Twist*–null heterozygous mice (Rice *et al.,* 2000).

In this study we report a detailed expression pattern of *cTwist* during chick development. Our observations also support a correlation between *Twist* expression and FGF and SHH activities. Moreover, our data from limb bud manipulations show that FGF released from the AER and SHH from the ZPA regulates *cTwist* expression in mesenchymal cells. In the limb bud, as well as in other embryonic tissues, Twist may be part of a positive feedback loop acting between FGFs and their receptors. The similarities observed in the phenotypes of mouse and human mutants for TWIST and for FGFRs (reviewed in De Moerlooze and Dickson, 1997; Gripp *et al.,* 2000), together with evidence that in *Drosophila* and *C. elegans* Twist regulates the expression of FGFRs (Shishido *et al.,* 1993; Harfe *et al.,* 1998), suggest that *cTwist* may function as a regulator of FGFR expression.

Results

Cloning and expression pattern of cTwist

In the screening of a stage 20-22 (Hamburger and Hamilton, 1951) chick limb bud cDNA library, we isolated a chick *Twist* cDNA sequence encoding a putative protein of 573 residues. The predicted amino acid sequence of chick Twist is 79, 79, 61, 58, 25% identical to the human, mouse, *Xenopus*, zebrafish and *Drosophila* homologues, respectively (Fig. 1). The homology is higher in the bHLH domain, with amino acid identities of 100, 100, 93, 96 and 78%, respectively.

Chick *Twist* (*cTwist*) transcripts were first detected at stages 11-12 in the medial region of the somites (Fig. 2A). A very dynamic pattern of expression is seen during somite differentiation. At stages 13-15, *cTwist* expression is very strong in the ventral medial portion (presumptive sclerotome; Fig. 3 A,B; arrows) and weak in the dorsal portion (presumptive dermomyotome; Fig. 3 A,B; arrowheads). At stages 20-23, *cTwist* expression in the somites is

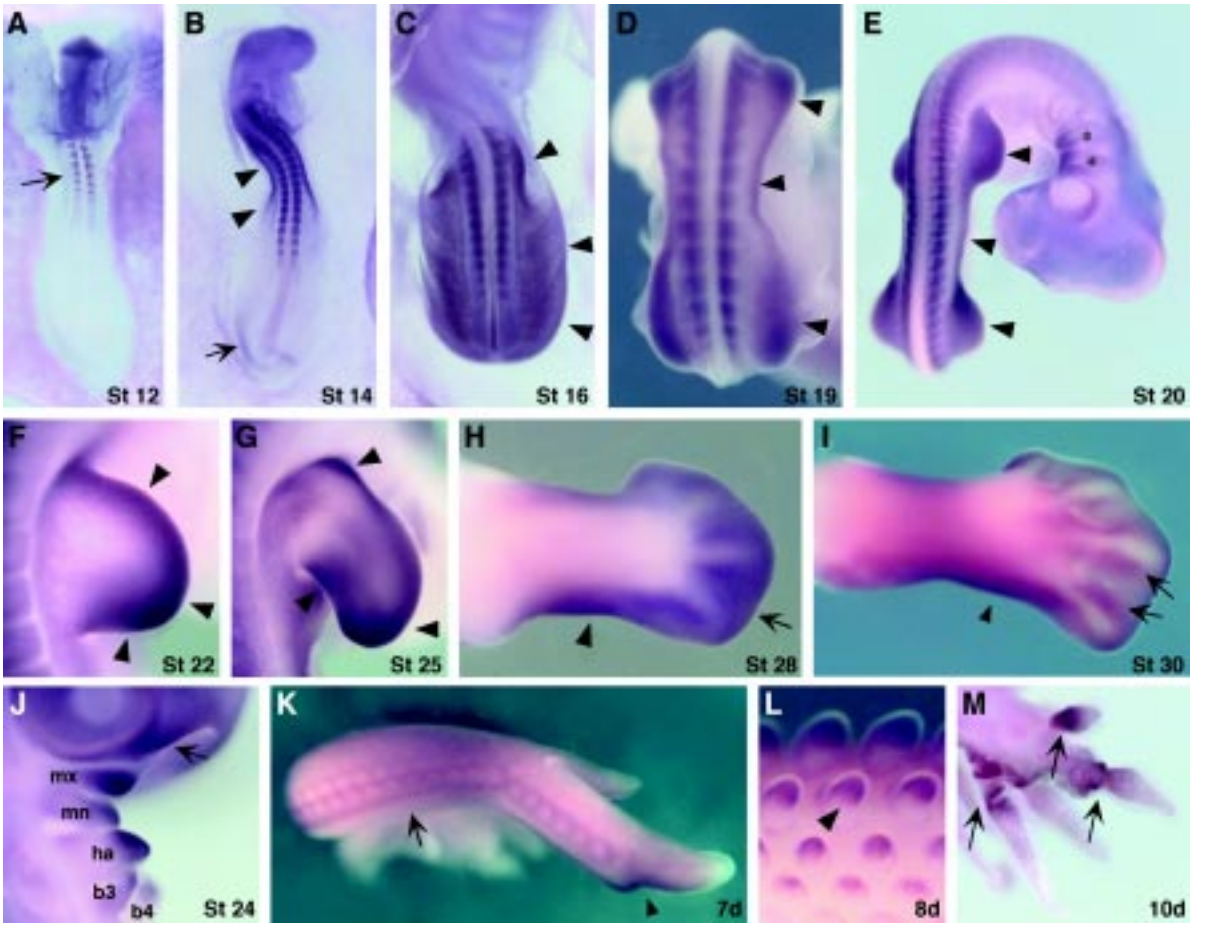

Fig. 2. Whole mount *in situ* hybridization analysis of *cTwist* in the chick embryo. (A) *At stage 12, cTwist transcripts are detected in the medial portion of the somites (arrow).* **(B)** *At stage 14, expression is found in the lateral plate mesoderm. The staining is stronger in the anterior (arrowheads) than in the posterior (arrow) LPM.* **(C)** *At stage 16, the expression of cTwist in the LPM has a broader domain spanning the limb and flank fields (arrowheads).* **(D)** *At stage 19, the expression is detected in the limb bud mesenchyme and in the flank (arrowheads).* **(E)** *At stage 20, cTwist expression remains in the flank and allover the mesenchyme of the limb buds (arrowheads). cTwist transcripts can also be detected in the branchial arches (asterisks).* **(F)** *At stage 22, expression in the limb buds is excluded from the central portion, remaining in the anterior, distal and posterior mesenchyme (arrowheads). In a stage 25 limb bud* **(G)**, *cTwist expression is stronger in the anterior, distal and posterior mesenchymal regions (arrowheads).* **(H)** *At stage 28, transcripts are detected in the posterior mesenchyme (arrowhead) and in the interdigital spaces (arrow) of the limb bud.* **(I)** *cTwist expression remains in the posterior mesenchyme of the limb bud (arrowhead) and in the interdigital mesenchyme close to the differentiating digits (arrows).* **(J)** *In the developing face, transcripts are found in the head mesenchyme (arrow), in the maxillary processes (mx), mandibular processes (mn), hyoid arch (ha), third branchial arch (b3) and fourth branchial arch (b4).* **(K)** *At day 7 of development, cTwist expression in the limb bud is found in a discrete posterior region (arrowhead) and in the area inbetween the feather buds (arrow).* **(L)** *At day 8 of development, cTwist transcripts are localized throughout the entire mesenchyme of the early feather buds (arrowhead).* **(M)** *At later stages (10-day old embryo), expression in the feather filaments is confined to the proximal mesenchyme (arrows).*

localized to the dermatome (higher levels) and sclerotome (lower levels), and excluded from the myotome (Fig. 3 C,D; arrows). *cTwist* transcripts could be detected in the somites until the last stage analyzed (stage 28; data not shown).

At stages 13-14, *cTwist* transcripts are detected in the anterior lateral plate mesoderm (Fig. 2B; arrowheads) and faintly in the posterior lateral mesoderm (Fig. 2B; arrow). A transverse section of these embryos shows that transcripts are found in the somatic component of the lateral mesoderm (somatopleura) but not in the splanchnic component (splanchnopleura; Fig. 3 A,B). At stage 16, *cTwist* transcripts are found all over the lateral plate mesoderm (Fig. 2C; arrowheads). *cTwist* expression remains in the flank mesoderm until stage 24 (Fig. 2 D,E, and data not shown). In the developing limb, *cTwist* is expressed throughout the limb bud mesenchyme at stages 19-20 (Fig. 2 D,E; Fig. 3C; arrowheads). From stage 22 on, *cTwist* expression in the limb bud is excluded from the mesenchyme of the central and proximal regions and is detected in the anterior, distal and posterior mesenchyme (Fig. 2 F-H; Fig. 3D; arrowheads). At stages 28-30, transcripts are present at higher levels in the posterior mesenchyme (Fig. 2 H,I; arrowheads) and in the interdigital regions (Fig. 2 H,I; arrows). The pattern of expression in these regions becomes restricted to areas in the vicinity of the developing digits at stage 30 (Fig. 2I, arrows). At 7 days of development, *cTwist* expression is still detected in a discrete region of the distal posterior mesenchyme (Fig. 2K; arrowhead). *cTwist* expression is similar in both the forelimb and hindlimb buds (data not shown).

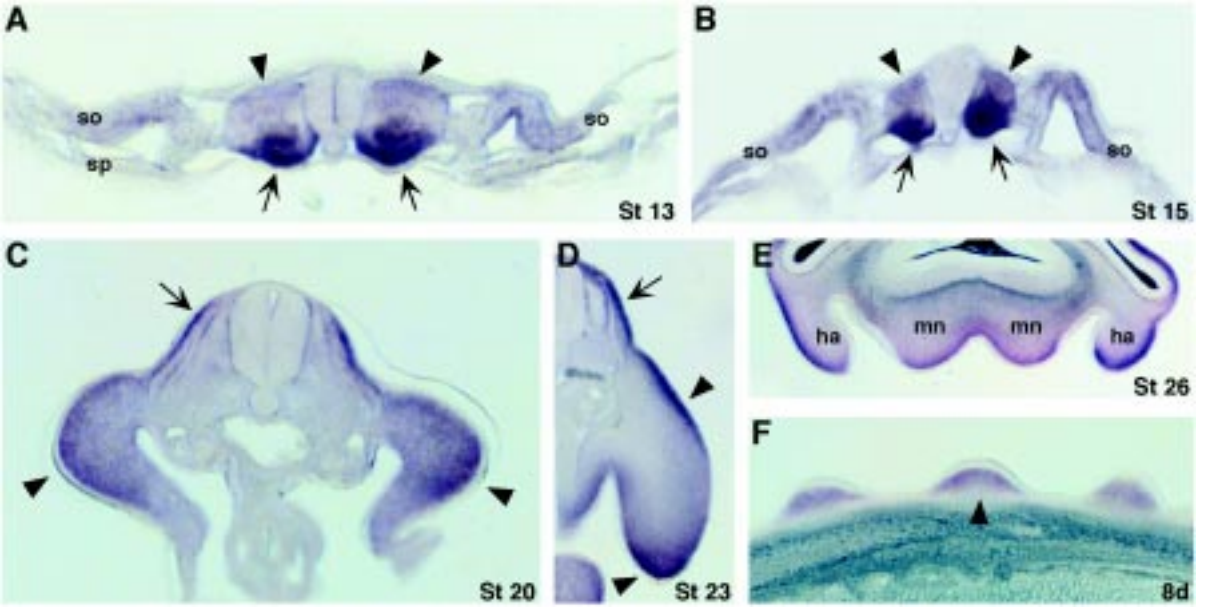

Fig. 3. Sections of chick embryos stained for *cTwist* by whole mount *in situ* hybridization. (A) *Transverse section of a stage 13 chick embryo showing cTwist expression in the ventral regions of the somites (arrows) and, less intensely, in the dorsal somites (arrowheads) and somatic lateral mesoderm (so). cTwist transcripts are not found in the splancnopleura (sn).* **(B)** *At stage 15, expression remains in the ventral (arrows) and dorsal (arrowheads) somites, and becomes more intense in the somatopleura (so).* **(C)** *In a transverse section of a stage 20 embryo, cTwist expression can be seen in the mesenchyme (arrowheads) and in the somites (arrow). Transcripts are found in the dermatome (higher expression) and sclerotome but excluded from the myotome.* **(D)** *At stage 23, expression is detected in the distal and dorsal limb mesenchyme (arrowheads) and maintained in the same regions of the somites (arrow).* **(E)** *Section through a stage 26 embryo showing cTwist expression in the distal mesenchyme of the mandibular (mn) and the hyoid (ha) arches.* **(F)** *In a section of the feather buds of an 8-day old embryo, cTwist transcripts can be seen in the mesenchyme (arrowhead) and are excluded from the ectoderm.*

During craniofacial development, chick *cTwist* is expressed initially in the branchial arches (Fig. 2E; asterisks) and later in the maxillary and mandibular processes, hyoid arch, and third and fourth branchial arches (Fig. 2J), where it is restricted to the distal mesenchyme (Fig. 3E). In addition, *cTwist* transcripts are found in the head mesenchyme (Fig. 2J, arrow).

cTwist expression is also found in the developing feathers. At day 7 of development, transcripts are found in the interfollicular areas (Fig. 2K; arrow). By embryonic day 8, *cTwist* expression moves to the mesenchyme of the feather buds (Figs. 2L, 3F arrowhead), and as the feather buds elongate, expression remains in the proximal mesenchyme (Fig. 2M; arrows).

Regulation of cTwist *expression by the AER, retinoic acid and SHH*

Since the expression of *cTwist* is detected in limb areas influenced by the apical ectodermal ridge and the zone of polarizing activity**,** we analyzed the role of several molecules that mediate their biological activities.

Surgical removal of the AER was performed in order to determine its implication in maintaining *cTwist* expression. At 6-8 hours after AER ablation, *cTwist* expression is downregulated and by 12 hours after removal cTwist transcripts are almost undetectable (n=12; Fig. 4A). The growth of the limb bud along the proximal-distal axis is controlled by FGFs released from the AER. To test the influence of FGFs in *cTwist* expression, beads soaked in FGF-4 and FGF-8 were implanted after AER removal (n=16). The expression domain of *cTwist* is maintained after FGF-8 treatment and is enlarged when compared with the contralateral limb (Fig. 4B). Implantation of FGF-4 soaked beads is followed by maintenance of the *cTwist* expression domain, but this domain is restricted to the posterior part of the limb bud (Fig. 4C).

The expression of *cTwist* is also observed in the ZPA. Anterior treatments with beads incubated in RA sustain the anterior domain of *cTwist* (n=20; Fig. 4D), which is maintained during limb bud duplication (data not shown). To verify if *cTwist* is induced in response to SHH, we implanted clumps of SHH expressing cells in the anterior mesenchyme (n=14). Cells expressing SHH induce an ectopic area of *cTwist* in the anterior part of the limb bud (Fig. 4E), also observed in the mirror image duplication obtained 48 hours after treatment (Fig. 4F).

In order to elucidate if SHH signalling is sufficient to maintain *cTwist* expression, clumps of SHH expressing cells were implanted following removal of the AER. After 12 hours, no *cTwist* expression is detected in the manipulated limb, suggesting that SHH signalling acts indirectly by maintaining *cTwist* expression (data not shown).

Discussion

During embryonic development, the expression patterns of chick *Twist* coincide with those reported for the mouse homologue (Wolf *et al.,* 1991; Fuchtbauer, 1995; Gitelman, 1997; Bourgeois *et al.,* 1998), with the obvious exceptions of *MTwist* expression during odontogenesis and *cTwist* expression in the developing feathers. In general, the pattern of *cTwist* expression in the developing chick

coincides with mesenchymal regions that are under the control of FGFs and SHH.

In the somites, *cTwist* is expressed first in the ventral medial portion and later in the dermatome and sclerotome compartments. *Fgf-4* (Niswander and Martin, 1992), *Fgf-5* (Haub and Goldfarb, 1991), *Fgf-6* (deLapeyrière *et al.,* 1993) and *Fgf-8* (Vogel *et al.,* 1996) are also expressed in the somites, and *shh* expression in the notochord and floor plate was shown to be required for somitic cell survival (Teillet *et al.,* 1998, Marcelle *et al.,* 1999).

In the branchial arches, *cTwist* is expressed in the mesenchyme, whereas *Fgf-3* (Mahmoon *et al.,* 1996), *Fgf-8* (Heikinheimo *et al.,* 1994) and *shh* (Wall and Hogan, 1995) transcripts are detected in the ectoderm. On the other hand, *cTwist* and *Fgf-8* are also co-expressed in the head mesenchyme (Heikinheimo *et al.,* 1994).

During feather development, *cTwist* transcripts are detected initially in the interfollicular region and later in the bud mesenchyme. Conversely, *Fgf-2*, *Fgf-4*, *Fgf-18* and *shh* are expressed in the overlying ectodermal placodes (Nohno *et al.,* 1995; Song *et al.,* 1996; Ohuchi *et al.,* 2000).

In limb buds, *cTwist* transcripts are initially detected throughout the limb bud mesenchyme and then become restricted to the anterior, distal and posterior mesenchyme. This expression pattern is very similar to that of *Fgf-10* (Ohuchi *et al.,* 1997) and partially overlaps with *Fgf-18* (Ohuchi *et al.,* 2000) *Fgf-12* (*cFhf-1*) and *Fgf-13* (*cFhf-2;* Munoz-Sanjuan *et al.,* 1999) expression domains. Moreover, several *Fgfs* are expressed in the AER: *Fgf-2* (Savage and Fallon, 1995), *Fgf-4* (Niswander and Martin, 1992; Savage *et al.,* 1993), *Fgf-8* (Heikinheimo *et al.,* 1994, Ohuchi *et al.,* 1994, Crossley and Martin, 1995), and *Fgf-9* (Colvin *et al.,* 1999). The posterior domain of *cTwist* is also overlapped in the limb bud mesenchyme with *shh* expression (Echelard *et al.,* 1993, Krauss *et al.,* 1993, Riddle *et al.,* 1993).

These closely tied expression domains suggest the existence of a relationship between *Fgfs* and *cTwist.* In here we present data that further reinforce this relationship. Removal of the AER results in truncated limbs (Summerbell, 1974; Rowe *et al.,* 1982; Todt and Fallon, 1987) and downregulation of *Fgf-10* and *Fgf-18* expression in the mesenchyme (Ohuchi *et al.,* 1997, Ohuchi *et al.,* 2000), phenotypes that can be rescued by application of FGFs (Niswander *et al.,* 1993; Fallon *et al.,* 1994; Crossley *et al.,* 1996; Vogel *et al.,* 1996, Ohuchi *et al.,* 1997, Ohuchi *et al.,* 2000). Ablation of AER downregulates *cTwist* expression. The normal expression is restored when a bead releasing either FGF-4 or FGF-8 is implanted after removal of the AER. The maintenance of *cTwist* expression through FGF signalling is in agreement with several studies showing that *Twist* expression is induced by FGFs, not only *in vitro* (Fang *et al.,* 2001) but also *in vivo* (Isaac *et al.,* 2000).

It is also noteworthy that expression domains of *cTwist* and *shh* are closely related during development. In limb buds, the expression of *cTwist* is also observed in the ZPA, overlapping with that of *shh* expression. In addition, implants of SHH expressing cells or beads soaked in RA (that are able to induce *shh* expression; Riddle *et al.,* 1993) at the anterior margin of the limb bud induce *cTwist* expression. However, the regulation through SHH seems to be indirect since SHH expressing cells are not able to maintain *cTwist* expression after AER removal. The induction of *cTwist* by SHH in the anterior mesenchyme may be mediated by the upregulation of FGFs in the overlying AER.

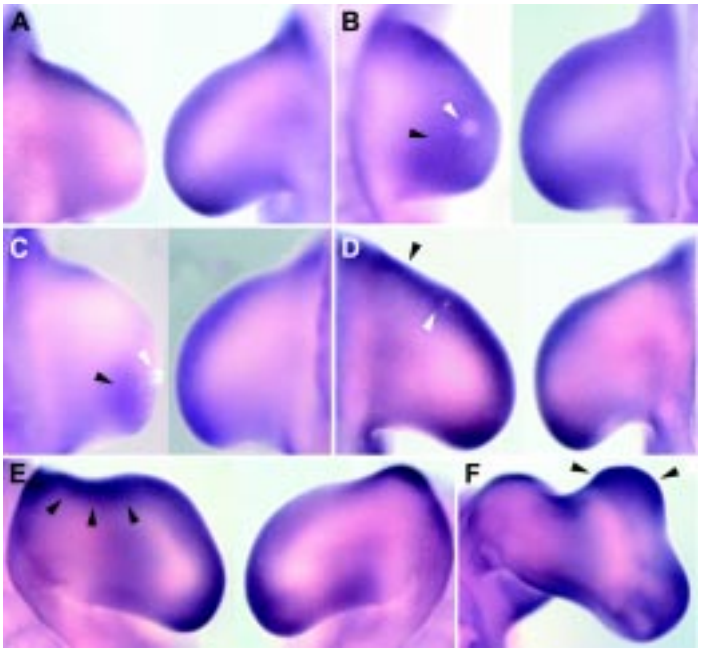

Fig. 4. Whole mount *in situ* hybridizations of *cTwist* after different treatments. *The manipulated limb (right) is on the left side of each panel and the control one (left) is on the right side.* **(A)** *cTwist expression 12 hours after AER removal.* **(B-C)** *Maintenance of cTwist expression 12 hours after AER ablation followed by application of a bead soaked in FGF-8* **(B)** *or FGF-4* **(C)**. **(D)** *Ectopic expression of cTwist 16 hours after implantation of a bead carrying RA.* **(E-F)** *Anterior expression of cTwist transcripts is detected 16 hours after grafting SHH expressing cells in the anterior part of limb bud* **(E)**. *This ectopic expression is maintained 48 hours after implantation, when duplication is evident* **(F)**. *White arrows indicate beads. The induced expression domains of cTwist are noted by black arrows.*

Some of the possible downstream targets of Twist are the FGF receptors. In *Drosophila* and *C. elegans*, the expression of their *Fgfr* homologues appears to be regulated by *Twist* during mesodermal patterning (Shishido *et al.,* 1993; Harfe *et al.,* 1998). In vertebrates, *Fgfr1* and *-2* expression during embryonic development coincides with that of *Twist* in several tissues, such as the limb bud mesenchyme (Orr-Urtreger *et al.,* 1993; Deng *et al.,* 1997), head mesenchyme and branchial arches (Walshe and Mason, 2000), and mesenchyme of interfollicular region and feather buds (Noji *et al.,* 1993). In addition, and as also shown here, during chick limb development, expression of *cTwist* in the interdigital mesenchyme is coincident with the expression domains of several *Fgfr* (Noji *et al.,* 1993, Marcelle *et al.,* 1995).

Taken together, these results suggest that Twist may be implicated in the response of several mesenchymal tissues to FGF signalling, possibly as a mediator in the maintenance of *Fgfr* expression.

Materials and Methods

cDNA cloning

Chick *cTwist* full length clones were identified in the screening of a stage 20-22 chick limb bud λZAPII cDNA library with a 311 bp chick *cTwist* RT-PCR fragment as a probe (nucleotides 262-573; 5' primer: CCGCAGTCCTACGAGGAGCTG;

3' primer: GTGGGATGCGGACATGGACCA; from G-Twist gene sequence in Genbank with accession number Y08261). The positive clones were sequenced with an automated sequencer. The largest clone identified was 1683 bp long, included 573 bp of coding sequence and, by comparison with homologues of other species, appeared to be a full length *Twist* clone. The *cTwist* mRNA sequence has also been reported in Genbank by Isaac *et al.* under accession number AF093816.

Sequence comparison was performed using Clustal W (version 1.8). The SwissProt accession numbers of the protein sequences used in the alignment are: P26687 (mouse Twist), Q15672 (human Twist), P13903 (*Xenopus* Twist), Q9PTE3 (Zebrafish Twist), and P10627 (*Drosophila* Twist).

Whole-mount in situ *hybridization and histology*

Chicken embryos were obtained from MacIntyre Poultry (San Diego, U.S.A.) and Avipronto (Benavente, Portugal). Eggs were incubated at 38°C and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Whole-mount *in situ* hybridization was carried out as described (Wilkinson, 1993). The riboprobe was synthesized as a digoxigenin (Boehringer) labelled probe from the whole sequence of chick *cTwist* (1.6kb) cloned in pSlax vector (EcoRI digestion; T7 polymerase transcription). For histology, the stained embryos were dehydrated in 30% sucrose, embedded in gelatine, frozen in an isopenthane bath, and sectioned in a cryostat.

Experimental manipulation of limb buds

Application of RA was performed with AG1-X2 ion exchange beads (BIO-RAD), and FGF-4 and FGF-8 proteins (R&D Systems) were implanted in heparin acrylic beads (Sigma). Beads were soaked in PBS (as a control) or in a solution of the desired molecule and were implanted in the limb mesenchyme. Eggs were windowed and beads were implanted using a tungsten needle. The AER was surgically removed from the wing buds with a thin tungsten needle. In some experiments this treatment was followed by implantation of FGF soaked beads or cell clumps. Clumps of control and SHH expressing cells were grafted in the anterior mesenchyme of wing buds using a tungsten needle.

Manipulations were performed at stages 20-22 in the right wing bud, using the left one as a control. After each manipulation, the eggs were closed and reincubated during different time periods. Embryos were sacrificed, fixed in 4% paraformadehyde and processed for whole mount *in situ* hybridization with *cTwist* probe.

Preparation of beads and cell clumps

Heparin acrylic beads (Sigma) were used to apply FGF proteins (R&D Systems). Beads between 100 and 150 μm were selected, washed in PBS and then incubated for 1 hour at room temperature in the protein solution (0.5 mg/ml for each FGF). AG1-X2 ion exchange beads (BIO-RAD) between 100 and 150 μm were used for RA treatment. Beads were soaked in RA, diluted in DMSO (1 mg/ml) for 20 minutes and then washed in PBS-Phenol Red solution to remove the excess DMSO and for staining. Control beads were incubated in PBS.

QT6 control and QT6 SHH-expressing cell lines were obtained from Dr. D. Duprez. Clumps of 100 μm were selected and their preparation was performed as described in Duprez *et al.* (1998).

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