

## Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments

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### SUMMARY

Little is known about the genesis and patterning of tendons and other connective tissues, mostly owing to the absence of early markers. We have found that Scleraxis, a bHLH transcription factor, is a highly specific marker for all the connective tissues that mediate attachment of muscle to bone in chick and mouse, including the limb tendons, and show that early scleraxis expression marks the progenitor cell populations for these tissues. In the early limb bud, the tendon progenitor population is found in the superficial proximomedial mesenchyme. Using the scleraxis gene as a marker we show that these progenitors are induced by ectodermal signals and restricted by bone morphogenetic protein (BMP) signaling within the mesenchyme.

Application of Noggin protein antagonizes this endogenous BMP activity and induces ectopic scleraxis expression. However, the presence of excess tendon progenitors does not lead to the production of additional or longer tendons, indicating that additional signals are required for the final formation of a tendon. Finally, we show that the endogenous expression of noggin within the condensing digit cartilage contributes to the induction of distal tendons.

Key words: Tendon, Scleraxis, Musculoskeletal system, Chick, Mouse, Cell fate

### INTRODUCTION

The functional integrity of the musculoskeletal system requires precise and elaborate attachment of muscles to their respective skeletal elements in order for the force generated during muscle contraction to be transmitted faithfully to the skeleton. The collagen-rich muscle attachments range from narrow bands of connective tissue in some axial muscles to the long limb tendons (Benjamin and Ralphs, 2000). Most studies of differentiation and patterning of the musculoskeletal system in vertebrates have focused on the skeletal elements, which can be easily seen in cleaned and stained preparations, and on the muscles, whose differentiation have been particularly well studied on a molecular level. However, while tendons and other muscle attachments are of equal functional importance, surprisingly little is known about their genesis.

To date, most developmental studies of tendon formation have concentrated on the limb tendons, and in particular, on the morphogenesis of the distal autopod tendons (reviewed by Benjamin and Ralphs, 2000). The patterning of specific distal tendons is preceded by an extracellular matrix (ECM) scaffold

that is present in the hand plate from stage 27 onwards (Hurle et al., 1989). This scaffold is found on both the dorsal and ventral sides of the limb and consists of a 'mesenchymal lamina' (ML), which is parallel to and in contact with the ectodermal basement membrane. A complex fibrillar system extends in a dorsoventral orientation from the ML and serves as a scaffold for the forming tendons (Hurle et al., 1990). During these stages, a number of tendon-specific genetic markers have been described, all of which are expressed in a broad sub-ectodermal layer, in cells above and below the developing cartilage. These include *Epha4* (Patel et al., 1996), TGF $\beta$ 2 (Merino et al., 1998), follistatin (D'Souza and Patel, 1999), *Six1* and *Six2* (Oliver et al., 1995), and *Eya1* and *Eya2* (Xu et al., 1997). In the case of the *Six* and *Eya* genes, distinct family members are expressed in the forming dorsal extensor and ventral flexor tendons. As the morphogenesis of the tendons proceeds, these genes exhibit complex and dynamic changes in their expression patterns, ultimately resolving to specific regions of the tendons, for example, follistatin is found near the insertion of the tendon, while *Epha4* becomes localized to the body of the tendon (D'Souza and Patel, 1999).

Morphogenesis of proximal limb tendons, by contrast, differs significantly from that of the distal tendons (Kardon, 1998). In particular, a 'mesenchymal lamina' is never detected in the proximal limb bud, and none of the molecular markers mentioned above is expressed in the proximal tendons. However, Tenascin, an extracellular protein, is a good marker for all tendon blastema once they form, and has been used to describe early events in the formation of proximal tendons (Chiquet and Fambrough, 1984; Kardon, 1998). While all limb tendons are initially derived from lateral plate mesenchyme (Christ et al., 1979; Kieny and Chevallier, 1979; Shellswell and Wolpert, 1977), the proximal tendon blastema develops in close proximity to the muscle precursors, and the morphogenesis of this tissue is tightly coupled to that of the early limb muscles. Thus, although the early tendon precursors can form even in the absence of limb muscles, the proximal tendons fail to individuate in muscleless limbs and the tendon primordia disintegrate (Kardon, 1998). Conversely, distal limb tendons develop in separation from their respective muscles, and their morphogenesis is instead tightly coordinated with that of the forming skeletal elements. However, while the distal limb tendons form in the absence of muscles (Shellswell and Wolpert, 1977; Kieny and Chevallier, 1979; Christ et al., 1979), they nevertheless require muscles for their survival and degenerate in their absence (Kieny and Chevallier, 1979; Kardon, 1998).

Although some of these later stages of tendon morphogenesis are thus starting to be elucidated, fundamental aspects of the earliest steps in the process remain unclear. For example, it is currently not known whether a committed tendon progenitor cell population exists prior to the recruitment of mesenchymal cells to a specific tendon or whether mesenchymal cells assume the tendon cell fate only when they are recruited into a growing tendon. If relatively little is known about the formation of limb tendons, virtually nothing is known about the embryonic origin and morphogenesis of the attachments of axial muscles to their respective skeletal elements. One reason so little is known about the early steps of tendon formation is that a molecular marker that labels specifically and exclusively both the early tendon primordia and the later differentiated tendons has not been described to date. Tenascin, one of the best known markers for tendons, does label both the tendon primordia and the differentiated tendons. However, it also labels other cell types, such as glia and cartilage, thus complicating its use as a tendon marker (Chiquet and Fambrough, 1984; Kardon, 1998). All the other tendon markers described above are specific to the distal tendons, and moreover, are either initially found in broad complex domains and only later restricted to the forming tendons (e.g. *EphA4*; Patel et al., 1996), or are not expressed during the earlier stages when the tendon primordia are presumably forming (e.g. *Eya1* and *Eya2*; Xu et al., 1997).

The mouse scleraxis gene was isolated in a search for novel tissue-specific basic helix-loop-helix (bHLH) proteins, taking advantage of the propensity of these proteins to preferentially heterodimerize with ubiquitous bHLH partners (Cserjesi et al., 1995). Strikingly, embryos homozygous for a targeted disruption of the scleraxis gene do not form mesoderm, fail to undergo gastrulation and die at E8.5 (Brown et al., 1999). Scleraxis expression is also observed at later stages in various mesodermally derived tissues (Cserjesi et al., 1995).

We show that the later expression of scleraxis is specific to the developing connective tissue that mediates the attachment of muscle to bone including tendons, as well as in ligaments mediating the connection between bones. Scleraxis is continuously expressed in a population of cells from early somitic and limb bud stages to the eventual formation of tendons and other muscle attachments, suggesting that the early scleraxis-expressing cells represent a tendon progenitor pool. Using scleraxis as a marker, we find that the induction of the tendon primordium in the limb requires positive signals from the overlying ectoderm. The region where cells are able to adopt this fate is limited, in turn, by the negative influence of bone morphogenetic protein (BMP) signaling from surrounding areas of the limb bud mesenchyme. At later stages, the repressive effects of high level BMP expression in the interdigital domains of the autopod are overcome, in part, by the local activity of the BMP antagonist Noggin, whose expression is associated with the forming digits. Ectopic application of Noggin protein at earlier stages leads to an expanded domain and increased levels of scleraxis expression within the developing limb; however, a normal tendon pattern is still observed, demonstrating that additional signals are involved in controlling where individual tendons will eventually emerge.

## MATERIALS AND METHODS

### Cloning of chick scleraxis

Chick scleraxis was cloned from a stage 18-24 chick limb bud Clontech Lambda-Zap phage library, by low stringency hybridization with a probe for murine scleraxis (Cserjesi et al., 1995). A single clone containing the full-length scleraxis cDNA was isolated (pBScScx).

### Whole-mount and section in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Riddle et al., 1993) with minor modifications. Embryos at E10 and older were skinned and eviscerated to improve probe penetration. Proteinase-K concentrations were increased for older embryos up to 100 µg/ml for E10 embryos. For mouse embryos, 1% Tween was used in all buffers and Boehringer blocking reagent was added to the antibody block. Section in situ hybridization was performed on 5-10 µm paraffin sections. The hybridization protocol can be found at <http://axon.med.harvard.edu/~cepko/protocol/ctlab/ish.cn.htm>

Double in situ hybridization was performed essentially as described previously (Dietrich et al., 1998).

To generate a probe for chick scleraxis, pBScScx was digested with *Bgl*III and *Eco*RV and re-ligated. The resulting plasmid, pBScScxΔ, includes a 450 bp insert, which is directed mostly to the 3'UTR of scleraxis. Probe templates: chick scleraxis (pBScScxΔ, *Hind*III, T3), murine scleraxis (Brown et al., 1999), *Bmp2*, *Bmp4* and *Bmp7* (Laufer et al., 1997), *Pax1* (Qp1, *Hind*III, T7), *MyoD* (pCMDmyoD, *Hind*III, T7), autotaxin (ScATX, *Nco*I, T7), and noggin (Capdevila and Johnson, 1998). Noggin knockout mice were kindly provided by Andy McMahon.

### In vivo manipulations

Viral preparation and infection was performed as previously described (Logan and Tabin, 1998). Construction of the retroviral constructs was previously described: mouse *Bmp4* (Duprez et al., 1996); chick noggin (Capdevila and Johnson, 1998). The BMP2, BMP4, BMP7 and Noggin proteins were obtained from Genetics Institute. Affigel Blue agarose beads (BioRad) were washed in phosphate-buffered saline (PBS), and soaked in 50-100 ng/ml BMP protein and 700 ng/ml

Noggin on ice. Dorsal wing mesenchyme was slit at the desired positions by a tungsten needle and the beads were inserted. Ectoderm was removed as previously described (Yang and Niswander, 1995). Only medial ectoderm was removed to minimize effects on Sonic hedgehog expression in the zone of polarizing activity (ZPA).

## RESULTS

### Scleraxis is expressed in all connective tissue that forms muscle attachments

To study the role of the bHLH transcription factor Scleraxis in the experimentally accessible chick embryo system, we isolated a chicken homolog of mouse scleraxis. Chick scleraxis is fully identical to the mouse protein in the bHLH domain and flanking residues, the region responsible for mediating DNA binding and protein heterodimerization (Fig. 1). The two proteins also share extensive identity in the C and N termini of the protein, with intervening regions of lower similarity, including two nine amino acid stretches that are missing in the chick protein.

Whole-mount in situ hybridization of developing chick embryos using a scleraxis probe revealed a unique expression pattern. In 9-10 day embryos, Scleraxis transcripts are found in all the muscle-to-bone attachment sites (Fig. 2). Notably, scleraxis expression is apparent in all the limb tendons, both proximal and distal (Fig. 2A,D,E). In a dissected autopod flexor tendon, staining of the entire tendon can be seen, from the myotendinous junction to the bifurcated insertion of the tendon into the phalangeal joint (Fig. 2F). Scleraxis is also expressed in a wing aponeurosis, a tendinous element arranged in flattened bands (red arrowhead in Fig. 2D), and in some of the flattened sheets of connective tissue (fascia) associated with muscle (yellow arrowhead in Fig. 2D). The association of scleraxis expression with all muscle attachments is even more pronounced in the trunk and neck (Fig. 2B,C). In particular, the complex network of muscle attachments to the neck vertebrae is distinctly marked by scleraxis expression (Fig. 2C), and, in the trunk, the attachment and fascia of the scapular and pelvic muscles, as well as the longitudinal axial muscles, are also sites of scleraxis expression (Fig. 2B).

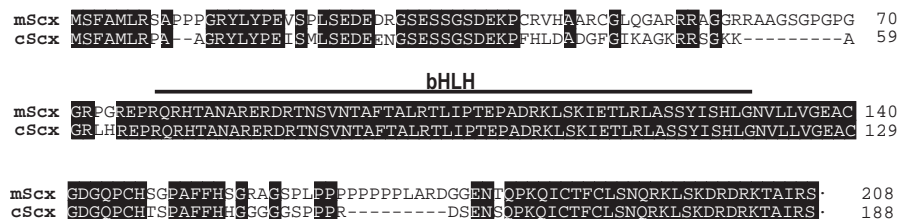
Scleraxis is thus a unique marker for tendons and other connective tissue elements mediating the attachment of muscle to bone. For simplicity, we will refer in the rest of this manuscript to all muscle-to-bone attachments as tendons. Interestingly, in sections through a knee of an E14 chick embryo, scleraxis expression was detected in cells of the ligaments as well (data not shown). Ligaments are dense connective tissue elements that connect bone to bone, thereby securing the functional integrity of the joints. While ligaments are similar to tendons in both biochemical composition and structure (Benjamin and Ralphs, 2000), they differentiate later than tendons during embryogenesis.

### Scleraxis is expressed in axial connective tissue progenitors

Early scleraxis expression is complex and dynamic; we therefore analyzed the

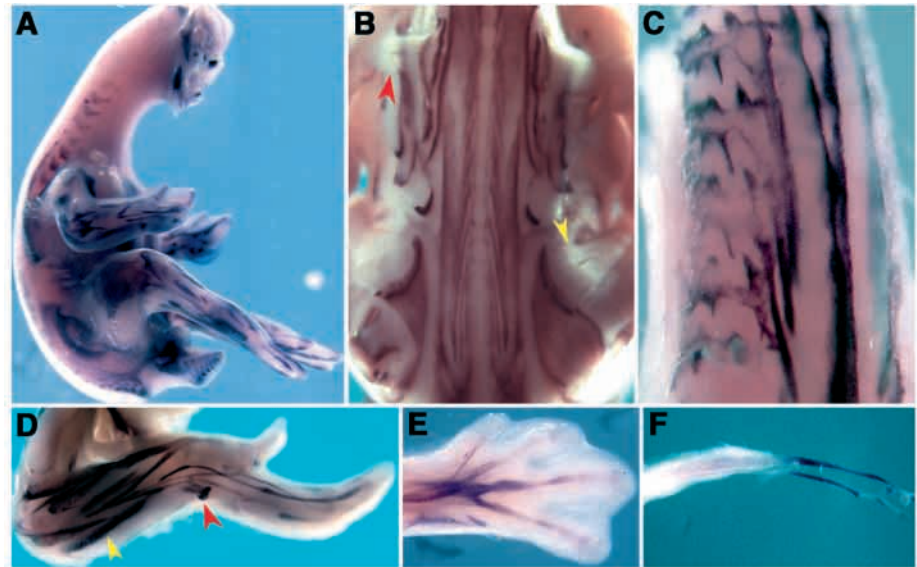
expression in detail to see if it correlated with tendon progenitors in the various tissues discussed above. Scleraxis transcripts are first detected in limbs (see below, Fig. 4) and somites (data not shown) of stage 21 embryos (staging according to Hamburger and Hamilton, 1992). In the somites, scleraxis is expressed in the mesenchyme lining the intersomitic boundary from stage 21 through stage 26 (Fig. 3A and data not shown). The expression was analyzed by section in situ hybridization to study the spatial relationship between the domain of scleraxis expression and the related somitic compartments: the sclerotome, origin of the axial cartilage and the myotome, the origin of axial musculature (Fig. 3I, Christ and Ordahl, 1995). While the sclerotome occupies the ventromedial region of the somite, the myotomal cells differentiate at the edges of the dermomyotome, which occupies the dorsolateral region of the early somite (Kalcheim et al., 1999). These cells gradually form the myotome as a layer of differentiated myogenic cells directly underneath the dermomyotome (Fig. 3I). In longitudinal coronal sections through the back of stage 25 embryos, the differentiated myofibrils in the myotome can be identified both morphologically (red arrowhead in Fig. 3C-E) and by expression of *MyoD*, a myogenic marker (Fig. 3E, Pownall and Emerson, 1992). The scleraxis-expressing cells are located directly medial to the junction between the myotomes of consecutive somites, thus aligning at the edges of the forming muscles and possibly prefiguring the eventual formation of myotendinous junctions (Fig. 3D). The proximity of scleraxis-positive cells and the edge of the myotome persists throughout the somite from dorsal to ventral sections (data not shown). The abutting but non overlapping nature of the expression domains of scleraxis and *MyoD* is further accentuated in a two-color in situ hybridization of a stage 26 embryo (Fig. 3F-H).

Conversely, the sclerotome, marked here by expression of *Pax1*, is found only in the ventral parts of the somite (Fig. 3C, Christ and Ordahl, 1995). Within the sclerotome, *Pax1* is expressed at low levels in the intersomitic mesenchyme, while high levels of expression and early cartilage condensations are found only in the body of the somite at this stage (Fig. 3C). It appears, therefore, that the scleraxis-expressing cells are a distinct population of cells aligned in relation to the myotome; they are not part of the sclerotome, as was previously suggested (Cserjesi et al., 1995). The subsequent differentiation and early patterning of the cartilage and muscles seen by stage 29 are accompanied by rapid and complex changes in the scleraxis expression pattern (Fig. 3B), which eventually resolves into the

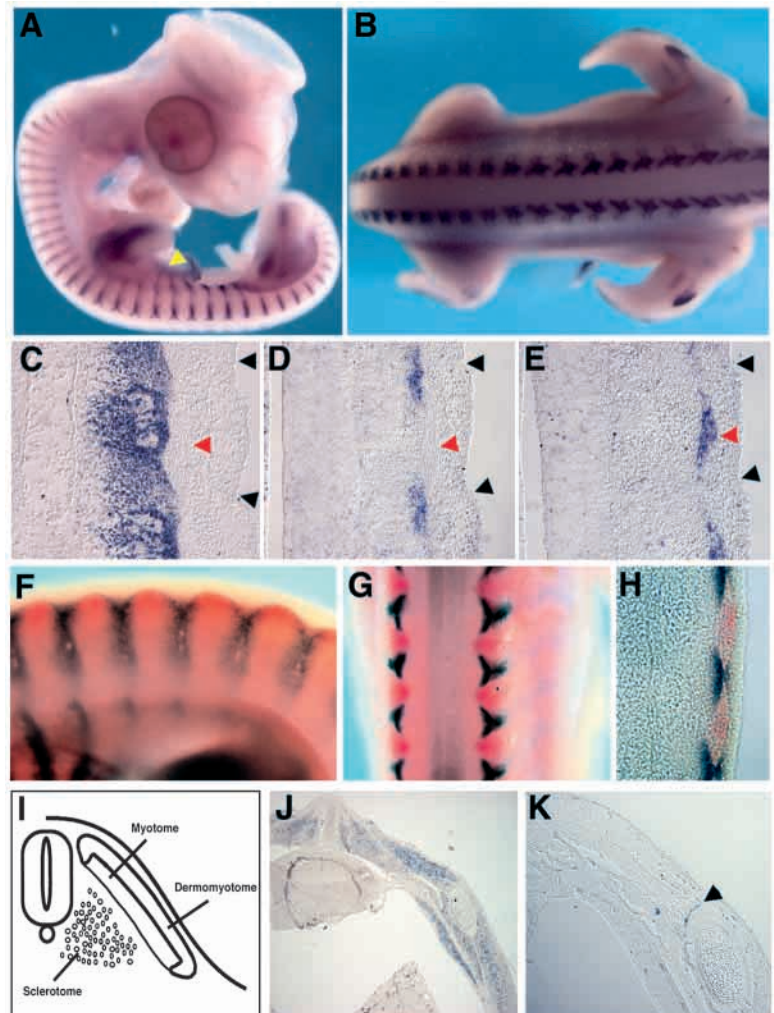


**Fig. 1.** Comparison of chick and mouse Scleraxis proteins. Alignment of the putative amino acid sequences of the chick and mouse Scleraxis proteins. The two proteins are identical in the bHLH domain and flanking amino acids. Long stretches of identity are also found both in the N-terminal region and in a 31 amino acid stretch at the C terminus. The overall amino acid identity between the two proteins is 75%.

**Fig. 2.** Scleraxis is expressed in all axial and limb tendons. Expression of scleraxis in chick embryos at day 9 (stage 35; A,E,F) and day 10 (stage 36; B,C,D) of development, visualized by whole-mount in situ hybridization. In all panels, anterior is upwards. (A) Scleraxis expression at stage 35 marks the complex network of limb tendons. (B,C) Scleraxis expression in tendons of the primary axis. In the trunk (B), the attachments of the scapular (red arrowhead) and pelvic (yellow arrowhead) muscles, which include the broad fasciae of these muscles express scleraxis. Similarly, long axial muscles and related fasciae are also stained. (C) In the neck (ventral view), scleraxis marks the forming tendons at the anterior and posterior edges of each vertebrae. (D-F) Details of scleraxis expression in limb tendons. (D) In a stage 36 wing, scleraxis expression marks all tendons, including an aponeurosis (red arrowhead) and the muscle-associated wing fascia (yellow arrowhead). (E) All details of the foot tendons are also marked by scleraxis. (F) A single dissected flexor tendon from a stage 35 foot highlights scleraxis expression throughout the tendon including the myotendinous junction.

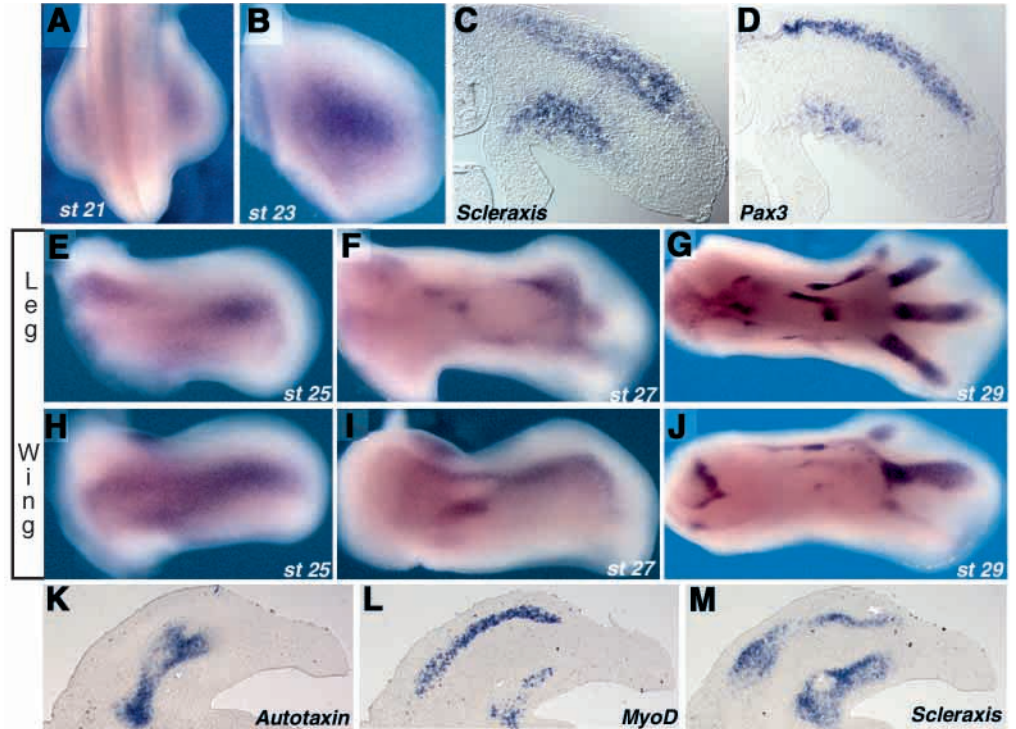


**Fig. 3.** Scleraxis is expressed in putative progenitors of the axial tendons. (A) Scleraxis is expressed in the intersomitic mesenchyme at stage 26. The yellow arrowhead points to the extension of scleraxis expression into the rib primordium. (B) Scleraxis expression becomes more elaborate after the initial patterning of the axial muscles and cartilage at stage 29. (C-E) The domain of scleraxis expression in the somite was analyzed by comparison with other probes in hybridization to alternating coronal longitudinal sections through the back of a stage 25 embryo. Black arrowheads mark somite edges and red arrowheads point to the morphologically distinct myotome. (C) *Pax1* is expressed in the sclerotome but excluded from the early cartilage condensations. Although expression extends to the intersomitic mesenchyme expression levels are higher in the center of the somite. (D) Scleraxis is expressed in cells adjacent and medial to the junction between the myotomes of consecutive somites. (E) *MyoD* is expressed specifically in the myotome. (F-H) Two color in situ hybridization for scleraxis and *MyoD*. Scleraxis (black) and *MyoD* (red) mark two adjacent but non overlapping cell population seen in a lateral view (F) and a dorsal view (G). (H) Scleraxis expression at the junction of adjacent myotomes is demarcated in a longitudinal coronal section (25  $\mu$ m) of the stained embryos. (I) Schematic representation of a transverse section through a late somite. The sclerotome, the origin of axial cartilage, is at the ventromedial region of the somite. The myotome, composed of differentiated myofibrils, which will give rise to the axial muscles, lies directly underneath the dermatome. (J,K) In situ hybridization to transverse sections through the trunk at a thoracic level of a day 12 chick embryo. (J) *MyoD* marks all the axial and intercostal muscles. (K) In an adjacent section, scleraxis is expressed specifically only in a single row of cells connecting an intercostal muscle to a rib.



**Fig. 4.** Scleraxis is expressed in putative limb tendon progenitors. Scleraxis expression in developing wings and legs was analyzed by whole-mount and section in situ hybridization. All limbs are shown in dorsal view. (A,B) Scleraxis is first detected in stage 21 leg buds in a superficial proximomedial domain and expression in this domain is enhanced by stage 23. (C) In situ hybridization to sections of a leg bud at stage 23 illustrates that the expression is superficial in both the dorsal and ventral mesenchyme. (D) This expression domain overlaps partially with the domain of migrating myoblasts, detected in an adjacent section by expression of *Pax3*. By stage 25, the scleraxis-expressing cells coalesce to form more discrete, limb-specific patterns in the leg (E) and wing (H). By stage 27, the dynamic expression of scleraxis continues to change in both leg (F) and wing (I). The first fibrous tendon elements can be seen in the proximal leg bud (F),

concurrent with the onset of scleraxis expression in the forming autopod. The expression is elaborated by stage 29 to include in both leg (G) and wing (J) much longer tendon fibers and the phalangeal tendon blastemas. (K-M) To determine the spatial relationship between the scleraxis-expressing cells and the related tissues alternating transverse limb bud sections were hybridized with probes to scleraxis, a muscle marker, *MyoD*, and an early cartilage marker, autotaxin. In stage 27 leg buds, the domain of scleraxis-expressing cells (M) is still largely overlapping with that of the now differentiating *MyoD*-expressing cells (L). However, neither overlaps with the differentiating cartilage elements in the deeper limb mesenchyme (K).

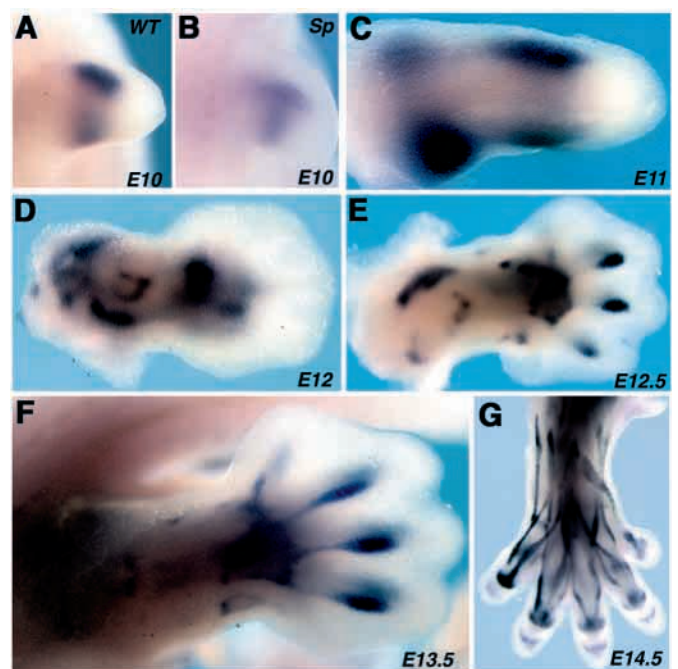


complex trunk and neck expression described earlier (Fig. 2B,C).

Continuity of scleraxis expression within a given population of early tendon progenitors through subsequent stages of differentiation of this tissue is apparent in the development of the intercostal muscles, the muscles that connect the ribs. In the chick, the somitic expression of scleraxis appears to extend into the rib primordium by stage 26 (arrowhead in Fig. 3A). By day 12 (stage 38) of development, *MyoD* expression in transverse trunk sections reveals the fully patterned axial and intercostal muscles (Fig. 3J). In an adjacent section, we find that scleraxis expression in this stage is detected only in the muscle-to-rib attachment, and not in the muscle or cartilage elements themselves

(Fig. 3K). The scleraxis-expressing cells form a monolayer lining the attachment site. We therefore suggest that the early intersomitic, scleraxis-positive, mesenchyme represents a connective tissue progenitor pool that contributes to the formation of all the axial tendons.

**Fig. 5.** Scleraxis is expressed in mouse limb tendons and their progenitors. Scleraxis expression detected by whole-mount in situ hybridization is shown in a developmental series of mouse forelimbs. The expression is shown in a dorsal view (A,B,D-G) and lateral view (C). As in chick, scleraxis expression is first detected in the proximomedial (A) and superficial (C) limb mesenchyme between E10 and E11. (B) Scleraxis expression is not altered in E10 splotch mutant embryos. (D,E) By E12 the expression is much more complex with distinct fibrous elements, and by E12.5 further elaboration of the proximal pattern and the early autopod expression can be detected. (F,G) At E13.5 the digit related expression extends over the whole length of the growing digits and by E14.5 the mature and complex tendon pattern including the phalangeal insertions can be detected.



### ***Scleraxis* expression coincides with limb tendon progenitors**

Because of its accessibility for experimental manipulation, we next focused our attention on *scleraxis* expression in the limb bud. The limb bud expression of *scleraxis* is maintained from stage 21 onwards, and can best be described in four distinct phases. *Scleraxis* expression is first detected in proximomedial domains of stage 21 limb buds (Fig. 4A-C). The expression is punctate and superficial in both the dorsal and ventral mesenchyme of the limb bud (Fig. 4C). Previously, early tendon blastema were identified by following the accumulation of Tenascin, an extracellular matrix protein (Kardon, 1998). Both markers identify the subectodermal mesenchyme as the initial site of limb tendon formation. *Scleraxis* expression, however, precedes that of Tenascin, which is first detected at stage 25 (Kardon, 1998).

*Scleraxis* expression at these early stages is also similar to that of the migrating myoblasts, and indeed, section in situ hybridization reveals a partial overlap between the expression domains of *scleraxis* and *Pax3*, a myoblast marker (Fig. 4C,D). It is well established that unlike the somitically derived muscles, limb tendons are derived from lateral plate mesenchyme (Kieny and Chevallier, 1979; Christ et al., 1979; Shellswell and Wolpert, 1977). Furthermore, early formation of the tendon primordium is not dependent on the presence of myoblasts (Kardon, 1998). Therefore, to be certain that *scleraxis*-expressing cells do indeed represent a tendon progenitor population, it was important to verify that the mesenchymal expression of *scleraxis* is neither in myoblasts nor dependent on the presence of myoblasts in the limb bud. We found that the expression of *scleraxis* in the early mouse limb bud was very similar to that observed in the chick (see Fig. 5, below). We therefore chose to address this issue by looking at *scleraxis* expression in limb buds of splotch mice, which carry a mutation in the *Pax3* gene and are completely devoid of limb musculature (Bober et al., 1994). We find that *scleraxis* expression is similar in limbs from *Pax3* mutant and wild-type embryos (Fig. 5A,B), consistent with the hypothesis that *scleraxis* expression marks tendon progenitors in the limb.

In the second phase, from stage 25 to 27, the *scleraxis*-expressing cells coalesce to form complex and dynamic patterns that differ between dorsal and ventral mesenchyme and between the wing and leg buds (Fig. 4E,F,H,I). The *scleraxis*-expressing cells are found both dorsal and ventral to the newly formed cartilage element marked by the expression of autotaxin (an early marker for cartilage; R. S. and C. J. T., unpublished), and still overlap with the domain of differentiating myoblasts, detected at this stage by the expression of *MyoD* (Fig. 4K-M).

The third phase of *scleraxis* expression, beginning at stage 28, is marked by formation of the first tendon fibers (Fig. 4G,J). *Scleraxis* expression is first detected in small fibrous elements that subsequently elongate to form the more mature tendons. At these stages of limb development, the autopod enlarges in both limbs and cartilage condensations of the digits begin to form. With the initial formation of digit tendons, *scleraxis* is expressed in broad mesenchymal stripes located both dorsal and ventral to the forming digits and directly underneath the ectoderm. This aspect of *scleraxis* expression is similar to the expression of previously reported distal tendon markers like *Epha4* and *follistatin* (Patel et al., 1996; D'Souza and Patel,

1999). Interestingly, these other markers are restricted to autopod tendons, and their expression is not similar to that of *scleraxis* in other limb domains.

Finally, at stages 31 and later, *scleraxis* marks all the limb tendons, and serves as a very good marker both for the elaboration of tendon pattern and for the specific insertions of tendons into their respective skeletal elements (Fig. 2A,D,E).

Since the chick *scleraxis* gene was cloned as a homolog of the mouse gene, we further analyzed its expression in the mouse, both to see whether *scleraxis* expression faithfully marks the tendons in mouse and chick, and to see whether the location of the tendon progenitor populations is similar in the two organisms. In the somites, mouse *scleraxis* expression is indeed similar to chick in the intersomitic mesenchyme and in the rib primordium (not shown). Likewise, the limb expression shows a high degree of similarity between the two species. In early limb buds of E10 and E11, *scleraxis* is expressed in the proximomedial and subectodermal limb bud mesenchyme, in a pattern very similar to the early expression in chick (Fig. 5A,C). By E12, the expression resolves into limb-specific patterns and small tendon fibers can be detected throughout the limb bud (Fig. 5D,E). Concomitantly, and again similar to the expression in chick, expression is induced in mesenchymal condensations both dorsal and ventral to the forming digits, and by E13.5 the hand plate expression resolves into tendinous fibers. Throughout these stages, a broad domain of expression located dorsal and ventral to the forming wrist is much more pronounced in the mouse than in similarly staged chick embryos (Fig. 5D-F). Finally, by E14.5, the complex network of distal limb tendons is completely marked by *scleraxis* expression (Fig. 5G), and this expression in limb tendons persists even as late as E19 (data not shown).

In summary, expression of *scleraxis* is an excellent marker for limb tendons and other muscle-to-bone attachments in both chick and mouse. Furthermore, early *scleraxis* expression in limbs and somites defines cell populations that are the putative tendon progenitors for these tissues.

### **Tendon progenitors are induced by ectodermal signals**

The early pattern of *scleraxis* expression in the limb suggests that cells within the superficial proximomedial limb bud mesenchyme serve as the progenitor population for limb tendons. This marker, in turn, presents us with a unique opportunity for studying the signals that define these putative tendon progenitors. The subectodermal location of early *Scleraxis* expression suggested to us that the ectoderm might play a role in regulating early *Scleraxis* expression. To test this, dorsal wing bud ectoderm was removed at stage 21, before the onset of *scleraxis* expression in the wing bud. When harvested 16-24 hours later, *scleraxis* expression could not be detected within the dorsal mesenchyme (Fig. 6A,B), while the ventral expression of *Scleraxis* was not affected. Moreover, *Tbx5*, a marker for all wing bud mesenchymal cells, was still expressed in the exposed mesenchyme (Fig. 6E), indicating that the exposed mesenchyme retained its viability. Partial ectoderm healing was also observed in about half of the operated limbs. In these cases, partial to full restoration of *scleraxis* expression was observed, indicating that the experimental procedure of removing the ectoderm did not directly interfere with *scleraxis* expression (data not shown). In addition, when embryos were

allowed to mature for more than 24 hours, the ectoderm in most cases healed and tendons could be found (data not shown).

The experiments described above suggested that the ectoderm might be required either for induction or for maintenance of scleraxis expression in the early limb bud. To test whether it is also required for continued scleraxis expression, dorsal ectoderm was removed from wing buds at stages 23-24, after the initial induction of scleraxis expression had occurred, and the embryos were again harvested after 24 hours and processed for whole-mount *in situ* hybridization. After late ectoderm removal, scleraxis expression could still be detected in the exposed mesenchyme, indicating that the ectoderm is not strictly required for maintenance of scleraxis expression (Fig. 6C,D). Significantly, the operated limbs were smaller than the contralateral control limbs (Fig. 6A,C), consistent with previously described roles for the ectoderm in limb outgrowth (Yang and Niswander, 1995) and proliferation of the limb mesenchyme (Amthor et al., 1998; Martin and Lewis, 1986). These size differences were also reflected in a smaller domain of scleraxis expression in these limbs (Fig. 6D).

### The progenitor cell fate can be repressed by BMP signaling

While the ectoderm thus appears to be necessary for early scleraxis expression in the limb, there are regions of the superficial mesenchyme that do not express scleraxis, suggesting that other factors are involved in refining the scleraxis expression domain. Accordingly, we noted that the early scleraxis expression domain appears to be mutually exclusive with the combined expression domains of *Bmp2*, *Bmp4* and *Bmp7* (Fig. 6 F-I), suggesting that BMP signaling might play an endogenous role in restricting scleraxis expression. To test this hypothesis, BMP4 protein was misexpressed in both the limb buds and trunk of chick embryos using a BMP4-expressing retrovirus. In embryos infected at stage 10 in the precursors of both the leg and wing buds and harvested at stage 26, a significant reduction in scleraxis expression was detected (Fig. 7A,B). The protocol we used also targeted the infection to the somitic region adjacent to the limb buds, and in such cases scleraxis expression was repressed in these regions as well (Fig. 7A).

As the use of viral vectors involves a delay of a few days between infection and analysis, we were concerned that the downregulation of scleraxis expression might represent a secondary effect of exposure to BMPs, especially as BMP signaling has the potential to redirect cells to the chondrogenic cell fate (Zou et al., 1997). Therefore, to assess the BMP effects more directly, we applied recombinant BMP proteins directly to the limb bud using BMP-soaked agarose beads, and analyzed the effect on scleraxis at various time points. Following short-term exposure to BMP4, BMP2 or BMP7 protein, scleraxis expression was indeed downregulated in the manipulated limbs (Fig. 7C,D and data not shown). Significantly, this effect could be seen as early as 3 hours after implanting the bead, suggesting that the repression by BMP signaling is direct. Scleraxis repression by BMP4 protein was highly symmetrical around the bead, suggesting that it was limited only by the range of BMP protein diffusion. In addition, repression of scleraxis was detected both at stage 22 limbs and as late as stage 25, when limbs are already in the second phase

of scleraxis expression (Fig. 7C,D). At all stages analyzed, application of recombinant BMP2, BMP4 and BMP7 protein resulted in similar effects.

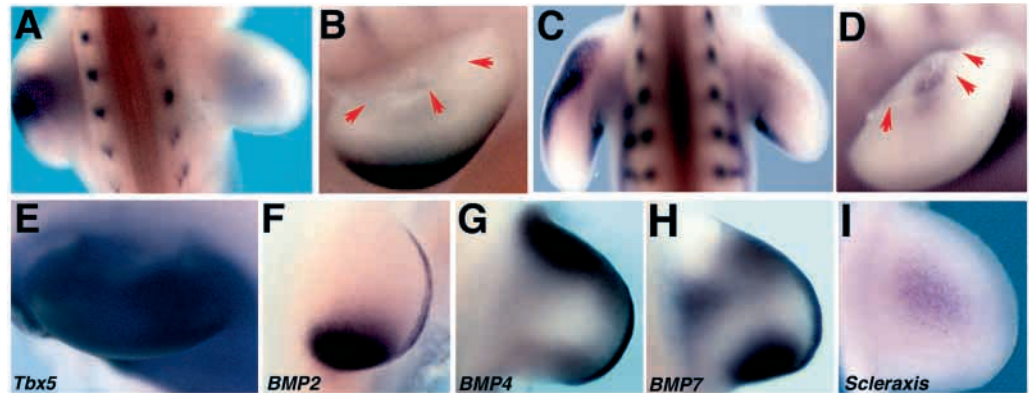
### Antagonizing BMP signaling induces scleraxis expression

Having shown that ectopic BMP signaling is sufficient to downregulate scleraxis expression, we next wished to determine whether BMP signaling acts endogenously to restrict scleraxis expression. We therefore tried to block endogenous BMP signaling using the BMP antagonist Noggin (Zimmerman et al., 1996). For broad misexpression, we again used a retrovirus, this time encoding the noggin message (Capdevila and Johnson, 1998). Strikingly, infection of the presumptive limb mesenchyme at stage 10 resulted in a dramatic upregulation of scleraxis throughout the limb bud as early as stage 22 (Fig. 7E). When allowed to mature further, the previously reported effects of Noggin on limb formation were manifested (Capdevila and Johnson, 1998; Pizette and Niswander, 1999), from total limb loss to dramatic limb malformations (data not shown). In all cases, however, scleraxis expression was maintained in the affected limbs. Interestingly, scleraxis expression in the somites was never affected by noggin misexpression (data not shown), indicating that although BMP signaling is capable of repressing scleraxis in this region, it does not play an endogenous role in limiting axial scleraxis expression.

In order to verify that the effects of Noggin misexpression were direct and were not due to its pleiotropic effects on the limb bud, we again applied recombinant Noggin protein directly to the limb bud. Similar to the BMPs, Noggin effects were dramatic and immediate. As early as 3 hours after bead implantation into a stage 22 wing bud, clear upregulation of scleraxis could be seen (Fig. 7G). Longer incubations of up to 24 hours resulted in a broad intense upregulation of scleraxis expression (Fig. 7F); however, the pattern of scleraxis upregulation was variable and dependent on both the timing and location of the bead implant (see Fig. 7F inset), unlike the symmetrical downregulation of scleraxis produced by the BMP4-soaked beads. This variability might reflect local differences in the levels of BMP signaling (and hence the ability of the Noggin protein to sufficiently antagonize it), or alternatively, it might reflect the influence of other localized factor(s) involved in scleraxis induction or repression. In addition to this variability seen at early stages (stage 21-24), we also observed that implanting Noggin beads at later stages (stage 25 and onwards) did not result in ectopic scleraxis induction (data not shown). The competence of the limb mesenchyme to induce scleraxis expression is therefore limited to the first phase of scleraxis expression at stages 21-24; once the second phase is reached, the competence of the mesenchyme to induce *de novo* scleraxis expression is lost.

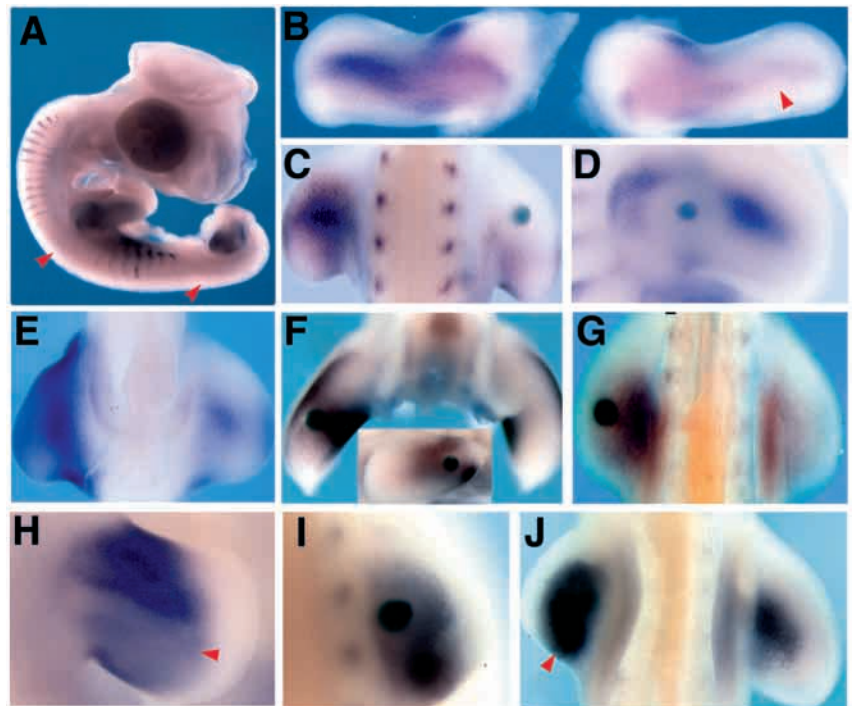
The ability of early application of Noggin to induce scleraxis expression also provided us with the opportunity to test whether the ectopic induction of scleraxis would result in the formation of ectopic tendons. To this end, Noggin-soaked beads were implanted into stage 21 wing buds and the embryos were harvested 1, 2 and 3 days later. As before, scleraxis overexpression was induced within 1 day of bead application; however, expression was at or near wild-type levels by stage 26, 2 days after bead application, and no ectopic tendons were

**Fig. 6.** The ectoderm and mesenchymal BMPs define the early domain of scleraxis expression. (A,B) Early ectoderm removal results in a complete loss of scleraxis expression. The medial ectoderm was removed from stage 21 right wing buds, and the embryos were harvested 14-20 hours later and processed for whole-mount in situ hybridization using a scleraxis probe. While scleraxis expression is clearly detected in the control left wing bud, expression is not seen in the operated wing bud (A). The area



where the ectoderm was removed is easy to detect in a lateral view of the operated limb bud (arrows in B). Note the normal expression of scleraxis in the ventral limb bud and lack of expression in the dorsal mesenchyme. (C,D) To determine whether the ectoderm is required for maintenance of scleraxis expression, ectoderm was removed from wing buds at stage 24, after the onset of scleraxis expression, and the embryos again harvested after 14-20 hours. Low scleraxis expression is detected in the operated wing bud compared with the robust expression in the control left wing bud (C). In a lateral view of the operated wing bud (D), the region of ectoderm removal can again be easily detected (arrows in D) and residual scleraxis expression in the exposed mesenchyme can be seen. (E) *Tbx5* expression is maintained in the exposed mesenchyme of a stage 22 wing bud 14 hours after ectoderm removal. (F-I) The early, proximomedial scleraxis expression domain in the wing bud is mutually exclusive to that of BMPs. Comparison of the early expression of *Bmp2*, *Bmp4*, *Bmp7* and scleraxis; in wing buds from stage 22 embryos.

**Fig. 7.** BMP signaling restricts scleraxis expression. BMP protein (A-D) and the BMP antagonist Noggin (E-J) were applied to wing buds and the effects on scleraxis expression were monitored by whole-mount in situ hybridization. (A-D) Scleraxis expression is repressed by BMP signaling. (A,B) Presumptive limb and adjacent somites of stage 10 embryos were infected with a BMP4 retrovirus. Repression of scleraxis is apparent in the somites (arrowheads in A) and in the limbs – compare in B the expression in the infected right wing bud (red arrowhead) and the control left wing bud. (C,D) Beads soaked in BMP4 protein (75 µg/ml) were implanted into the right wing bud of a stage 23 embryo and the embryos harvested after 3 hours (C) or into stage 25 embryos, which were harvested after 6 hours (D). A dramatic down regulation of scleraxis expression is detected in both cases. (E-G) Upregulation of scleraxis expression by antagonizing BMP signaling. (E) Embryos were infected at stage 10 with a Noggin-expressing retrovirus and harvested after 2 days at stage 22. Scleraxis expression, seen here in ventral view, was induced throughout most of the limb bud mesenchyme. (F) Beads soaked in recombinant Noggin protein were implanted into stage 23 wing buds, and the embryos were harvested after 6 hours. Scleraxis is induced, but only in the ventral mesenchyme adjacent to the bead (see inset in F). (G) Weak but distinct induction of scleraxis can be seen 3 hours after a Noggin-soaked bead was implanted into a stage 21 wing bud. (H-I) Noggin cannot replace the ectodermal inducing signal. (H) The right wing buds of a stage 10 embryo was infected with a Noggin retrovirus at stage 10. The ectoderm was subsequently removed at stage 21 and the embryos harvested after 24 hours. Scleraxis expression is not detected in the exposed mesenchyme (arrowhead), but broad induction of scleraxis can be seen in the regions where ectoderm healing had occurred. (I,J) The ectoderm was removed from the right wing bud of a stage 21 embryo and a bead soaked in Noggin protein was implanted into the exposed mesenchyme. The embryos were harvested after 10 hours. (I) Scleraxis expression was not induced in the dorsal exposed mesenchyme. The dark background around the bead is a reflection of scleraxis induction by the Noggin protein in the ventral mesenchyme, which is clearly seen in a ventral view of the same embryo (arrowhead in J).

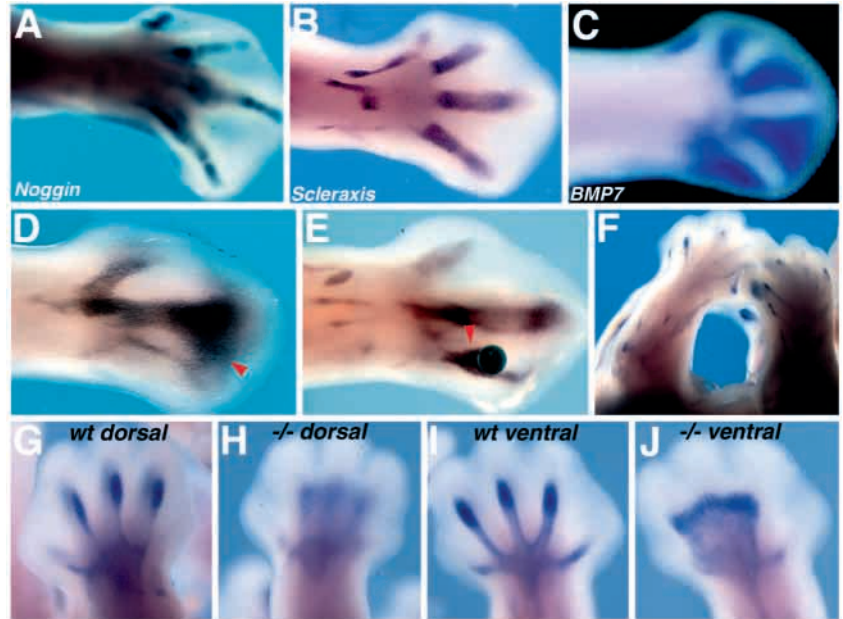


found in the embryos harvested at later stages (data not shown). Similarly, Tenascin C expression in the wing buds of stage 27 embryos was not altered by an early application of Noggin protein (data not shown). Discrete Tenascin expression is first

detected in stage 26 wing buds. It therefore, appears that the onset of Tenascin expression does not coincide with the earliest tendon progenitors, but rather with a later stage in the differentiation of these tissues.



**Fig. 8.** Endogenous *noggin* contributes to the induction of autopod tendons. Induction of scleraxis expression in the autopod (B) is concurrent with *noggin* expression in the condensing digit cartilage (A) and high BMP expression in the interdigital mesenchyme (C). (D) Mild infection of limb buds at stage 19 with the *Noggin* retrovirus, resulted in limited spread of the virus by stage 29. In such limbs, which appear grossly normal, ectopic induction of scleraxis in the autopod was seen (see arrowhead in D). (E) Beads soaked in *Noggin* protein were implanted in the autopod at stage 28 and the embryos were harvested 24 hours later. Limited scleraxis induction (arrowhead in E) was detected in roughly half of the embryos ( $n=10$ ). (F–J) Comparison of scleraxis expression in wild-type and mutant mouse embryos homozygous for a targeted deletion in the *noggin* gene. (G–J) Expression is shown in E13.5 hind limbs. While dorsal autopod expression of scleraxis is already broad and extends along the forming digits of wild-type limbs (G), the expression is very low in the dorsal side of a mutant autopod (H). In the ventral side of mutant forelimbs scleraxis is expressed and its domain is broader than the expression in the ventral autopod of a wild-type hindlimb (compare I with J). (F) By E14.5, dorsal tendons do form in the *noggin* mutant, though they are thinner and less developed compared with wild type (compare F with Fig. 5G).



These results imply that while the scleraxis-positive cells may serve as a tendon progenitor pool, actual tendon formation is regulated by additional late signals, such that the early presence of excess progenitors does not alter the final tendon pattern in these limbs.

#### **Noggin cannot rescue the loss of scleraxis expression after ectoderm removal**

As BMP antagonism led to scleraxis induction, we wanted to see next whether BMP antagonism could substitute for the endogenous ectodermal scleraxis-inducing activity. To examine this possibility, we tested whether *Noggin* misexpression could rescue the loss of scleraxis expression after ectoderm removal, again using both retroviral misexpression and direct protein application. In the first case, wing buds were infected at stage 10 with the *noggin* retrovirus, and then the ectoderm was removed at stage 21, and the embryos were harvested 24 hours later (Fig. 7H). After these manipulations, broad ectopic scleraxis induction was detected in the mesenchyme still covered by ectoderm; however, scleraxis transcripts were never found in the exposed mesenchyme (Fig. 7H, arrowhead). Alternatively, ectoderm was removed at stage 21 and a bead soaked in *Noggin* protein was directly implanted into the mesenchyme (Fig. 7I,J). *Scleraxis* was again absent in the exposed dorsal mesenchyme (Fig. 7I), while its expression was induced by the *Noggin* protein on the ventral side of the operated wing bud (which retained its ectoderm Fig. 7J, arrowhead). We therefore conclude that the ectoderm produces a scleraxis-inducing signal that is not related to the mesenchymal BMP signal, restricting scleraxis induction to the proximomedial mesenchyme.

#### **Noggin is partially responsible for the early induction of autopod tendons**

Tendon morphogenesis in the autopod is significantly different

from the morphogenesis of proximal tendons (Kardon, 1998). In particular, the distal tendons develop in spatial isolation from their respective muscles, and unlike their proximal counterparts, they can initiate tendon formation even in muscleless limbs (Kieny and Chevallier, 1979; Shellswell and Wolpert, 1977). Nonetheless, comparison of the earliest expression of scleraxis in the autopod with the previously reported interdigital domains of BMP expression (Fig. 8B,C and data not shown) suggested that BMP signaling might act to restrict the domain of scleraxis induction in the autopod, as it does in the rest of the limb. In confirmation of this hypothesis, viral misexpression of *Noggin* did indeed result in broad scleraxis induction in the autopod at stage 29 (Fig. 8D). Recombinant *Noggin* protein could also induce scleraxis expression at this stage (Fig. 8E), but the effect was much weaker than in the earlier stages and induction could only be detected in ~50% of the embryos, suggesting that the high interdigital expression of BMPs poses a higher barrier for *Noggin* to overcome in inducing ectopic scleraxis expression.

In the experiments described thus far, *Noggin* was used as an exogenous tool to manipulate BMP signaling. In the developing limb, the endogenous expression of *noggin* was seen in the condensing cartilage beginning at stage 25 (Capdevila and Johnson, 1998). Proximal scleraxis expression at chick stage 22, precedes cartilage condensation and is therefore not likely to be influenced by *noggin*. The sequence of events is changed, however, in the autopod, where scleraxis expression appears concurrently with the early cartilage condensations and the onset of *noggin* expression (Fig. 8A,B). Moreover, it has previously been shown that experimental induction of an extra digit results also in the generation of a tendinous element, suggesting that the distal cartilage has the ability to induce tendon formation (Hurle et al., 1990). We therefore reasoned that the expression of *noggin* in the condensing cartilage elements of the autopod may play an endogenous role in the induction of distal tendons. As scleraxis

and noggin expression are also induced concurrently in the autopod of the mouse (data not shown), we decided to address the possible role of endogenous Noggin in distal tendon formation by analyzing scleraxis expression in mice carrying a targeted deletion of the *noggin* gene (Brunet et al., 1998). While excess cartilage and loss of joints were previously reported in embryos carrying the *noggin* mutation, an effect on tendons had not been documented.

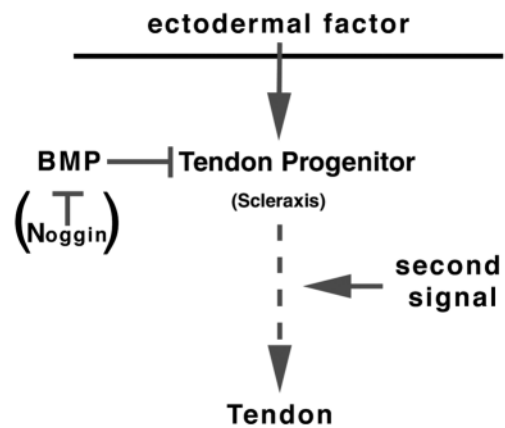
We observed a significant delay in scleraxis expression in the autopods of *noggin* mutant embryos. In the wild-type autopod, scleraxis expression is first detected at E12, later resolving into actual tendon-like elements by E13.5. Conversely, in littermate *noggin* mutants, scleraxis expression was not detected at all in the dorsal autopod until E13.5 (Fig. 8G,H). Scleraxis expression was, however, detected in the ventral autopod of the *noggin* mutants. Surprisingly, this ventral expression was notably broader than in wild-type littermates (Fig. 8I,J), possibly reflecting the reported expansion of the autopod cartilage in *noggin* mutant embryos. In spite of these early alterations in the scleraxis pattern, however, tendons do form in the mutant embryos by E14.5, extending into both the dorsal and ventral hand plates (Fig. 8F). Although these tendons do appear abnormal, this may be due to a lack of normal patterning cues in the autopod. Thus, while the delay of dorsal scleraxis expression suggests that *Noggin* does play an endogenous role in tendon induction, the fact that both dorsal and ventral tendons ultimately form in *noggin* mutants clearly demonstrates that other signals must also be involved, including, most probably, additional signal(s) from the cartilage.

## DISCUSSION

Surprisingly little is known about the genesis and patterning of tendons and ligaments, largely owing to the lack, up until now, of a good marker for these tissues. Scleraxis, which is highly specific to tendons and ligaments, and is expressed in these cells from the putative early progenitor stage right through to the final differentiated elements, represents just such a marker. The use of scleraxis as a marker therefore provides a unique opportunity to advance our knowledge of the cellular origin of these tissues, and to examine the effects of mutations and other embryonic manipulations on the early patterning of tendons and tendon progenitors.

### Early *scleraxis* expression marks tendon progenitors

Analysis of the tendon cell fate requires the identification of the early progenitors for this tissue. The continuity of scleraxis expression from early mesenchymal populations to the final specific expression in tendons allowed us to define three putative connective tissue progenitor pools in the early embryo: the intersomitic mesenchyme, which gives rise to axial tendons, the superficial proximomedial limb mesenchyme, which produces the proximal limb tendons, and the previously described subectodermal mesenchyme surrounding the forming digits, which prefigures the distal tendons. While compelling, the continuity of scleraxis expression does not prove that the early scleraxis-expressing cells are indeed tendon progenitors. This hypothesis can, however, be directly



**Fig. 9.** Regulation of the tendon cell fate. The tendon progenitors, represented by the early limb expression of scleraxis, are found in the superficial proximomedial limb bud mesenchyme. Scleraxis expression is induced by a ubiquitous signal emanating from the ectoderm. However, the induction of progenitors is restricted to the proximomedial domain by the presence of localized BMP signaling, which represses scleraxis expression in other regions. Antagonizing the BMP signal by misexpression of *Noggin* results in a much broader induction of tendon progenitors. Nevertheless, the presence of excess progenitors does not lead to the production of excess tendons, suggesting that other signals regulate mature tendon formation. The same set of signals also appear to be required for distal tendon formation, where endogenous *Noggin* expressed by the condensing cartilage appears to contribute to the induction of the tendon cell fate.

tested using recombinase-mediated fate mapping (Zinyk et al., 1998), in which Cre recombinase expression in a domain of interest activates expression of a histochemical marker, thereby marking the Cre-expressing cells and all cells descendent from them. Mice expressing Cre in the scleraxis domain will thus allow us to directly ask if the early scleraxis-expressing cells are indeed tendon progenitors, and if all tendon cells are derived from these progenitor pools.

### Regulation of the tendon cell fate

In our studies, we have identified a set of signals that direct the limb mesenchyme to assume the tendon cell fate (Fig. 9). In the early limb bud, the ectoderm is required for the induction of the tendon cell fate, indicating that the ectoderm is either the source for tendon progenitor inducing signal(s), or that the ectodermal signals are permissive, allowing the inductive activity of a different signal to be manifested. At the same time, repression by mesenchymal BMPs assures that only the proximomedial cells actually assume this cell fate. The ectodermally derived signal is probably expressed throughout the ectoderm, as broad retroviral misexpression of *Noggin* resulted in ubiquitous expression of scleraxis in the early limb bud. Although the molecular nature of the ectodermal signal is not known, we have established that it is not an effector of BMP signaling, as *Noggin* misexpression could not rescue the expression of scleraxis after ectoderm removal. Experiments to identify the signals that represent the ectodermal inducing activity are under way. Interestingly the mesenchymal and somitic expression of *Paraxis*, a bHLH protein that is highly related to *Scleraxis*, is also regulated by signals from the ectoderm (Sosic et al., 1997).

Significantly, while late ectoderm removal did not result in a complete loss of *Scleraxis* expression, the domain of *Scleraxis* expression was much smaller when compared with the contralateral control limb (Fig. 6D), suggesting an additional role for the ectoderm in stimulating the proliferation of early tendon progenitors. Direct regulation of limb mesenchyme proliferation by signals emanating from the ectoderm has been previously demonstrated (Amthor et al., 1998; Martin and Lewis, 1986). It has further been demonstrated that limbs, when left to mature after complete removal of the dorsal ectoderm, lose all of the dorsal soft tissue (Martin and Lewis, 1986), making it difficult to examine experimentally the specific effects of the ectoderm on the actual generation of limb tendons.

In contrast to the positive influence of the ectoderm, the tendon progenitor pool appears to be limited in the limb by the activity of members of the BMP family. The early *scleraxis* domain in the limb bud is mutually exclusive with the combined expression domains of *Bmp2*, *Bmp4* and *Bmp7*. As such, early *scleraxis* expression in the limb may simply be governed by the diffusion gradient of these secreted signals, such that high circumferential BMP signaling prevents *scleraxis* expression, while lower medial signaling allows *scleraxis* to be expressed. Alternatively, it is possible that a BMP antagonist expressed specifically in the relevant *scleraxis* domain helps to assure the proper *scleraxis* expression pattern. The ability of ectopic Noggin protein to upregulate *scleraxis* expression even within its endogenous expression domain indicates that at least low level BMP signaling does indeed occur in the *scleraxis*-positive domain, presumably by diffusion from surrounding BMP-expressing cells.

While the distal autopod tendon progenitors originate later in development and in a distinct mesenchymal population, they appear to require a similar set of signals for their induction. Although dependence on an ectodermal signal has not been formally demonstrated, the subectodermal location of the 'mesenchymal lamina' and the distal tendon markers suggests that the ectoderm is involved in inducing the distal progenitors. Furthermore, the tendon progenitors are again induced in a domain that is mutually exclusive with the high interdigital BMP expression. Follistatin, produced by the differentiating mesenchymal cells, and Noggin, secreted from the cartilage, may thus be essential for antagonizing this high BMP activity and allowing the subsequent differentiation of tendon progenitors. Interestingly, sensitivity to BMP signaling appears to be lost in the differentiated tendons, as *Bmp4* and the gene for transforming growth factor  $\beta 2$  are themselves expressed in differentiated distal tendons (D'Souza and Patel, 1999; Merino et al., 1998).

In contrast to what is observed in the limb, the intersomitic expression of *scleraxis* was not expanded after Noggin misexpression. Nevertheless, excess BMP signaling can repress the intersomitic expression of *scleraxis*, and has also been shown to exert the same activity in tissue culture cells (Liu et al., 1997). It therefore appears that while BMP signaling may be a general repressor of the early tendon cell fate, restriction of *scleraxis* expression in the primary axis is mediated by other signals.

The limb bud mesenchyme is competent to respond to the *scleraxis*-inducing signals only during a narrow developmental window from stage 21 to 24. By stage 25, the proximal limb

mesenchyme has lost the capacity to respond. However, it is interesting to note that the competence to respond to the same signals is regained by stage 27 or 28 in the distal autopod mesenchyme, resulting in de novo induction of *scleraxis* expression in these cells, and their subsequent commitment to the tendon progenitor cell fate.

### Analysis of *scleraxis* function

*Scleraxis* is a bHLH transcription factor expressed in tendons from the early progenitor stage to the formation of mature tendons. This raises the intriguing possibility that *scleraxis* itself might regulate the tendon cell fate. In preliminary experiments, we have studied the possible role of *scleraxis* in tendon specification by using retroviral misexpression in the chick limb bud. However, massive infection using these retroviruses has not led to any obvious phenotypic consequences: the tendons remain intact and endogenous *Scleraxis* or *Tenascin* expression is not affected (R. S. and C. J. T., unpublished). Alternative methods for analyzing *scleraxis* function include loss-of-function studies in the mouse. However, embryos with a homozygous mutation in *scleraxis* die at E8.5 (Brown et al., 1999), precluding the analysis of any later *scleraxis* functions in these mutants. Therefore, we are now in the process of generating a conditional mutant allele at the *scleraxis* locus in order to directly analyze the role of *scleraxis* in the formation of tendons.

### Tendon morphogenesis is a multistep process

The patterning signals that direct specific muscles to their designated skeletal insertion sites are not known. Considering the tendon as a third participant in the patterning of the musculoskeletal system introduces new challenges to our understanding of this complex patterning process. What are the interactions between these tissues that lead to formation of the final pattern? In agreement with previous studies in the limb using *Tenascin* to mark the tendon progenitors (Kardon, 1998), our data suggest that in both the primary axis and the proximal limb, the tendon progenitors are at early stages spatially associated with the myogenic cells. During subsequent stages, it seems likely, therefore, that the muscles and tendons together identify their proper skeletal insertion sites. This, however, is not a necessary sequence of interactions, as the distal autopod tendons develop first in close association with their cartilage elements and in complete separation from their respective muscles. Only once they are fully formed are these tendon elements connected to the emerging proximal portion of the tendons (Kardon, 1998).

Tendon morphogenesis occurs in a number of discrete steps that coincide with the distinct phases of *scleraxis* expression described herein. Tendon progenitors are first specified in the superficial proximomedial limb mesenchyme at stages 21-24. Coincident with the termination of mesenchymal competence to activate *scleraxis* expression at stage 25 is the second phase of *scleraxis* expression, during which the progenitor cells form specific patterns in response to tissue-specific signals. These signals are probably not derived from the muscle masses since some of the specific patterns of *scleraxis*-positive cells in the mouse can be detected even in limbs of *Pax3* mutant embryos, which lack limb muscles (data not shown). By contrast, the third step, the induction of actual tendon fibers, which is initiated at stage 28, probably does rely on signals from

differentiating muscles, since individuating proximal tendons were not detected in muscleless limbs (Kardon, 1998). Our observation that the induction of excess scleraxis-positive, putative tendon progenitors by application of Noggin protein did not result in the production of extra tendons is consistent with the multiple step model for tendon morphogenesis. Specifically, while excess progenitors were present in these limbs, the subsequent signals required for tendon maturation were not changed, and therefore no new tendons were induced. Instead, the excess scleraxis-expressing cells may have been incorporated into the normal tendon blastemas, which subsequently regulate their cell number, or alternatively, the cells not recruited into the forming tendons may turn off scleraxis expression under the influence of continuing BMP exposure and adopt an alternate fate or undergo apoptosis. Further use of scleraxis as a marker provides the necessary tool for answering these questions in more detail.

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