Developmental Biology **247,** 351–366 (2002)
doi:10.1006/dbio.2002.0707

Fgf4 Positively Regulates Scleraxis and Tenascin Expression in Chick Limb Tendons

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In vertebrates, tendons connect muscles to skeletal elements. Surgical experiments in the chick have underlined developmental interactions between tendons and muscles. Initial formation of tendons occurs autonomously with respect to muscle. However, further tendon development requires the presence of muscle. The molecular signals involved in these interactions remain unknown. In the chick limb, *Fgf4* transcripts are located at the extremities of muscles, where the future tendons will attach. In this paper, we analyse the putative role of muscle-Fgf4 on tendon development. We have used three general tendon markers, *scleraxis*, tenascin, and *Fgf8* to analyse the regulation of these tendon-associated molecules by Fgf4 under different experimental conditions. In the absence of *Fgf4*, in muscleless and aneural limbs, the expression of the three tendon-associated molecules, *scleraxis*, tenascin, and *Fgf8*, is down-regulated. Exogenous implantation of Fgf4 in normal, aneural, and muscleless limbs induces *scleraxis* and tenascin expression but not that of *Fgf8*. These results indicate that *Fgf4* expressed in muscle is required for the maintenance of *scleraxis* and tenascin but not *Fgf8* expression in tendons.

Key Words: **Fgf4; Fgf8; scleraxis; tenascin; tendons; limb bud; chick embryo.**

INTRODUCTION

Tendons consist of dense fibrous connective tissues that link muscle to cartilage. They serve to transmit the force generated during muscle contraction to the skeleton. The myotendinous junction, where force is transferred from muscle to tendon, links cells of different embryological origins. Myogenic cells forming the striated skeletal muscles of the limb originate from the somites, while tendons originate from the lateral plate (Christ *et al.*, 1977; Chevallier *et al.*, 1977).

During limb development, the morphogenesis of muscle and tendon occurs in close spatial and temporal association (Kieny and Chevallier, 1979; Kardon, 1998). Myogenic precursor cells migrate from the lateral part of somites into the limb bud from E2 (Ordahl and Le Douarin, 1992; Christ and Ordahl, 1995). As early as E4, myoblasts aggregate into dorsal and ventral muscle masses on both sides of the cartilage-forming regions (Schramm and Solursh, 1990). From E5, these two muscle masses split along the proximo-distal axis of the limb and subsequently form the three pairs of muscle masses of the stylopod (arm and thigh), zeugopod (forearm and shank), and autopod (digits). Then, these three pairs of muscle masses will split progressively and form the individual muscles of the limbs (Shellswell and Wolpert, 1977; Pautou *et al.*, 1982; Robson *et al.*, 1994; Kardon, 1998; Duprez *et al.*, 1999). In parallel, between E4.5 and E5, three pairs (dorsal and ventral) of tendon primordia form in association with the three major joints of the limb, in between the three pairs of muscle masses (Kardon, 1998). They are named proximal, intermediate, and distal tendon primordia (Kardon, 1998). The successive segregation of tendon primordia occurs in tandem with that of the muscle masses (Kardon, 1998).

Most studies of tendon differentiation concentrate on distal autopod tendons in which three main stages of maturation have been distinguished (Ros *et al.*, 1995): (1) thickening of the ectoderm–mesenchyme interface, (2) the appearance of cellular condensation in mesenchyme identifiable as a tendon blastema, and (3) final organisation of tendon corresponding to the encirclement of the tendon

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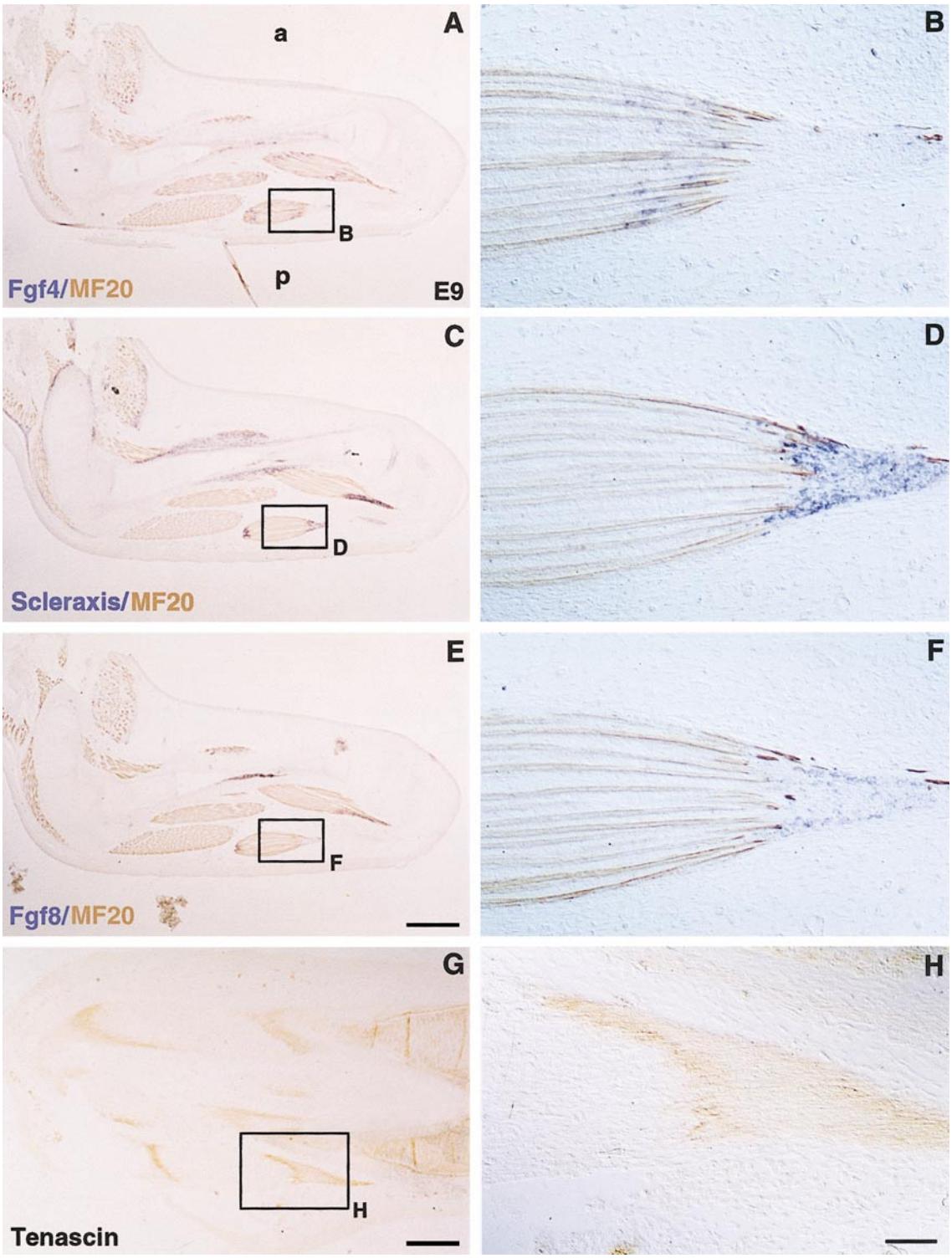

FIG. 1. *Fgf4* transcripts are located at the extremities of muscles close to the attachment sites of tendons in the embryonic chick wing. Adjacent longitudinal sections were hybridised with DIG-labelled antisense probes for *Fgf4* (A, B), *scleraxis* (C, D), and *Fgf8* (E, F) (blue) at embryonic day 9 (E9) and then incubated with the MF20 antibody (brown) recognising all isoforms of myosin heavy chains. (G, H) Longitudinal sections were incubated with the M1B4 antibody recognising tenascin. (B, D, F, H) Higher magnifications of one extremity of forearm muscle from pictures (A), (C), (E), and (G), respectively. Scale bars: (A, C, E) 890 μm; (B, D, F, H) 90 μm; (G) 444 μm.

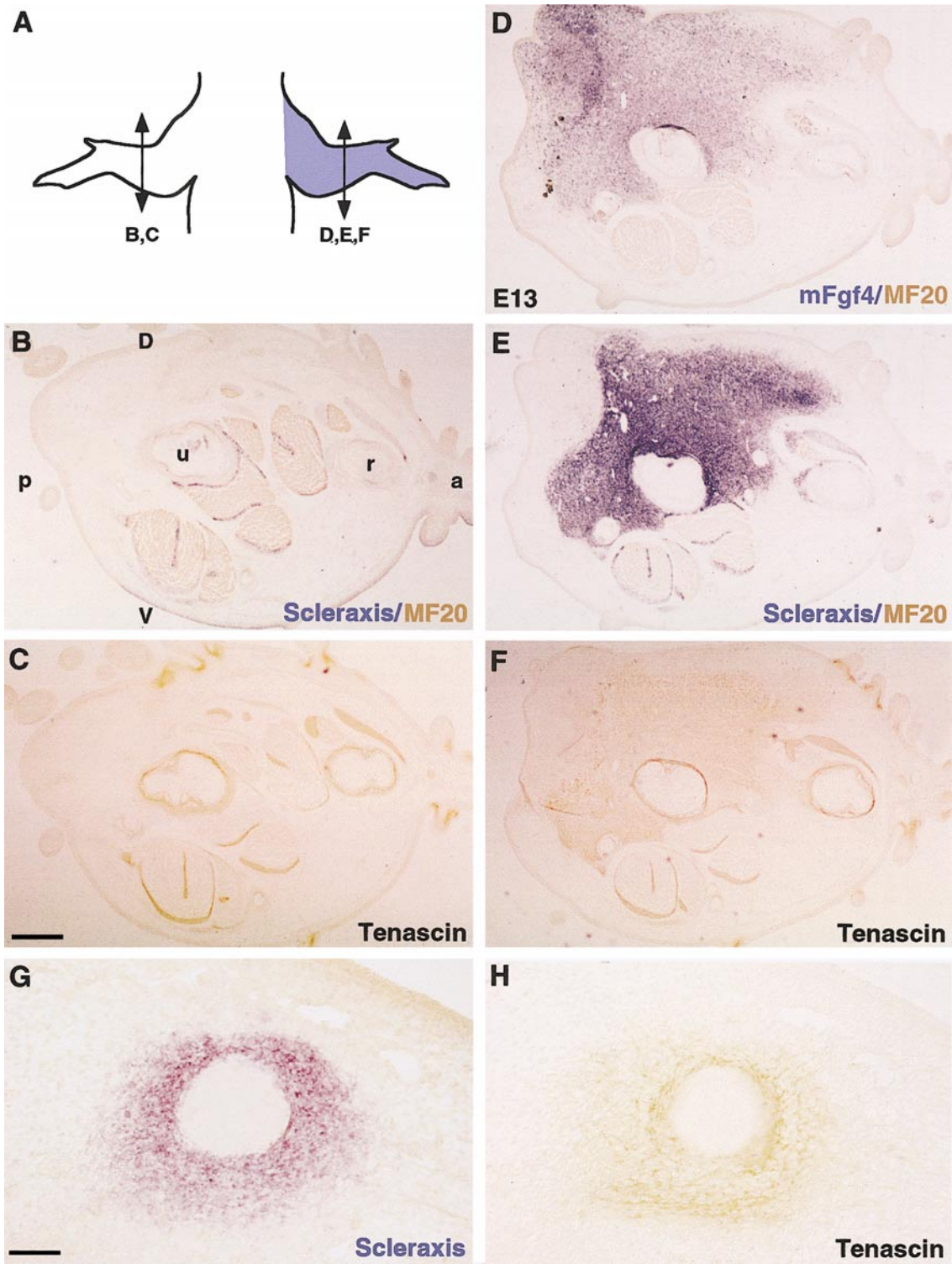

FIG. 2. Overexpression of *mFgf4* up-regulates *scleraxis* and tenascin in chick wing buds. Right wings were grafted with mFgf4/RCAS-expressing cells at E4 and reincubated for another 9 days. (A) The diagram shows the levels of the sections in control-left and grafted-right wings. Transverse sections of the nongrafted left wing hybridised with *scleraxis* (blue) and labelled with the MF20 antibody (brown) (B) or labelled with the tenascin antibody (C) display the normal tendon and muscle pattern. Transverse sections from the right grafted-wing hybridised with *mFgf4* (D) show the extent of the virus spread. Consecutive sections hybridised with *scleraxis* probe (blue) and incubated

blastemas by a peripheral layer of flattened cells. The molecular steps driving tendon differentiation are not understood. Several types of molecules have been reported to be specifically associated with distal tendon in the chick limbs: extracellular matrix components such as collagen type I, IV, elastin (Hurle *et al.*, 1989; Ros *et al.*, 1995), and the tyrosine kinase receptor, *Eph-A4* (Patel *et al.*, 1996; D'Souza and Patel, 1999). Transcription factors have also been described in distal tendons during mouse limb development, such as *Six 1* and *2* (Oliver *et al.*, 1995) and *Eya 1* and *2* (Xu *et al.*, 1997). *Six2* and *Eya1* are restricted to ventral flexor tendons, while *Six1* and *Eya2* are associated with the dorsal extensor tendons, at the digit level. The transforming growth factorβ 2 (Tgfβ2) has also been described as being located in digit tendons (Merino *et al.*, 1998). However, the correlation between all these molecules and the successive steps of tendon formation (Ros *et al.*, 1995) has not been well established.

Three molecules have been described as being located in proximal, intermediate, and distal tendons in the limb. The extracellular protein tenascin is classically used as a general tendon marker (Chiquet and Fambrough, 1984; Hurle *et al.*, 1989; Ros *et al.*, 1995; Kardon, 1998). Its expression in hindlimb tendon primordia can be detected at E4.5 (Kardon, 1998). However, tenascin has been reported to be associated with other sites, such as nerves and perichondrium (Chiquet and Fambrough, 1984). The recent description of the expression pattern of the bHLH transcription factor *scleraxis* provides another general marker of tendons in chick and mouse embryos (Schweitzer *et al.*, 2001). *Scleraxis* transcripts are detected from E3 in the chick limb, so it can serve as an early tendon marker (Schweitzer *et al.*, 2001). Finally, we have recently presented the secreted factor *Fgf8* as a late marker (from E7) for tendons in the chick limb (Edom-Vovard *et al.*, 2001a). *Scleraxis* and *Fgf8* mutants are not informative concerning tendon formation since they fail to gastrulate (Meyers *et al.*, 1998; Brown *et al.*, 1999). No tendon phenotype has been reported for tenascin mutants (Mackie and Tucker, 1999). The only relationship between the tendon-associated markers we can establish to date is based on the timing of their endogenous expression in the chick limb tendons (Kardon, 1998; Schweitzer *et al.*, 2001; Edom-Vovard *et al.*, 2001a): first *scleraxis* followed by tenascin and then *Fgf8*.

Facilitated by the distinct embryological origin of myogenic and tendon cells, surgical studies in the chick have underlined interactions between these two tissues. Despite the linked morphogenesis of muscles and tendons (Kardon, 1998), tendons can initiate their development independently of muscles. Initial tendon formation occurs normally in muscleless limbs (Shellswell and Wolpert, 1977; Kieny and Chevallier, 1979; Brand *et al.*, 1985; Kardon, 1998). However, in the absence of muscle, further tendon development is altered if no proper junctions to muscle fibres are established (Shellswell and Wolpert, 1977; Kieny and Chevallier, 1979; Brand *et al.*, 1985; Kardon, 1998). These experiments suggest that late tendon development requires the presence of muscle. However, the degree of muscle dependence varies along the proximodistal axis. Muscles are required for segregation and maintenance of intermediate and proximal tendons but only for the maintenance of distal tendons (Kardon, 1998). The molecular aspects of the interactions between muscles and tendons have not been explored.

Recently, we have shown that the Fibroblast growth factor-4 (*Fgf4*) expression domain is restricted to the extremities of muscle cells (Edom-Vovard *et al.*, 2001b). Interestingly, *Fgf4* transcripts are located in the muscle fibres in a region that is in contact with the developing tendons. In addition, *Fgf4* transcripts are first detected at E6, a time when muscle and tendon primordia lie adjacent to one another (Kardon, 1998). Fgf4 is a good candidate to be a muscle factor involved in further tendon development. In order to clarify the role of Fgf4 in muscle and tendon interactions, we observed the consequences of the absence of Fgf4, in muscleless and aneural conditions, for the expression of the tendon-associated molecules, tenascin, *scleraxis*, and *Fgf8*. We also implanted a source of Fgf4 using a replication-competent virus, RCAS, in normal, aneural, and muscleless limbs. These experiments show that Fgf4 in muscle positively regulates *scleraxis* and tenascin expression but not that of *Fgf8* in tendons.

MATERIALS AND METHODS

Chick Embryos

Fertilised eggs from commercial sources [JA 57 strain, Institut de Sélection Animale (ISA), Lyon, France] or (White Leghorn from HAAS, Strasbourg) were incubated at 37°C. At E2, embryos were staged according to somite number (Christ and Ordahl, 1995). Young embryos were staged according to Hamburger and Hamilton (1951) (HH), while old embryos were staged according to days *in ovo*. To facilitate comparisons, somite and HH stages are reported with the number of days of incubation.

with the MF20 antibody (brown) (E) or labelled with tenascin antibody (F) show the up-regulation of the tendon markers in the infected areas. Beads soaked in Fgf4 protein were implanted into E5 right wing bud. Three days after grafting, consecutive longitudinal sections of the grafted wings were hybridised with *scleraxis* (blue) (G) or labelled with the M1B4 antibody (brown) (H). An up-regulation of *scleraxis* and tenascin is detected around the Fgf4-beads. a, anterior; p, posterior; D, dorsal; V, ventral; u, ulna; r, radius. Scale bars: (B–F) 444 μm; (G, H) 100 μm.

Ceolomic Wing Graft Surgery

The lateral plate areas corresponding to the future wing buds, level of somites 14–21, were isolated from chick embryos at 17–25 somites (E2). Depending on the stages of the embryos, somites 14–21 adjacent to the wing lateral plate were not always completely segmented. The wing lateral plate fragments were placed into coelomic cavities of E3.5 host embryos (stage HH21) that were incubated for an additional 3–8 days. The muscleless wings were harvested between 5 and 10 days with respect to their own total development and processed either for *in situ* hybridisation or for grafting experiments.

Neural Tube Ablation

To get aneural limbs, neural tube excision was performed on embryos at 15–23 somites (E2), before the exit of ventral root fibres. The neural tube and notochord were separated from the paraxial mesoderm and from the endoderm along the whole length of the neural axis, from somite 3 (Teillet and Le Douarin, 1983; Rong *et al.*, 1992). The axial organs were sectioned at the anterior and posterior ends of the segment to be removed and were sucked out by using a calibrated micropipette. The operated embryos were reincubated for various times depending on the type of analysis (*in situ* hybridisation or grafting). This type of ablation experiment produced complete aneural wings.

Production and Grafting of Fgf4/RCAS-Expressing or Control RCAS-Expressing Cells

Fgf4-expressing cells and control cells were prepared for grafting as described by Edom-Vovard *et al.* (2001b). Briefly, chicken embryonic fibroblasts (CEFs) were isolated from 10-day-old 0 line embryos (BBSRC, Institute for Animal Health, Compton, Berkshire, UK) and grown in DMEM (Gibco/BRL) containing 8% (v/v) foetal calf serum and 2% (v/v) chick serum supplemented with antibiotics. CEFs were transiently transfected with retroviral recombinant DNA (Fgf4/RCAS or RCAS alone) at the concentration of 1 μg/μl by using Transfectam (Gibco/BRL) according to the manufacturer's instructions.

Pellets of approximately 50 μm in diameter were grafted into the right wings of normal embryos at stage HH22 (E4) or into the right aneural limbs at stage HH26 (E5). At different times after grafting, embryos were harvested and processed for *in situ* hybridisation to whole mount or tissue sections and immunohistochemistry. The left wing is used as internal control.

The muscleless wings were isolated at E5 from the coelomic cavity of the host embryos 3 days after the coelomic grafts. These muscleless wings were grafted with mFgf4/RCAS-expressing pellets in a petri dish and then implanted into the chorioallantoic membrane (CAM) of E7 host embryos. The host embryos (and the mFgf4-grafted muscleless wings) were left for another 5 days of development and then processed for *in situ* hybridisation. We assume that the resulting manipulated wings have 10 days of development.

Fgf4 Bead Implantation

The Fgf4 recombinant protein was obtained from R&D Systems. Heparin beads (Sigma) were washed in PBS and soaked in 1 mg/ml Fgf4 for 1 h, in ice. Beads were grafted into the right wings of normal embryos at stage HH26 (E5). Three days after grafting, embryos were harvested and processed for *in situ* hybridisation to tissue sections and immunohistochemistry.

In Situ Hybridisation to Whole Mounts and Tissue Sections

Embryos were fixed in 4% (v/v) formaldehyde and processed for *in situ* hybridisation to whole mounts and tissue sections as previously described (Edom-Vovard *et al.*, 2001b). The digoxigenin-labelled mRNA probes were prepared as described: *cFgf4*, *mFgf4*, and *MyoD* (Edom-Vovard *et al.*, 2001b), and *scleraxis* and *Fgf8* (Edom-Vovard *et al.*, 2001a).

Immunohistochemistry

Differentiated muscle cells were detected on sections by using the monoclonal antibody against sarcomeric myosin heavy chain MF20 (Developmental Hybridoma Bank) and a horseradish peroxidase (HRP)-conjugated secondary antibody and diaminibenzidine (DAB) as substrate. The nerve courses were visualised by using the HNK1 monoclonal antibody (Developmental Hybridoma Bank) as described by Catala *et al.* (2000). The tendons were visualised by using the M1B4 antibody against tenascin (Developmental Hybridoma Bank).

RESULTS

Fgf4 Transcripts Are Located at the Extremities of Muscles Near the Attachment Sites of Tendons

Fgf4 transcripts are first detected in the wing on the sixth day of chick embryonic life (stage HH 28/29) (Edom-Vovard *et al.*, 2001b). At that stage, proximal, intermediate, and distal tendon primordia are already apparent on both ventral and dorsal sides of the limb, using tenascin as a molecular marker for tendons (Kardon, 1998). *Fgf4* expression is detected after the initiation of tendon development. We compared the expression of *Fgf4* in muscles with that of the tendon-associated molecules *scleraxis* (Cserjesi *et al.*, 1995; Brown *et al.*, 1999; Schweitzer *et al.*, 2001), *Fgf8* (Edom-Vovard *et al.*, 2001a), and tenascin (Kardon, 1998) by performing *in situ* hybridisation or immunohistochemistry on longitudinal sections of wing muscles at E9. The MF20 antibody recognises the myosin heavy chains (MyHC) expressed only by differentiated myogenic cells. These experiments show that *Fgf4* transcripts (Figs. 1A and 1B) are expressed at the muscle extremities close to the area where the tendon-associated molecules, *scleraxis* (Figs. 1C and 1D), *Fgf8* (Figs. 1E and 1F), and tenascin (Figs. 1G and 1H), are located. This close association between the expression of Fgf4 in muscle and that of the tendon-associated molecules is also observed in the digits (data not shown). Muscle *Fgf4* is thus a possible regulator for tendon development.

Overexpression of Fgf4 Induces Ectopic Expression of scleraxis and tenascin in Wing Buds

In order to determine the consequences of *Fgf4* misexpression for tendon development, we grafted mFgf4/RCAS-

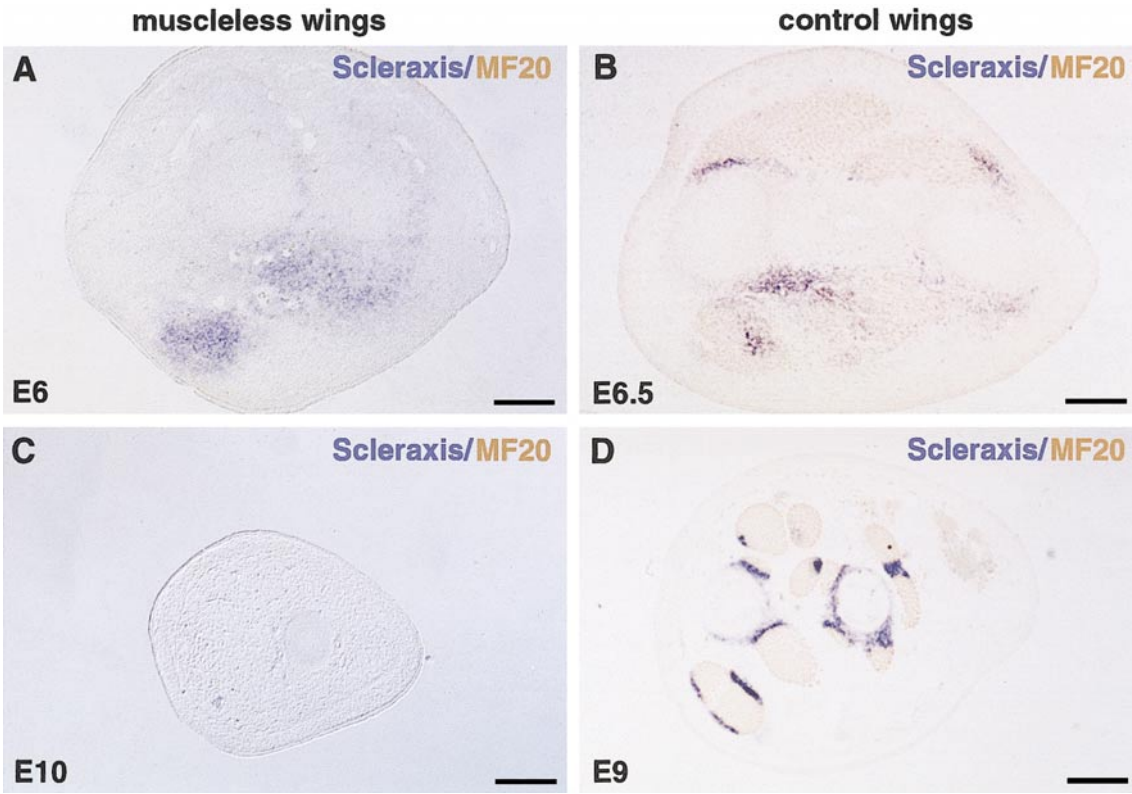

FIG. 3. Evolution of *scleraxis* expression in muscleless wings. Four days after coelomic wing lateral plate grafts, transverse sections from muscleless wings (E6) hybridised with *scleraxis* probe (blue) and incubated with MF20 antibody (brown) show the presence of the tendon marker *scleraxis* around cartilage in the absence of muscle (A). Eight days after coelomic wing lateral plate grafts, transverse sections of E10 muscleless wings show the down-regulation of *scleraxis* expression (C). The absence of muscle is confirmed by the absence of MF20 staining (A, C). Transverse sections of normal wings hybridised with *scleraxis* (blue) and labelled with MF20 (brown) display the normal tendon and muscle pattern at E6.5 (B) and E9 (D). Scale bars: (A) 173 μm; (B) 296 μm; (C, D) 444 μm.

expressing cells into E4 wings and observed tendon markers. *In situ* hybridisation to *scleraxis* transcripts in whole-mount preparations shows an up-regulation of *scleraxis* 4 days after grafting (n = 4 out of 4; data not shown). The operated embryos were cut transversely through the forelimb region, 6 and 9 days after grafting (Fig. 2A). *In situ* hybridisations with the *mFgf4* probe show the extent of the virus spread (Fig. 2D). Adjacent sections show a systematic and dramatic up-regulation of *scleraxis* transcripts in the dorsal area where *mFgf4* is present (Fig. 2E; n = 5 out of 5 at E10 and n = 2 out of 2 at E13). It should be noted that *scleraxis* expression is never induced close to the ectoderm, corresponding to the dermis (Fig. 2E) despite the presence of ectopic *Fgf4* in this region (Fig. 2D). We also observed an up-regulation of tenascin in the same regions and at the same time as the up-regulation of *scleraxis* (Fig. 2F; n = 1 out of 1 at E10; n = 2 out of 2 at E13). The up-regulation of *scleraxis* and tenascin can also occur at the digit level (data not shown). In contrast, the expression of another tendon-associated molecule, *Fgf8*, is not detected in the dorsal area where ectopic *mFgf4* is present (n = 2 out of 2 at E10; n = 1 at E13; data not shown). The up-regulation of *scleraxis* and tenascin is accompanied by a down-regulation of myosin expression as we already described (Edom-Vovard *et al.*, 2001b). The control left wing shows a normal tendon pattern, assayed by *scleraxis* expression (Fig. 2B, in blue), tenascin location (Fig. 2C, in brown), and a normal muscle pattern, assayed by myosin expression (Fig. 2B, in brown). Application of Fgf4 using beads as carrier shows similar results, i.e., induction of *scleraxis* (Fig. 2G) and tenascin (Fig. 2H) expression around the bead 3 days after bead implantation.

Analysis of scleraxis and Fgf8 Expression in Muscleless Wings

The muscle requirement for tendon development was previously observed by using histological criteria (Shellswell and Wolpert, 1977; Kieny and Chevallier, 1979; Brand *et al.*, 1985) and tenascin expression (Kardon, 1998). We analysed the expression of the tendon-associated molecules *scleraxis* and *Fgf8* in muscleless limbs. We obtained

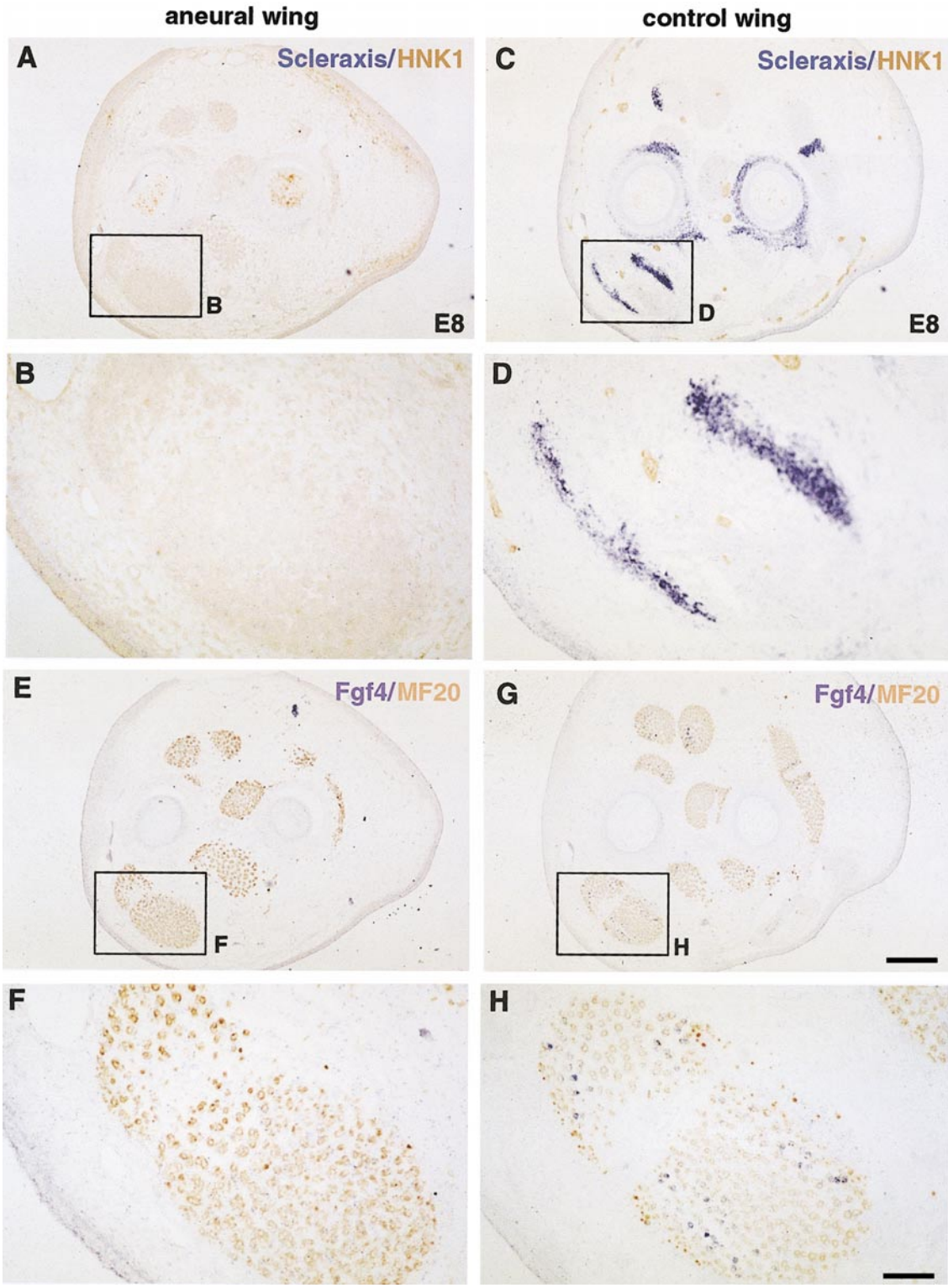

FIG. 4. The absence of *Fgf4* is accompanied by a down-regulation of *scleraxis* expression in aneural wings. Adjacent transverse sections from aneural (A, B, E, F) and normal (C, D, G, H) wings at E8 were hybridised with DIG-antisense probes (blue) for *scleraxis* (A–D) and *cFgf4* (E–H) and then incubated with the MF20 antibody (E–H) or HNK1 antibody recognising the nerves (A–D). (B, D, F, H) Higher magnifications of the ventral and posterior muscle FCU (flexor carpi ulnaris) in control and aneural wings (A, C, E, G). Scale bars: (A, C, E, G) 347 μm; (B, D, F, H) 87 μm.

TABLE 1

Correlation between *Fgf4* Expression in Muscle and Expression of the Tendon-Associated Molecules in Aneural Wings along the Proximodistal Axis

	Proximal (forearm level)				Distal (digit level)			
	Fgf4	*scleraxis*	tenascin	*Fgf8*	*Fgf4*	*scleraxis*	tenascin	*Fgf8*
E7	+++ (4)	+++ (2)	+++ (1)	n.d.	+++ (4)	+++ (2)	+++ (1)	n.d.
E7.5	+ (1)	+ (1)	n.d.	+ (1)				
E8	± (1)	± (3)	± (2)	± (1)	± (4)	+ (3)	+ (1)	
	− (4)	− (2)		− (3)	− (1)	± (2)	± (1)	− (4)
E9		± (2)	± (2)			+ (1)		
	− (3)	− (1)		− (2)	− (2)	± (1)	± (2)	− (2)
E11	− (1)	− (1)	n.d.	n.d.	− (1)	± (1)	n.d.	n.d.

Note. When *Fgf4* is present, the expression of the tendon-associated molecules is normal (+++). *Fgf4* and the tendon-associated molecules, *scleraxis*, tenascin, and *Fgf8*, are down-regulated progressively from E8, in muscles and tendons, respectively (+, detectable expression; ±, faint expression, no detectable expression). *Fgf4* transcripts are clearly down-regulated before the tendon markers, since we can observe the situation with no *Fgf4* expression and residual tendon marker expression, the opposite situation (Fgf4, +; and tendon markers, −) never being observed. The expression of *Fgf4* in distal muscles is down-regulated with a slight delay compared with the disappearance in proximal regions. There is also a delay in the down-regulation of the distal vs proximal tendon markers. All the cases of ± in proximal regions for tendon markers correspond to a residual expression in one particular tendon, located near the FCU. The number in brackets correspond to the number of wings analysed on sections; n.d., not determined.

muscleless limbs by transplanting the wing lateral plate, before the somitic myoblast migration, into the coelomic cavity of E3.5 chick embryos (see Materials and Methods). The absence of muscle cells in such experimental wings was verified by the absence of myosin expression using the MF20 antibody ($n = 7$ out of 7; Figs. 3A and 3C). In E6 muscleless wing, at the forearm level, *scleraxis* expression is less segregated ($n = 2$ out of 2; Fig. 3A) than in the control wing (Fig. 3B). This is consistent with what was observed for tenascin by Kardon (1998) and which led to the conclusion that the muscle is required for segregation of the proximal tendons (Kardon, 1998). At E8, *scleraxis* transcripts can still be detected, although at very low levels ($n = 1$; data not shown). *Scleraxis* transcripts are not detected from E10 ($n = 4$ out of 4; Fig. 3C) in the forearm region of muscleless wings, while *scleraxis* is expressed in normal E9 wings (Fig. 3D). However, in three of four cases, we could observe low amounts of *scleraxis* transcripts at the distal regions (digits) of those E10 muscleless wings (data not shown), consistent with the observation that the tendon degeneration occurs progressively along the proximal–distal axis (Kardon, 1998). These experiments show that the presence of muscle is necessary for the normal segregation and the maintenance of *scleraxis* expression in tendons at the forearm level, as is the case for tenascin expression (Kardon, 1998). In contrast, we failed to detect *Fgf8* transcripts in muscleless wings at all of the stages analysed [data not shown; E6 ($n = 2$); E8 ($n = 1$); and E10 ($n = 1$)], which indicates that the initiation of *Fgf8* expression in tendon requires the presence of muscles.

In Aneural Wings, the Down-Regulation of Fgf4 Expression Is Correlated with a Down-Regulation of Expression of the Tendon-Associated Molecules

We analysed the *Fgf4* expression in muscles in the absence of innervation, following early excision of axial organs (neural tube and notochord). We found that *Fgf4* expression was down-regulated from E8 in aneural limb muscles (see Figs. 4E and 4F). In the absence of innervation, muscles develop but start to degenerate progressively from E10 onward (Rong *et al.*, 1992). Thus, the aneural wing from E8 to E10 provides us with a model where *Fgf4* expression is absent from muscles. It has the advantage on the muscleless limb of having the muscle present. Although many factors could be altered by the absence of innervation, we used this system to analyse the possible effects of *Fgf4* removal from aneural muscles on tendon markers.

To assess the success of neural tube removal, sections were labelled with the HNK1 antibody, which specifically labels nervous tissues (Catala *et al.*, 2000). All 15 embryos analysed show complete absence of innervation (see examples in Figs. 4A, 4B, 5C, and 5D), while HNK1 labels the nerve courses in wings from nonmanipulated embryos; see examples, at E8, on transverse sections (Figs. 4C and 4D) and on longitudinal sections (Figs. 5A and 5B). From E8, aneural limb muscles can still be visualised by using MF20 staining, but generally no longer express *Fgf4* transcripts (transverse sections: Figs. 4E and 4F; longitudinal sections: Figs. 5G and 5H; Table1), in contrast to control wing muscles (Figs. 4G and 4H). Transverse sections of aneural wing show a complete absence of *scleraxis* at the forearm

level (Figs. 4A and 4B), while *scleraxis* transcripts are present close to the muscles in a normal wing at the same stage (Figs. 4C and 4D). Longitudinal sections also show an absence of *scleraxis* (Figs. 5C and 5D) and *Fgf8* transcripts (Figs. 5E and 5F) in the forearm, from E8 aneural embryos. In one case, at E8, in which faint *Fgf4* was detected in some muscles, we could still detect slight *scleraxis* and *Fgf8* expression near these muscles, suggesting that the progressive disappearance of *scleraxis* and *Fgf8* expression follows closely that of *Fgf4* expression (Table 1). We also analysed the evolution of another tendon-associated molecule, tenascin, in aneural condition. Tenascin expression has already been reported to be normal in tendons in denervated limbs at least until E7, the latest stage analysed by the authors (Wehrle-Haller *et al.*, 1991; Martini and Schachner, 1991). Analysis of E8 and E9 aneural wings shows a down-regulation of tenascin in tendons (Figs. 5I and 5J), similar to that of *scleraxis* and *Fgf8* (Table 1).

Longitudinal sections of E8 aneural wings show that *scleraxis* transcripts are still present at the digit level (Fig. 5C; Table 1). In order to visualise the degeneration of tendons along the entire proximodistal axis, we analysed the evolution of *scleraxis* in aneural limbs by whole-mount *in situ* hybridisation. Since axial structure ablations lead to two identical aneural wings, we used the right aneural wing for whole-mount *in situ* hybridisation to *scleraxis*, while the left aneural wing was used to control the absence of innervation and *Fgf4* expression (data not shown). At E7, in absence of innervation (Fig. 6B), but when *Fgf4* transcripts are still detected (data not shown), *scleraxis* expression is similar to a normal wing (Fig. 6A, see Fig. 4 in Schweitzer *et al.*, 2001) and detected at muscle extremities (Fig. 6B, arrows). At E9, *scleraxis* expression is not detected anymore in the forearm but can still be visualised in digits of aneural wings (Fig. 6C), although at a lesser intensity compared with the digit tendons of the stage-matched normal wing (Fig. 6D). At E11, we can still detect very low levels of *scleraxis* expression in the digit tendons compared with the expression of digit tendons in normal E11 wing (data not shown). This time course is also confirmed by the analysis in sections (Table 1). In digits, the expression of tenascin also follows that of *scleraxis* (Table 1). *Fgf8* transcripts are never detected in aneural digits (Table 1). However, *Fgf8* transcripts are normally expressed in normal digits from E9 (Edom-Vovard *et al.*, 2001a). The slight delay of the down-regulation of the digit tendon makers compared with the proximal region is correlated with a delay of the down-regulation of Fgf4 in digit (Table 1).

These results establish that the absence of *Fgf4* in aneural muscle is correlated with the down-regulation of the expression of the tendon-associated molecules *scleraxis*, *Fgf8*, and tenascin. This correlation is observed all along the proximodistal axis, that is in forearm and digit tendons.

Grafts of mFgf4-Expressing Cells in Aneural Limbs Rescue scleraxis and tenascin Expression but Not That of Fgf8

In order to confirm the relationship between Fgf4 and the tendon-associated molecules, we decided to reimplant a source of Fgf4 in the aneural limbs. The experimental procedure is illustrated in Fig. 7A. Ablation of the neural tube was performed at E2, leading to aneural wings. In a second step, the right aneural wings were grafted with mFgf4/RCAS-expressing cells at E5 *in ovo*. At this stage *scleraxis* and tenascin are normally expressed and endogenous expression of *Fgf4* and *Fgf8* has not started yet. The doubly operated embryos were incubated for another 5 days. Six embryos survived these two experimental steps. Right wings of such embryos were cut longitudinally and hybridised with the *mFgf4* probe. In five of six cases, we could detect ectopic *mFgf4* (Fig. 7D). Adjacent sections hybridised with the *scleraxis* probe show an up-regulation of *scleraxis* transcripts in the infected area (Fig. 7E; $n = 5$ out of 5), while *scleraxis* is not detected in the aneural left wing used as a control (Fig. 7B). In the *scleraxis*-induced region, myosin expression is absent. In contrast, *Fgf8* transcripts are not up-regulated where ectopic *mFgf4* is detected (Fig. 7F; $n = 3$ out of 3). Analysis of tenascin in such manipulated embryos at E8 shows an up-regulation that closely follows the *scleraxis* up-regulation (data not shown; $n = 2$ out of 2). In one case, the retroviral Fgf4 reached the digits and we could observe an up-regulation of *scleraxis* and tenascin in the digits, showing that Fgf4 can rescue *scleraxis* and tenascin along the entire proximodistal axis (data not shown).

Grafts of mFgf4-Expressing Cells in Muscleless Limb Rescue scleraxis and tenascin Expression

In order to determine whether Fgf4 was also able to rescue the tendon markers, *scleraxis* and tenascin, in the absence of muscle, we performed the equivalent experiments in the muscleless wings; that is we reimplanted a source of Fgf4 in muscleless wings. The successive steps of this experimental procedure are summarised in Fig. 8A. Muscleless wing grafts were excised from the coelomic cavity at E5 of development. mFgf4/RCAS-expressing cells were grafted at the forearm level of these muscleless wings, which were then implanted in the chorioallantoic membrane (CAM) of E7 host embryos and left for 5 days. Ectopic *mFgf4* was implanted at a time when endogenous *scleraxis* transcripts and tenascin (Kardon, 1998) are still present in muscleless wings. Three wings successfully passed all these experimental steps. The operated wings were cut transversely and hybridised with the *mFgf4* probe, showing the extent of the virus spread (Fig. 8B). Adjacent sections hybridised with the *scleraxis* probe show a clear up-regulation of *scleraxis* transcripts (Fig. 8C; $n = 3$ out of 3) and tenascin protein (Fig. 8D; $n = 2$ out of 3) in the area where m*Fgf4* is present, in absence of muscle as shown by the absence of MF20 immunoreactivity. In one case, the

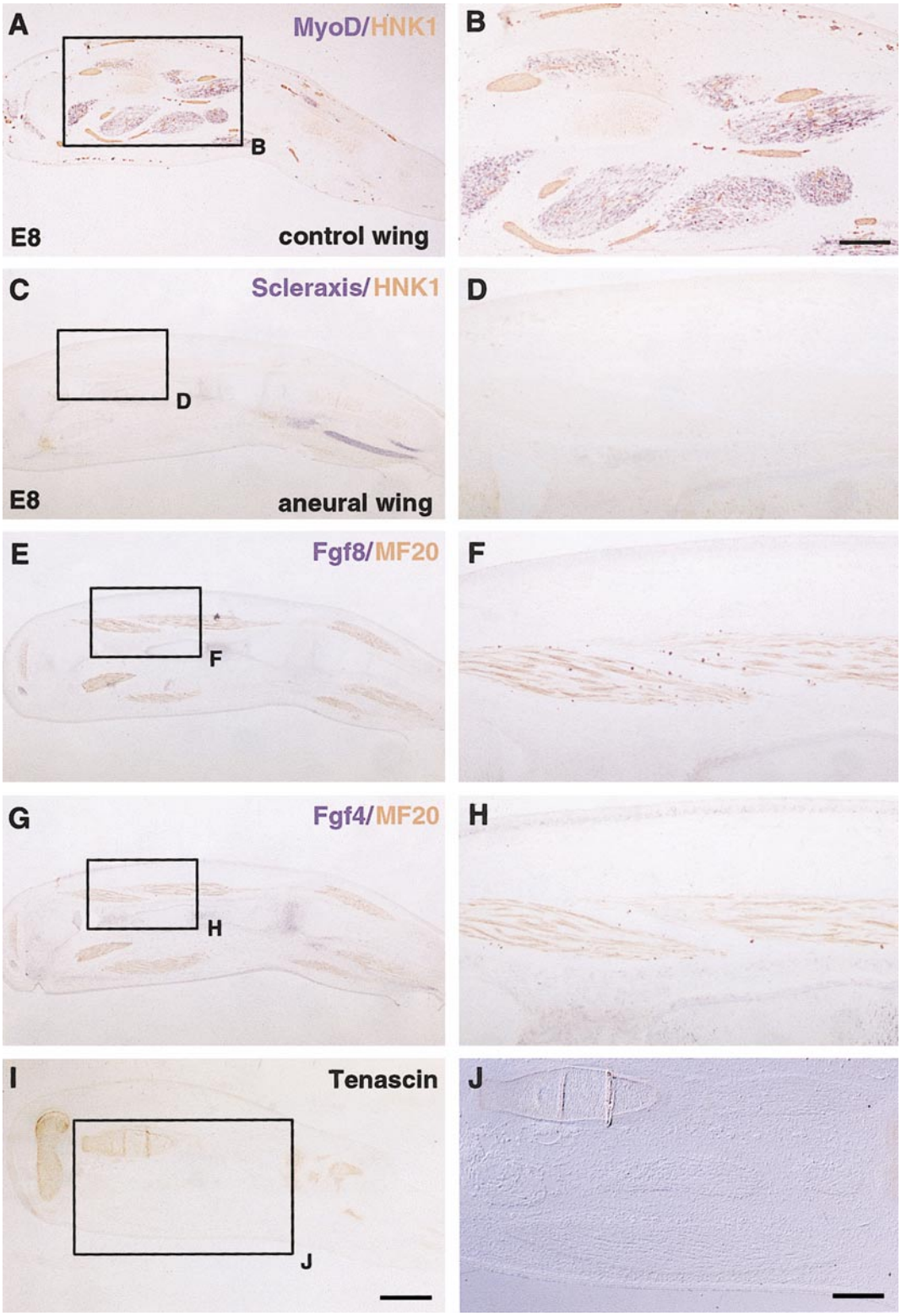

FIG. 5. The expression of the tendon-associated molecules, *scleraxis*, *Fgf8*, and tenascin, are simultaneously down-regulated at the forearm level in aneural wings. Longitudinal sections of a normal E8 wing hybridised with the *MyoD* probe (blue) showing the muscles and incubated with the HNK1 antibody (brown) revealing the normal nerve pattern (A, B). Adjacent longitudinal sections from E8 aneural wing

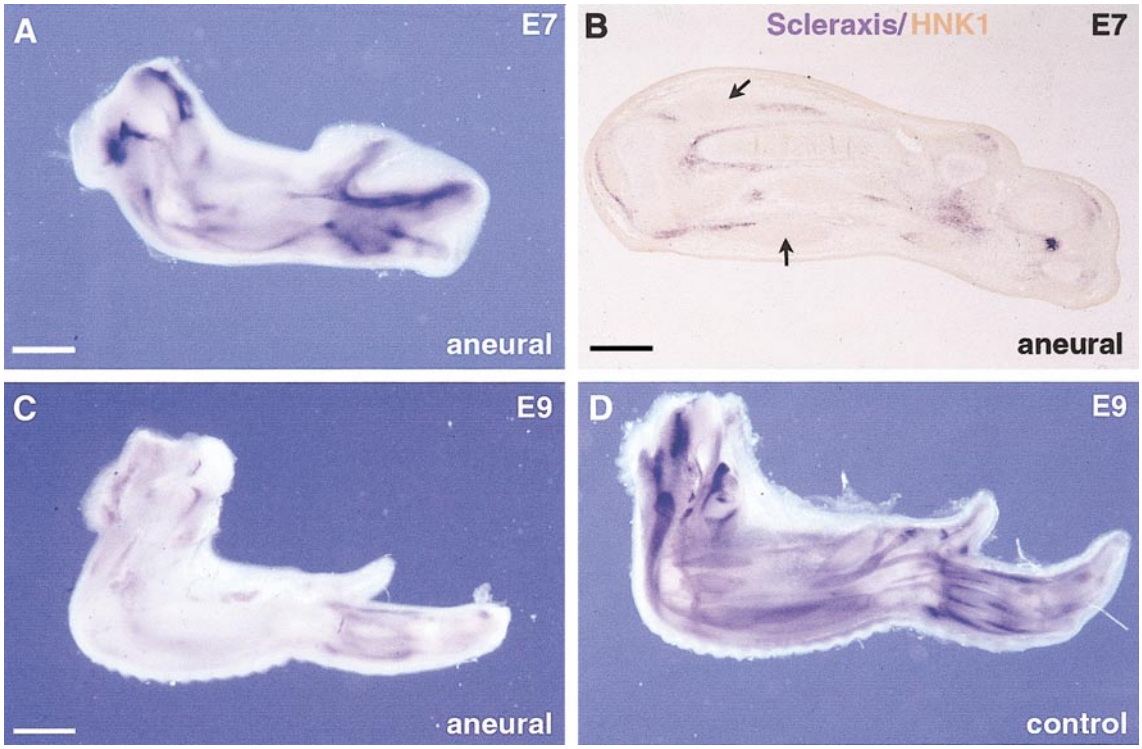

FIG. 6. Scleraxis expression is downregulated progressively along the entire proximodistal axis in aneural conditions. Ventral view of aneural (A, C) and normal (D) wings hybridised with *scleraxis* probe at E7 (A) and E9 (C, D). At E7, *scleraxis* expression is normally detected in the right aneural wing (A) and displays the normal pattern on longitudinal sections from the left wing (B) from the same experimental embryo, where HNK1 labelling shows the absence of innervation (B). At E9, when Fgf4 is absent, *scleraxis* transcripts are also absent at the forearm level, but can still be detected at the digit level of the aneural wing (C), although at lesser intensity compared with the digit tendons of the stage-matched normal wing (D). Scale bars: (A) 2.2 mm; (B) 444 μm; (C, D), 2.7 mm.

ectopic expression of *mFgf4* was very localised and we could not detect any clear up-regulation of tenascin despite an up-regulation of *scleraxis* expression. A picture of a control muscleless wing devoid of *scleraxis* expression at E9 is shown in Fig. 3C. These results show that the presence of muscles is not required for the maintenance of *scleraxis* and tenascin expression by Fgf4.

DISCUSSION

Ectopic Fgf4 Up-Regulates Two Tendon-Associated Molecules, scleraxis and tenascin

In the present paper, we have shown that ectopic Fgf4 positively regulates the expression of two tendon markers, *scleraxis* and tenascin, in the limb buds. After misexpression of *mFgf4* using retroviruses, we observe a clear reduction of myosin expression, concomitant with the extension of tendon markers (Fig. 2; see also Edom-Vovard *et al.*, 2001b). We have already shown that the inhibition of muscle markers is due to a diminution of muscle cell number (Edom-Vovard *et al.*, 2001b). This excludes the possibility of conversion of muscle cells to tendon cells. One explanation is that Fgf4 induces the proliferation of tendon progenitor cells. Consistent with this, we observe a clear increase of connective tissue compared with control wings (Fig. 2). Interestingly, using *in vitro* models, Fgf2 (or bFgf) has been shown to increase proliferation of rat tail tendon-derived fibroblasts (Stein, 1985) and rat patellar tendon-derived fibroblasts (Chan *et al.*, 1997, 2000). Our

were hybridised with DIG-antisense probes for *scleraxis* (C, D), *Fgf8* (E, F), and *cFgf4* (G, H) and then incubated with the HNK1 antibody, recognising the nerves (C, D), or with the MF20 antibody (E–H). Equivalent longitudinal sections from another E8 aneural wing were incubated with the M1B4 antibody (I, J). (B, D, F, H, J) Higher magnifications of forearm levels from the normal and nerveless wing framed in (A), (C), (E), (G), and (I). Scale bars: (A, C, E, G, I) 694 μm; (D, F, H) 173 μm; (B, J) 347 μm.

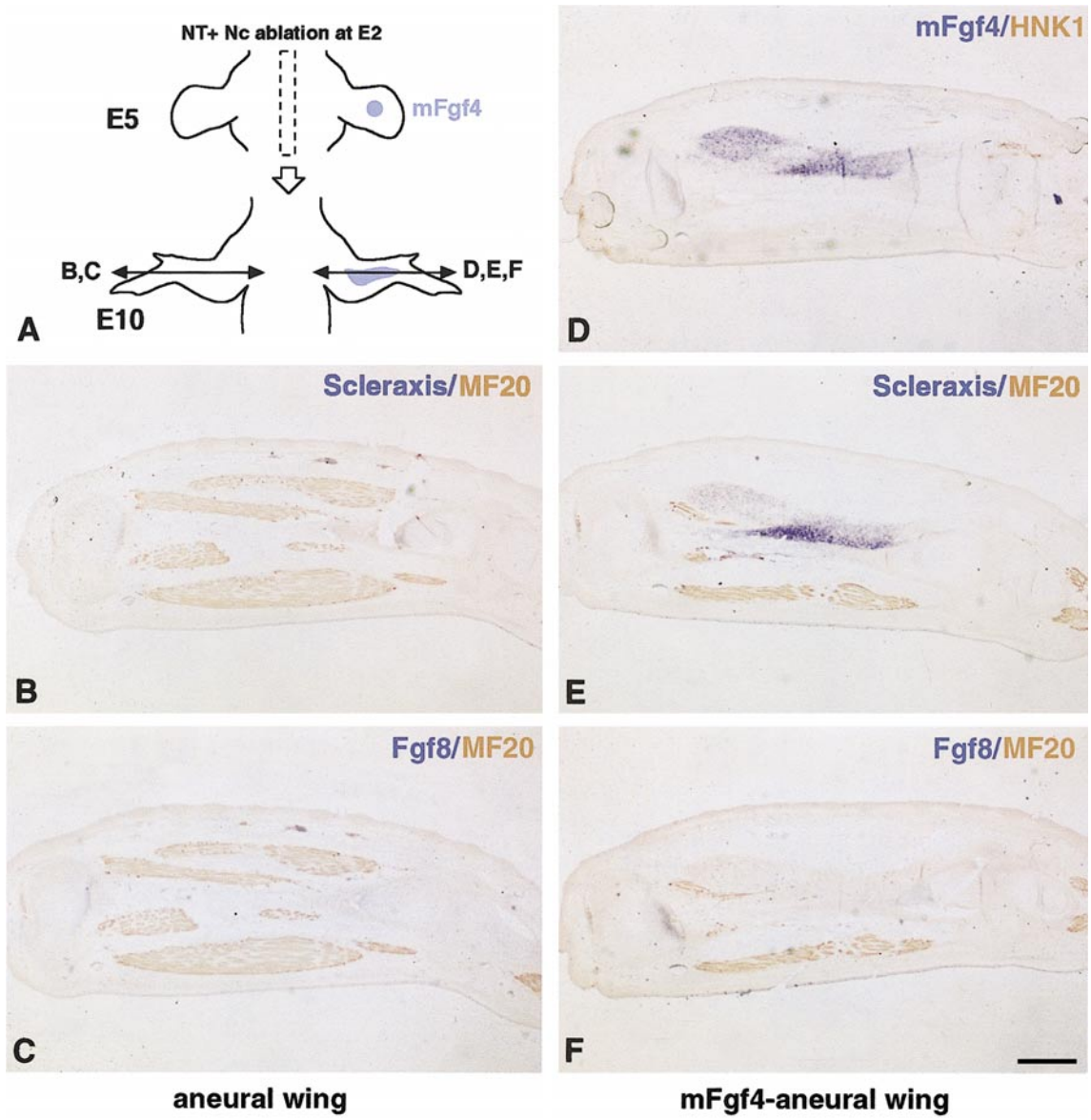

FIG. 7. Grafting *mFgf4*-expressing cells into aneural limbs rescues *scleraxis* expression but not that of *Fgf8*. The neural tube and notochord were removed from chick embryos at E2. Cells expressing mFgf4/RCAS were grafted dorsally into the middle of aneural right wings of the embryos at E5 (stage HH26). Five days after grafting, longitudinal sections of the left nongrafted wings show the down-regulation of *scleraxis* (B) and *Fgf8* (C) expression, following neural tube ablation. Longitudinal sections from the grafted right aneural wings were hybridised with *mFgf4* (blue), showing the extent of the virus spread, and then incubated with HNK1 antibody (brown), showing the absence of innervation (D). Adjacent sections hybridised with *scleraxis* (E) and *Fgf8* (F) probes (blue) and labelled with MF20 (B, C, E, F) show ectopic *scleraxis* expression but no *Fgf8* expression in the muscle-deprived regions. Scale bars: (B–F) 694 μm.

data raise the question of the final differentiation of the connective tissue, in which *scleraxis* and tenascin have been up-regulated under the influence of ectopic Fgf4. We noticed an increase of cell density in the regions that have up-regulated *scleraxis* and tenascin (Fig. 2). This cell condensation is reminiscent of the cell condensation occurring during tendon formation (Ros *et al.*, 1995; Kardon, 1998) and of the compact cellular aspect of mature tendons (Benjamin and Ralphs, 2000). However, ectopic Fgf4 application does not lead to ectopic expression of all the tendon markers, since *Fgf8*, a late tendon marker (Edom-Vovard *et al.*, 2001a), is absent. Moreover, *scleraxis*, although it is expressed early in tendon primordia, is not sufficient to drive complete tendon formation, since the up-regulation of

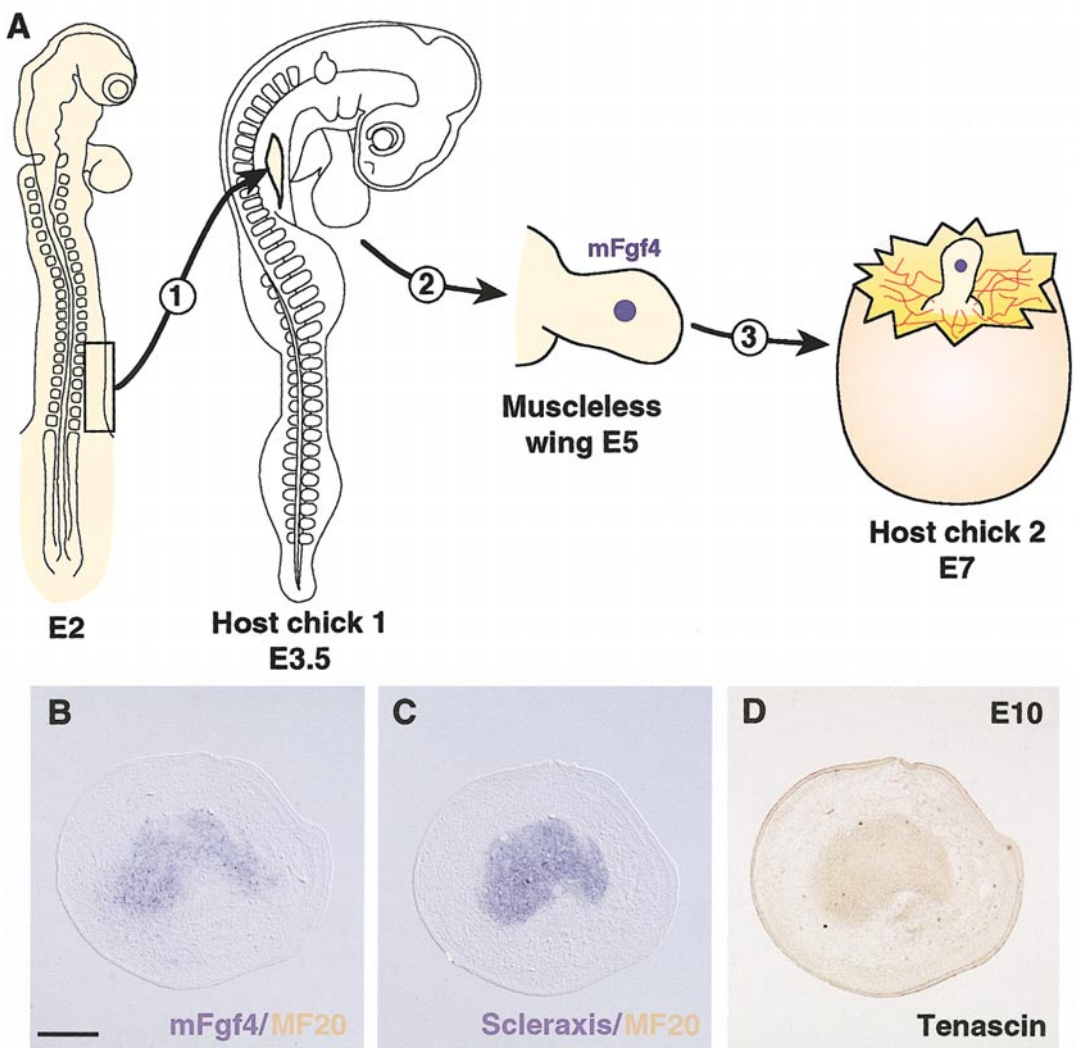

FIG. 8. A source of *mFgf4* prevents the disappearance of *scleraxis* and tenascin in muscleless limbs. The three-step experimental procedure is summarised in (A). (1) The lateral plate corresponding to the future wing was removed from a chick embryo at E2 (22 somites) and implanted into the coelomic cavity of a host embryo at E3.5 (stage HH21). (2) Muscleless wing was then excised from the host, 3 days later, at E5 with respect to its own total development (stage HH26). (3) After receiving a graft of cells expressing mFgf4/RCAS, the muscleless wing is implanted on the chorioallantoic membrane of another chick host at E7. Transverse sections from mFgf4-grafted muscleless wings at E10 hybridised with *mFgf4* (B) and *scleraxis* (C) probes (blue) and labelled with tenascin antibody; brown (D) shows ectopic *scleraxis* (C) and tenascin (D) expression where ectopic expression of *mFgf4* is detected (B). The *in situ* hybridisation with *Fgf4* and *scleraxis* was followed by an immunohistochemistry with the MF20 antibody (brown), showing the absence of muscle. Scale bars: (B–D) 347 μm.

scleraxis after ectopic expression of Noggin does not lead to ectopic tendons (Schweitzer *et al.*, 2001). However, in contrast to the up-regulation of *scleraxis* mediated by noggin (Schweitzer *et al.*, 2001), the up-regulation of *scleraxis* mediated by Fgf is accompanied by an up-regulation of tenascin.

It has been reported that the application of Fgf2 and -4 beads to the tips of the digits of chick leg buds at E6 inhibits the expression of two distal tendon-associated molecules, *Eph-A4* (D'Souza and Patel, 1999) and *Tgfβ2* (Merino *et al.*, 1998). The fact that this inhibition is accompanied by a loss of cartilage (D'Souza and Patel, 1999; Merino *et al.*, 1998) indicates that the inhibitory effect of Fgfs on distal tendon development may be related to a role of Fgfs in maintaining the distal mesoderm in an undifferentiated and proliferative state (Merino *et al.*, 1998). In contrast to these results, we observe an up-regulation of the tendon-associated molecules *scleraxis* and tenascin under Fgf4 exposure, all along

the proximodistal axis and with no modification of cartilage.

Fgf4 Involvement in Late Tendon Formation

Fgf4 transcripts are detected at the extremities of muscle fibres near the future myotendinous junction from E6, at a time when the interactions between muscle and tendon primordia occur (Kardon, 1998). Experiments in chick embryos suggested that only late tendon development requires the presence of muscles (see Introduction). The initiation of tendon formation and further degenerescence in muscleless limbs have been observed using histological criteria (Kieny and Chevallier, 1979; Brand *et al.*, 1985) and molecular markers, such as tenascin (Kardon, 1998) and *scleraxis* (Fig. 3). This muscle requirement for late tendon development could simply reflect a need for the physical presence of muscle. However, in our aneural limb, we observe the down-regulation of the tendon markers when muscles are still present from E8 to E10, suggesting that the muscle requirement involves a molecular aspect. The molecular signals involved in this muscle/tendon interaction are unknown. One attractive possibility is that Fgf4, which is located at the extremities of muscles close to the myotendinous junction and at the right time, is involved in tendon maintenance, by sustaining *scleraxis* and tenascin expression.

Interestingly, similar reciprocal interactions between muscles and tendons are found in *Drosophila* (Volk, 1999). Although *Drosophila* tendon cells differ in their ectodermal embryonic origin when compared with the mesodermal origin of vertebrate tendon cells, as in vertebrates, *Drosophila* tendons form independently of muscles but their late maturation requires the presence of the muscle (Becker *et al.*, 1997). The absence or ectopic presence of specific muscles leads to the disappearance or ectopic activation of tendon-specific gene expression, respectively (Becker *et al.*, 1997). Interestingly, the mutation of the *Drosophila* Fgf receptor, *heartless*, leads to a loss of specific muscles and to a consequent loss of the corresponding tendons (Becker *et al.*, 1997). The molecular aspect of these muscle/tendon interactions has started to be identified: Vein (a neuregulin-like ligand) is a muscle-derived signal that activates the EGF-receptor-signalling pathway in tendon cells (Yarnitzky *et al.*, 1997). Vein is considered to be required for the maturation of the tendon cells. It would be of particular interest to seek the vertebrate homologues of neuregulin-like genes present in muscles and study their interrelation with Fgf4.

Role of Fgfs in Tendon Development

We have shown that Fgf4, normally expressed in the muscle extremities close to the myotendinous junction, induces tendon markers such as *scleraxis* and tenascin when misexpressed in the limb bud. Ectopic mFgf4 activates Fgf signalling, probably through FgfR1, since *mFgf4* activates *FgfR1* expression (Edom-Vovard *et al.*, 2001b). We cannot completely exclude that ectopic mFgf4 mimics another Fgf. Moreover, an expansion of tenascin-expressing cells has also been observed *in vivo* after overexpression of human Fgf5 using retroviruses, in the chick limb (Clase *et al.*, 2000). *Fgf5* transcripts are detected in muscles (Haub and Goldfarb, 1991). There are quite a few Fgfs reported to be expressed in relation to muscles: *Fgf2* (Joseph-Silverstein *et al.*, 1989), *Fgf6* (deLapeyriere *et al.*, 1993; Han and Martin, 1993), *Fgf7* (Mason *et al.*, 1994), and *Fgf9* (Colvin *et al.*, 1999). However, with the exception of *Fgf2*, whose protein is clearly located all along the muscle fibres (Joseph-Silverstein *et al.*, 1989), and that of *Fgf4* (Edom-Vovard *et al.*, 2001b), the precise cellular locations (muscle fibres, myoblasts, or connective tissue) of the other *Fgfs* remain to be clarified. Ectopic mFgf4 could also mimic Fgf8 in tendon. Fgf8 bead implantation has been shown to induce tenascin-C expression in limb bud explants (Tucker *et al.*, 2001). However, the timing of the down-regulation of *scleraxis* expression in the aneural limb does not follow that of *Fgf8* very tightly as we might have been expecting if Fgf8 was maintaining *scleraxis* expression. For example, Fgf8 is hardly detectable in digit in aneural limb, while scleraxis is very slow to be down-regulated. In contrast, the down-regulation of *scleraxis* transcripts follows more closely the disappearance of *Fgf4* expression (see Table 1). Moreover, in muscleless limbs, *Fgf8* transcripts are never detected all along the proximodistal axis, while *scleraxis* expression is still detected until E10 in distal regions.

In conclusion, Fgf signalling regulates positively the expression of two tendon-associated molecules, *scleraxis* and tenascin. Based on this result and on the location and timing of *Fgf4* expression, we propose that *Fgf4* localised at the extremities of muscles is involved in tendon maintenance.

ACKNOWLEDGMENTS

We thank Marie-Claire Delfini and Thierry Jaffredo for critical reading of the manuscript. We are grateful to Francis Beaujean, Michel Fromaget, and Sophie Gournet for the illustrations. This work was supported by the "Association Française contre les myopathies" (AFM/Duprez), the "Association pour la Recherche contre le Cancer" (ARC/Teillet; ARC/Duprez), and the Centre National de la Recherche Scientifique (CNRS). F.E. is supported by the "Association pour la Recherche contre le Cancer" (ARC).

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Received for publication March 15, 2002
Revised April 19, 2002
Accepted April 19, 2002
Published online June 7, 2002