

# Muscle and tendon morphogenesis in the avian hind limb

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## SUMMARY

The proper development of the musculoskeletal system in the tetrapod limb requires the coordinated development of muscle, tendon and cartilage. This paper examines the morphogenesis of muscle and tendon in the developing avian hind limb. Based on a developmental series of embryos labeled with myosin and tenascin antibodies in whole mount, an integrative description of the temporal sequence and spatial pattern of muscle and tendon morphogenesis and their relationship to cartilage throughout the chick hind limb is presented for the first time. Anatomically distinct muscles arise by the progressive segregation of muscle: differentiated myotubes first appear as a pair of dorsal and ventral muscle masses; these masses subdivide into dorsal and ventral thigh, shank and foot muscle masses; and finally these six masses segregate into individual muscles. From their initial appearance, most myotubes are precisely oriented and their pattern presages the pattern of future, individual muscles. Anatomically distinct tendons emerge from three tendon primordia associated with the major joints of the limb. Contrary to previous reports, comparison of muscle and tendon reveals that much of their morphogenesis

is temporally and spatially closely associated. To test whether reciprocal muscle-tendon interactions are necessary for correct muscle-tendon patterning or whether morphogenesis of each of these tissues is autonomous, two sets of experiments were conducted: (1) tendon development was examined in muscleless limbs produced by coelomic grafting of early limb buds and (2) muscle development was analyzed in limbs where tendon had been surgically altered. These experiments demonstrate that in the avian hind limb the initial morphogenetic events, formation of tendon primordia and initial differentiation of myogenic precursors, occur autonomously with respect to one another. However, later morphogenetic events, such as subdivision of muscle masses and segregation of tendon primordia into individual tendons, do require to various degrees reciprocal interactions between muscle and tendon. The dependence of these later morphogenetic events on tissue interactions differs between different proximodistal regions of the limb.

Key words: muscle, tendon, limb, chick

## INTRODUCTION

In the tetrapod limb the arrangement of over forty muscles in a precise pattern of attachment via tendons to the limb skeleton provides an extraordinary range of motor activity. The proper development of this musculoskeletal system requires the coupled development of three tissues: muscle, tendon and cartilage. How are muscle, tendon and cartilage morphogenesis coordinated and a musculoskeletal system assembled from these tissues during development of the avian limb? In this paper I examine the morphogenesis of muscle and tendon and their relation to cartilage in the developing chick hind limb.

Embryological studies of chick indicate that the limb musculoskeletal system is derived from two mesodermal cell populations (Chevallier et al., 1977; Christ et al., 1977; Kieny and Chevallier, 1979; Ordahl and Le Douarin, 1992); muscle is derived from the lateral dermomyotome of somites adjacent to the limb bud, while tendon and cartilage are derived directly from the limb bud mesoderm. Beginning at st 17 (Hamburger and Hamilton, 1951), somitic myogenic precursors migrate into the hind limb bud (Hayashi and Ozawa, 1991; Jacob et al.,

1979) and then aggregate and differentiate into dorsal and ventral muscle masses (Hayashi and Ozawa, 1991). Within these muscle masses, segregation of individual, anatomically distinct muscles in a stereotyped temporal sequence and spatial pattern creates the basic muscle pattern (Pautou et al., 1982; Romer, 1927; Schroeter and Tosney, 1991; Wortham, 1948). Concurrently, cartilage cell precursors condense from the limb bud mesoderm and via a process of bifurcation, segmentation and proliferation produce the limb skeleton (Shubin and Alberch, 1986). Morphogenesis of tendon is the least understood aspect of musculoskeletal development. From studies of the distal-most tendons, tendon appears to first arise from the limb mesoderm as tendon primordia which subsequently divide into individual, anatomically distinct tendons (Hurle et al., 1989, 1990; Ros et al., 1995).

The temporal and spatial relationship between muscle, tendon and cartilage morphogenesis during limb development is unclear. Although mature myotendinous junctions do not begin to form until st 34 (Tidball, 1989), developing muscle and tendon may be closely associated as early as st 25-26. Studies of normal muscle and tendon development in the chick wing

and leg (Sullivan, 1962; Wortham, 1948) suggest that muscles and tendons individuate in contact and in tandem. However, other studies of mouse (Milaire, 1963) suggest that muscle and tendon initially develop in spatial isolation. The temporal and spatial relationship between developing tendon and cartilage have only been studied in the foot (Hurle et al., 1989, 1990; Ros et al., 1995). These investigations of distal tendon formation suggest that tendons develop separately from their cartilage origin and insertion sites and only attach relatively late (after st 30). Whether this is true for more proximal tendons is unknown.

What mechanisms specify the pattern and coordinate the morphogenesis of muscle and tendon? Quail-chick chimera studies have established that muscle pattern is not autonomously pre-specified within the somitic myogenic precursor cells (Chevallier et al., 1977; Jacob and Christ, 1980; but see Lance-Jones and Van Swearingen, 1995). Instead, muscle appears to be patterned by the somatopleural mesoderm of the limb bud (Jacob and Christ, 1980). Yet it is unclear what specific components of the somatopleure, whether the undifferentiated limb mesoderm, the developing tendons and/or the developing skeleton are the source of patterning information. The role of tendon in specification of muscle pattern was generally rejected because muscle and tendon were thought to develop in spatial isolation and only subsequently connect (e.g. Hauschka, 1994). Conversely, the role of muscle in specification of tendon pattern seemed unlikely in light of experiments showing that the distal tendons of the wing and leg develop in the absence of muscle (Brand et al., 1985; Kieny and Chevallier, 1979; Shellswell and Wolpert, 1977). These observations have lead researchers to dismiss the importance of muscle-tendon interactions in muscle and tendon pattern specification. Instead, Shellswell and Wolpert (1977) suggested that muscle and tendon develop completely autonomously from one another. They proposed that muscle and tendon patterning are ultimately coordinated because of their mutual use of a 'three-dimensional system of positional information', presumably present within the undifferentiated limb mesoderm.

To elucidate the mechanisms that pattern and coordinate muscle and tendon development in the limb, I have taken two approaches. First, I describe the temporal sequence and spatial pattern of muscle and tendon morphogenesis and their relation to cartilage in the developing chick hind limb. Unlike previous descriptions which relied on histological sections, this description is based on specimens doubly-labeled with molecular markers for differentiated myotubes and tendon and analyzed in whole mount with confocal microscopy. With this technique, an integrative description of the morphogenesis of muscles and tendons throughout the entire limb is presented for the first time. The close temporal and spatial association of muscle and tendon morphogenesis suggests that reciprocal interactions may be important for specifying and coordinating muscle-tendon pattern. Second, I test whether muscle-tendon interactions are necessary for correct muscle-tendon patterning or whether morphogenesis of each of these tissues is autonomous. Using experimental manipulations, I have examined tendon development in the absence of muscle and analyzed muscle development in limbs where tendon has been surgically altered. These experiments show that in the avian hind limb the initial morphogenetic events, initial differentiation of myogenic precursors and formation of tendon primordia, occur autonomously with respect to one another.

However, later morphogenetic events do require, to various degrees, reciprocal interactions between muscle and tendon. Interestingly, the dependence of these later morphogenetic events on tissue interactions differs between different proximodistal regions of the limb.

## MATERIALS AND METHODS

### Description of normal muscle-tendon pattern

To describe the normal development of muscle-tendon pattern, 50 chick (*Gallus gallus*) embryos were incubated at 37°C for 5-10 days and killed at stages 24-35 (Hamburger and Hamilton, 1951). Embryos were exsanguinated, eviscerated, and hind limbs separated from the rest of the embryo. The length of each fresh hind limb (from the posterior body-limb junction, parallel to the proximal-distal axis, to the distal limb tip) was measured with an optical micrometer. Limbs were doubly stained in whole mount with the monoclonal antibody F59 to adult myosin fast heavy chain isoforms and the polyclonal antibody HB1 to tenascin to follow muscle and tendon development, respectively, and analyzed on a confocal microscope (see below).

### Coelomic graft surgery

To analyze tendon development in the absence of muscle, 29 early st 16 hind limb buds (donor embryos had 28-30 somites) were isolated from migratory myogenic cells by placing them into st 16 host coelomic cavities. Grafted limbs were harvested at 6-10 days of total development (st 27-35), stained with the antibody F59 to confirm that muscle cells had not invaded the limbs and the antibody HB1 to follow tendon development, and analyzed on a confocal microscope. Twenty seven of the 29 limbs were muscleless.

### Tendon removal surgery

To assess the effect of tendon primordia on the formation of muscle masses, the dorsal proximal tendon primordium was removed from right hind limbs (fresh limb length 1.0-1.5 mm) of 16 st 24-25 embryos. Because of the temporal and spatial stereotypy of tendon development, tendon primordia could be located by comparison with maps of normal tendon development. The vitelline membrane was removed and a small incision in the chorion and amnion made above the surgery site. The tendon primordium and overlying ectoderm were removed using a combination of suction with a 400 µm (inner) diameter glass pipette and cutting with a fine tungsten needle. Limbs were harvested 2-3 days after surgery (st 28-30) by which time the ectoderm had generally healed over the surgery site. To check that the tendon primordium was removed, the excised primordium was stained with HB1 to confirm that it contained high levels of tenascin. Also in a small number of cases (5), limbs with excised tendon primordia were stained with HB1 directly after surgery, to confirm that the tenascin-positive primordia were removed.

Two sets of control experiments were also conducted. The effects of the surgical manipulation were assessed by sham removal of the dorsal proximal tendon primordium. The tendon primordium was completely removed as described above and then carefully replaced on 18 limbs. These limbs were harvested 2 days after surgery (st 28-29). The effects of the ectoderm above the tendon primordium were assessed by removing just the ectoderm above the dorsal proximal tendon primordium. Ectoderm was removed by local application via mouth pipette of 2% trypsin in CMF tyrodes and gentle scraping with a fine tungsten needle on 12 limbs. The limbs were harvested 2 days later (st 27-29) by which time the ectoderm had generally healed. All embryos were stained with the antibodies F59 and HB1 in whole mount and analyzed on a confocal microscope.

### Immunocytochemistry of whole mount and sectioned embryos

A modification of Klymkowsky and Hanken's (1991) whole-mount

immunocytochemical technique was used to stain muscle and tendon in whole normal and manipulated chick hind limbs. Limbs were fixed overnight at 4°C with 4% paraformaldehyde and then bleached overnight with Dent's bleach (50% methanol, 10% DMSO, 15% H<sub>2</sub>O<sub>2</sub>). Specimens were washed for 3 hours in PBS, stained overnight at room temperature with primary antibodies (in 5% serum, 20% DMSO), washed for 5 hours in PBS, stained overnight at room temperature with secondary antibodies (in 5% serum, 20% DMSO), washed for 5 hours in PBS, dehydrated, and cleared in Murray's clear (33% benzyl alcohol, 66% benzyl benzoate).

Muscle was stained with the monoclonal antibody F59 to adult myosin fast heavy chain isoforms (provided by FE Stockdale) which labels virtually all embryonic primary myotubes (Miller et al., 1985; Miller and Stockdale, 1986). All specimens (with the exception of st 34-35 limbs) contain only differentiated primary myotubes (Fredette and Landmesser, 1991). Tendon primordia and differentiated tendons were stained with the polyclonal antibody HB1 to tenascin (provided by H R Erickson). The monoclonal antibody M1B4 to tenascin (from the Developmental Studies Hybridoma Bank) gave staining patterns identical to those of HB1.

### Paraffin sections

For a finer level of analysis of normal muscle and tendon development, four st 27-28 embryos were embedded in paraffin, sectioned (either longitudinally along dorsal-ventral planes or along anterior-posterior planes) at 10 µm intervals and stained with F59 and HB1 antibodies. The protocol was similar to the whole-mount protocol except that the antibodies were incubated at 4°C, secondary antibodies were incubated for only 2 hours, and PBS washes were reduced to 1 hour.

### Analysis of muscle-tendon pattern

Muscle-tendon pattern in normal and manipulated chick limbs was analyzed with a Zeiss laser scanning confocal microscope. Specimens were mounted in clearing agent and optically sectioned longitudinally along dorsal-ventral planes at 20 µm intervals. Muscle-tendon pattern was examined on individual, digitally captured sections using a computer program for compositing stacks of sequential sections (developed on the Macintosh computer by M R Johnston). Identification of 18 thigh muscles was aided by descriptions by Romer (1927) and Schroeter and Tosney (1991) and identification of 15 shank and 7 foot muscles was aided by descriptions of Wortham (1948) and Pautou and co-workers (Pautou et al., 1982). Each muscle was identified by its characteristic shape, position, myotube orientation, origin and insertion. Identification of st 28-35 muscles was confirmed by microdissection of cleared specimens on a polarized light dissection microscope.

Anatomically distinct muscles were labeled as either 'identifiable' or 'segregated'. Identifiable muscles are not separate from neighboring muscles, but are distinguished by fibers whose angles are distinct from and discontinuous with neighboring fibers. Segregated muscles are separated from neighboring muscles by at least 10 µm and are physically separable by microdissection.

Tendons were identified by tenascin

staining that was significantly greater than and visibly discontinuous from neighboring tissue. Individual, anatomically distinct tendons were identified by their characteristic shape, position and cartilage attachment. More mature tendons were also distinguishable in whole mount by high concentrations of collagen (revealed by polyclonal collagen I antibodies and birefringent banding) and in paraffin sections by condensations of compact mesenchymal cells. Continuity of tenascin-labeled tendon with muscle constituted muscle-tendon contact, and continuity of tenascin-labeled tendon with cartilage (identifiable in whole mount by tenascin-labeled perichondrium) constituted muscle-cartilage attachment.

### Terminology

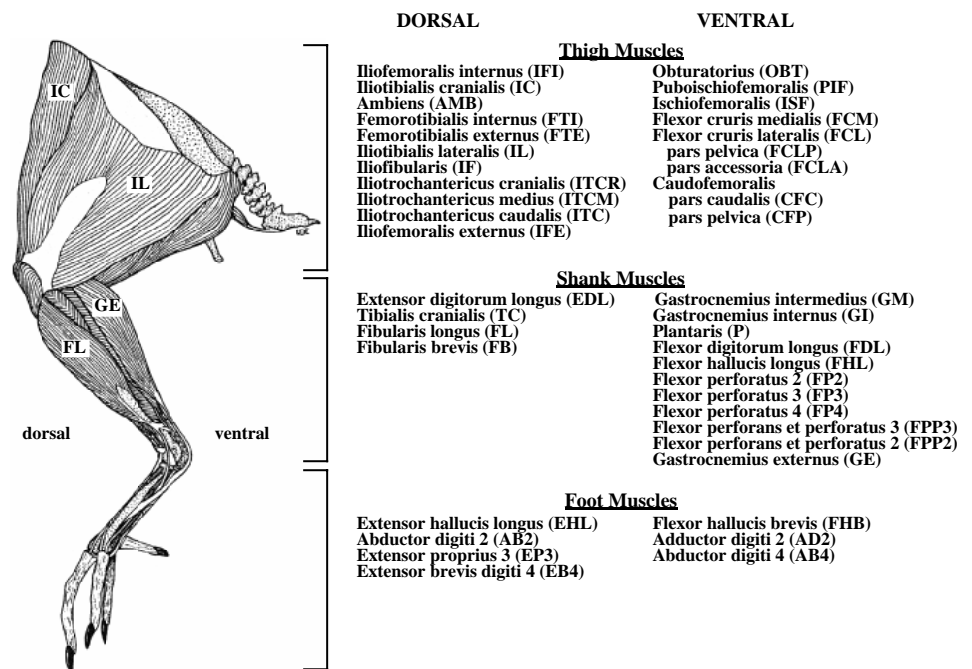
The 40 chick thigh, shank and foot muscles with their tendons examined are listed in Fig. 1. In the thigh, femorotibialis externus includes both lateral and medial parts, iliotibialis lateralis includes both pre- and post-acetabular parts, and puboischiofemoralis includes both medial and lateral parts. In the shank, the small popliteus muscle has not been included. Throughout the text, origin and insertion muscle heads refer to the muscle regions attaching to origin and insertion tendons. Origin tendons are always proximal, and insertion tendons distal.

## RESULTS

### Normal muscle and tendon development

#### Tenascin identifies tendon primordia and individual, anatomically distinct tendons

Tenascin labeled with the polyclonal HB1 antibody (or the monoclonal M1B4 antibody) was found to identify both the early tendon primordia and individual, anatomically distinct tendons arising from these primordia (Figs 2 and 3). This agrees with Hurle and colleagues' (Hurle et al., 1989, 1990; Ros et al., 1995)



**Fig. 1.** Dorsal and ventral muscles of the chicken leg. Names according to the nomina anatomica avium (Baumel et al., 1993). (left) Lateral view of a galliform bird leg (*Dendragapus obscurus*), modified from Hudson (1959).



finding that tenascin is the first identifiable extracellular matrix component of chick distal tendon blastemas and developing tendons. Also, reports by others (Chiquet and Fambrough, 1984; Swadison and Mayne, 1989) show localization of tenascin in the myotendinous junctions of adult chicken tendons.

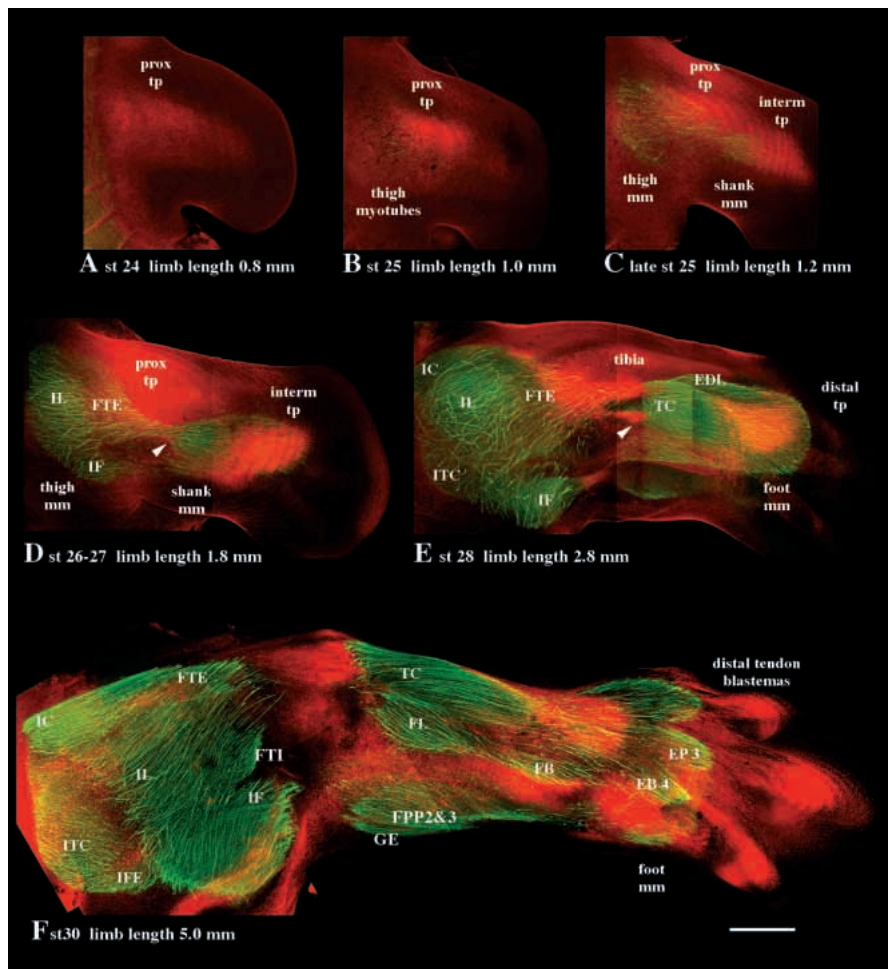
Within the developing limb, ligaments, perichondria of developing cartilages and satellite cells accompanying ingrowing nerves also contain tenascin (Chiquet and Fambrough, 1984; Martini and Schachner, 1991; Wehrle and Chiquet, 1990; Wehrle-Haller et al., 1991) and so may potentially obscure identification of tendon. However, centrally located ligaments and perichondria are readily distinguished from the generally peripherally located tendons. Tenascin-labeled nerves (particularly the fibularis and tibialis nerves) migrate adjacent to tendon primordia (Martini and Schachner, 1991; Wehrle-Haller et al., 1991), but are easily distinguishable from tendon in embryos younger than st 28 by examining stained specimens under polarized light (the collagen ensheathing nerves is birefringent, while immature tendon is not). In limbs older than st 28, most nerves are no longer tenascin positive (in agreement with Wehrle-Haller et al., 1991) and so do not complicate identification of tendon primordia and individuated tendons.

### Three pairs of tendon primordia form in association with the major joints of the hind limb

Between st 24 and 27, three pairs of tendon primordia appear in a generally proximal to distal sequence in the developing hind limb (Figs 2 and 3). One pair, here termed the proximal tendon primordia, appears dorsally and ventrally at the thigh-shank junction (the future knee). The second pair, here termed the intermediate tendon primordia, appears dorsally and ventrally at the shank-foot junction (the future intertarsal joint). The third pair, here termed the distal tendon primordia, appears dorsally and ventrally at the distal end of the foot at the future metatarsal-phalangeal and interphalangeal joints. Proximal and intermediate tendon primordia appear earliest on the dorsal side of the limb (st 24–25). On the ventral side, the intermediate tendon primordia is distinct early (by st 25), being both tenascin rich and morphologically identifiable as a pronounced swelling at the ventral base of the foot. The ventral proximal tendon primordia is not

apparent until st 27–28 (Fig. 3D,E). Distal tendon primordia are first visible at st 28–29 on both dorsal and ventral sides (Figs 2E and 3F).

The spatial relationship between the tendon primordia and the underlying cartilage and superficial ectoderm differs between the three pairs of primordia. Both the dorsal and ventral proximal tendon primordia extend from the ectodermal



**Fig. 2.** Dorsal view of muscle and tendon morphogenesis in the chick hind leg. Each panel is a dorsal projection of a stack(s) of optical dorsal-ventral sections showing only the dorsal side of a right hind limb. Anterior is at the top and distal is at the right of each panel. The F59 antibody to fast MHC is shown in green; the HB1 antibody to tenascin is shown in red. Limb lengths are measured in fixed limbs (approximately 75% of fresh limb length). Scale bar, 400  $\mu$ m. For muscle abbreviations see Fig. 1. (A) St 24. Dorsal proximal tendon primordium is visible in the limb before the differentiation of limb myotubes. Axial myotubes (left) have differentiated by this stage. (B) St 25. Myotubes of the thigh muscle mass are now visible just proximal to the tendon primordium. (C) Late st 25. Intermediate tendon primordium is now visible. The thigh muscle mass appears just proximal to the proximal tendon primordium; the shank muscle mass appears between the proximal and intermediate tendon primordia. (D) St 26–27. Within the shank muscle mass, the origin head of TC with its origin tendon is beginning to form (arrowhead). Within the still unsegregated thigh muscle mass, the orientation of myotubes precedes and predicts later compartments that will individuate into the IL, IF and FTE muscles. (E) St 28. Thigh and shank muscle masses have segregated into anatomically distinct muscles in tandem and in contact to the segregation of their individual tendons of origin and insertion. Arrowhead indicates origin head and tendon of TC. The foot muscle mass is now distinguishable. The dorsal distal tendon primordium is just barely visible, superficial to the metatarsals and distal to the foot muscle mass. (F) Most of the dorsal thigh, shank and foot muscle masses have clearly segregated into individual muscles in contact with their origin and insertion tendons.

basement membrane to the surfaces of the femur, tibia and fibula (Fig. 4A). In contrast, the dorsal and ventral intermediate and distal tendon primordia lie subjacent to the ectodermal basement membrane, but do not extend to the underlying cartilages (Fig. 4A).

The three pairs of tendon primordia also differ in fine scale structure. While tenascin appears to have an amorphous distribution in the proximal tendon primordia, tenascin in the intermediate and distal tendon primordia appears to be highly organized in a meshwork of radial and concentric fibers (Fig. 4B).

#### Thigh, shank and foot muscle masses differentiate in between the three pairs of tendon primordia

While muscle cell precursors populate the limb as the early bud forms (Goulding et al., 1994; Williams and Ordahl, 1994), they do not begin to form myotubes (and express F59) until just after the proximal (on the dorsal side) or intermediate (on the ventral side) tendon primordia can be seen (Figs 2 and 3). Myotubes differentiate in a roughly proximal-distal progression with differentiation of thigh myotubes beginning at early stage 25, shank myotubes at stage 25 and foot myotubes at st 26. The proximal-distal progression of differentiation occurs at approximately the same rate on the dorsal and ventral sides of the limb (dorsal differentiation slightly precedes ventral). In all three muscle-forming regions, muscle cells differentiate adjacent to the tenascin gridwork of tendon primordia (Fig. 4A,C).

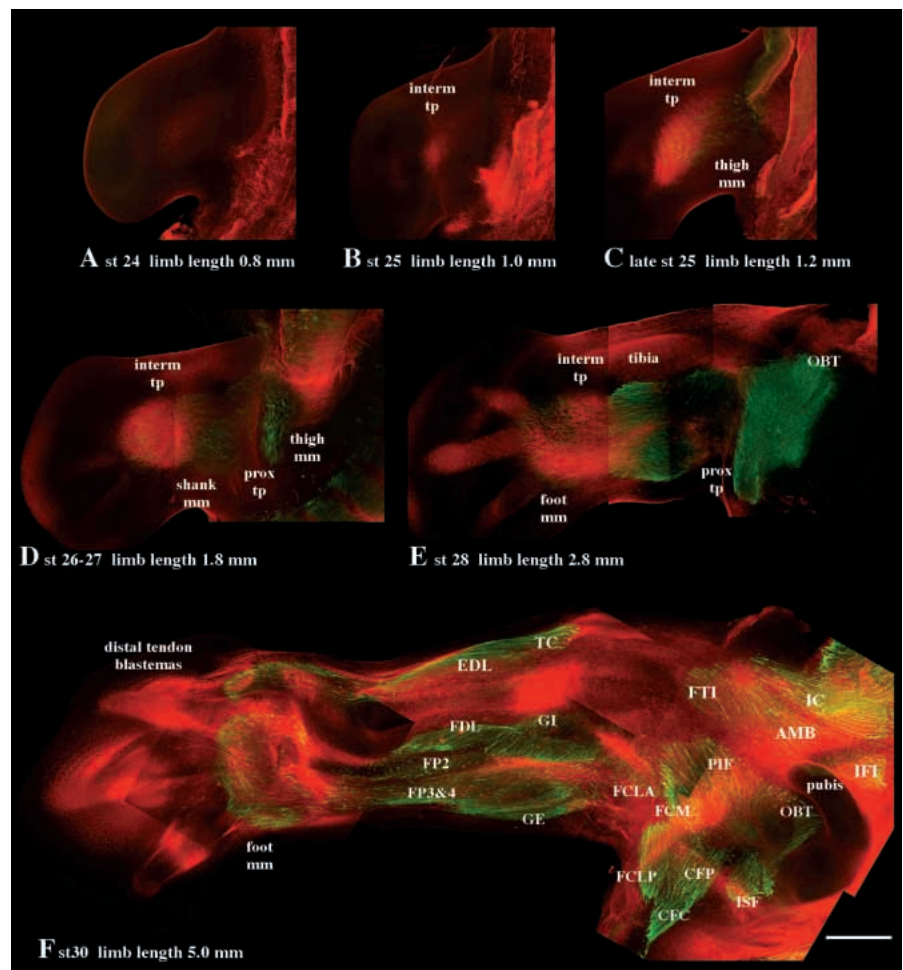
Most strikingly, the muscle masses differentiate in a highly stereotyped spatial pattern with reference to the tendon primordia: thigh myotubes differentiate just proximal to the proximal tendon primordia, shank myotubes form in between the proximal and intermediate tendon primordia, and foot myotubes form in between the intermediate and distal tendon primordia (Figs 2 and 3). This differentiation of myotubes within three particular limb regions bounded by tendon primordia sets up the fundamental division of muscle into thigh, shank and foot muscles. Although bounded by tendon primordia, the thigh, shank and foot muscle masses are initially somewhat continuous. Gradually these connections are lost and most of the muscle masses are spatially isolated from one another by st 30.

The tendon primordia not only initially bound the three muscle masses, but subsequently become the anatomical partners of the individuated thigh, shank and foot muscles. The proximal tendon primordia will give rise to the insertion

tendons of the thigh muscles and the origin tendons of the shank muscles, the intermediate tendon primordia will form the insertion tendons of the shank muscles and the origin tendons of the foot muscles, and the distal tendon primordia will form the insertion tendons of the foot muscles and part of the insertion tendons of shank muscles inserting into the phalanges.

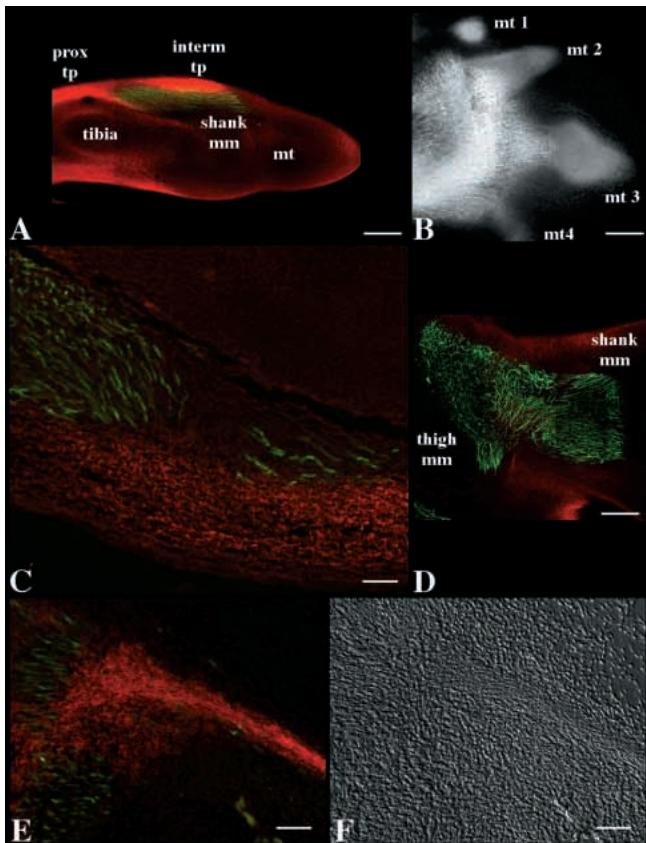
Myotubes initially differentiate aligned within the limb in the 'correct' orientation which presages the fiber direction of future individuated muscles

From their initial appearance, most myotubes are precisely oriented within the limb (Fig. 4D), and their orientation correctly predicts the fiber orientation of the future individuated



**Fig. 3.** Ventral view of muscle and tendon morphogenesis in the chick leg. (A-F) Projections of the ventral side of the limbs shown in Fig. 2A-F. Anterior is at the top and distal is at the left of each panel. The F59 antibody to fast MHC is shown in green; the HB1 antibody to tenascin, in green. Limb lengths are measured in fixed limbs. Scale bar, 400  $\mu$ m. For muscle abbreviations see Fig. 1. (A) St 24. No ventral tendon primordia are visible. (B) St 25. The ventral intermediate tendon primordium appears before the differentiation of limb myotubes. (C) Late St 25. Thigh muscle mass differentiation begins proximal to ventral intermediate tendon primordium. (D) St 26-27. Ventral proximal and intermediate tendon primordia appear with and contact thigh and shank muscle masses. (E) St 28. OBT muscle has segregated from the thigh muscle mass. Shank and foot muscle masses have formed in between the ventral proximal and intermediate tendon primordia, but the distal tendon primordium is not yet visible. (F) St 30. Most of the ventral thigh and shank muscle masses have segregated into individual muscles in contact with their origin and insertion tendons. Ventral foot muscle mass is still unsegregated.





**Fig. 4.** Detailed views of muscle and tendon morphogenesis in the chick hind limb. (A,C-E) The F59 antibody to fast MHC is shown in green; the HB1 antibody to tenascin in red. (A) The spatial relationship between the tendon primordia and the underlying cartilage differs between the different tendon primordia. Longitudinal optical section of st 27 limb (dorsal at top, distal at right) shows that the proximal tendon primordium extends between the ectoderm and the tibia but intermediate tendon lies just subjacent to the ectoderm. mt, metatarsal. (B) Ventral projection (anterior at top, distal at right) of ventral distal tendon primordium. Primordium appears as a highly organized network of radial and concentric fibers. St 29 limb stained in whole mount with HB1. mt, metatarsal. (C) Longitudinal paraffin section (dorsal at top, distal at right) of st 27 limb showing differentiation of shank myotubes (green) adjacent, but not within ventral intermediate tendon primordium (red). (D) Prior to the segregation of thigh and shank muscle masses, individual myotubes are aligned in a distinctive orientation which presages the fiber direction of future individuated muscles. Ventral optical section (anterior at top, distal at right) of st 27 limb stained in whole mount. (E,F) Individuated tendon of IC muscle is rich in tenascin and consists of condensations of flattened mesenchymal cells. Longitudinal paraffin section (anterior at top, distal at right) of st 28 limb viewed with fluorescence (E) and DIC (F). Scale bars (A,B,D) 200  $\mu$ m, (C) 50  $\mu$ m, (E,F) 25  $\mu$ m.

muscles of which the myotubes will be a part. This precise alignment of myotubes is particularly obvious in the thigh where adjacent myotubes may be oriented at 60-90° angles with respect to one another (Fig. 4D). The dramatic differences in myotube orientation in the thigh reflect the later, nearly radial arrangement of adult thigh muscles around the femur (Fig. 1). As a consequence of the early (st 26) distinctive array of myotubes in the thigh, many individual anatomically distinct

muscles are distinguishable long before the thigh muscle mass begins segregating at st 28-29 (Fig. 5). In the shank and foot, most myotubes are aligned initially along the proximal-distal axis, reflecting the generally longitudinal arrangement of adult shank and foot (Fig. 1). As a result, individual muscles in the shank and foot are not as readily identifiable before segregation of shank and foot muscle masses.

Although most myotubes are 'correctly' oriented, there is some imprecision in the orientation of early myotubes. A small percentage (less than 10%) of myotubes in st 26-29 limbs, are incorrectly oriented (Fig. 2E). By st 30 incorrectly oriented myotubes are rarely found.

#### Individual muscles and their origin and insertion tendons emerge in contact and in tandem

Beginning at st 26, muscle masses start to segregate into individual muscles adjacent to and in tandem with the segregation from tendon primordia of their individual origin and insertion tendons (Figs 2, 3, 5). The first indication of muscle mass segregation is the appearance of individual muscle origin and insertion heads (discrete regions from which myotubes appear to radiate; Fig. 2D,E). All individual muscles were found to cleave from adjacent muscle beginning at the muscle's origin or insertion end, in a proximal to distal or distal to proximal progression (in agreement with Schroeter and Tosney, 1991). No muscles were found to cleave from the center of the muscle outwards towards the origin and insertion ends (in contrast to Pautou et al., 1982).

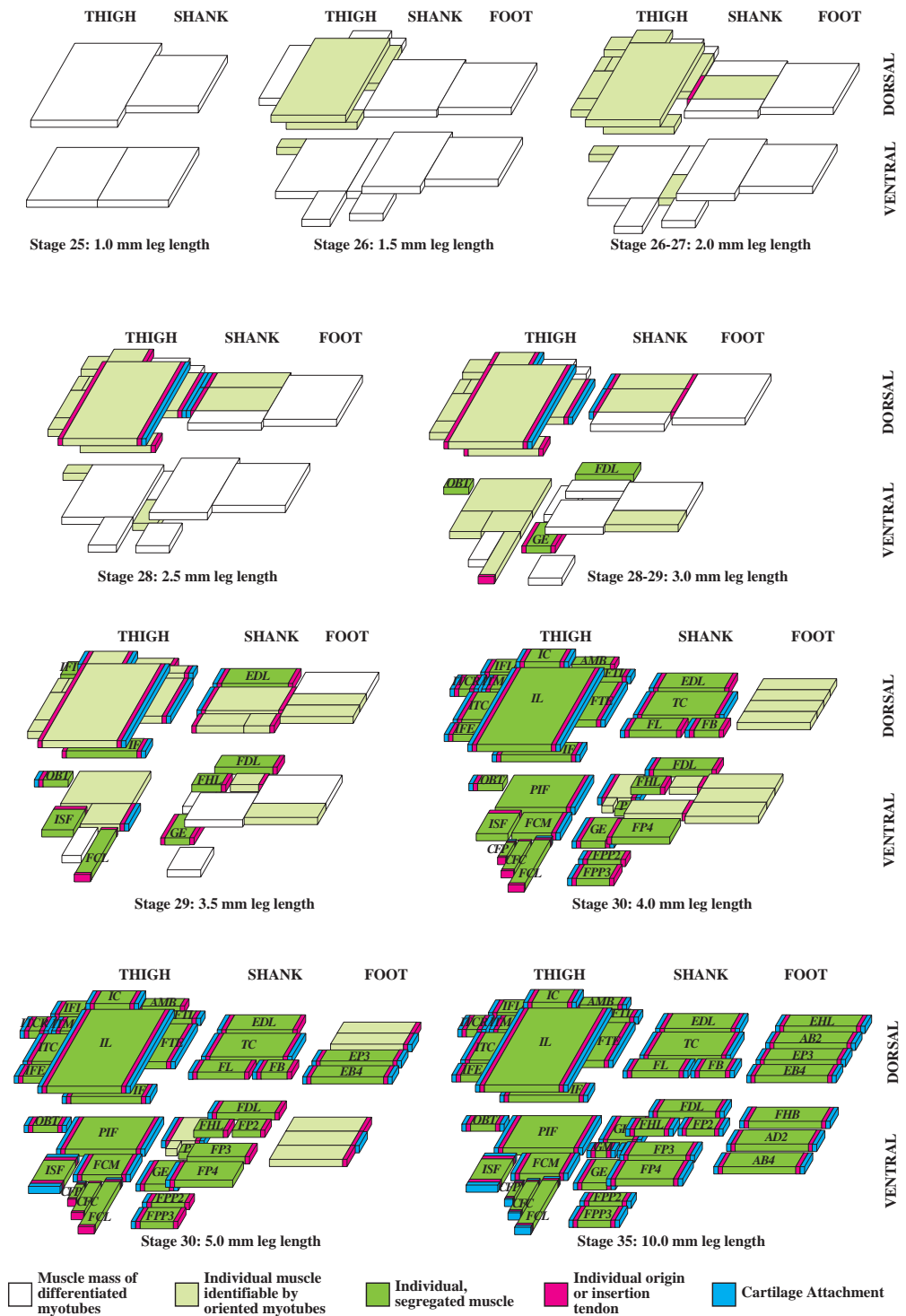
Individual tendons are first distinguishable as discrete regions of tenascin staining adjacent to their forming partner muscle origin or insertion heads (Fig. 2D,E). In paraffin sections, these tenascin-positive tendons are shown to correspond to regions of condensations of mesenchymal cells (Fig. 4E,F). In the case of the distal tendon primordia, individual tendons do not emerge directly from the primordia. Instead, these primordia subdivide into four tendinous blastemas associated with the four digits. At later stages, these blastemas segregate into individual tendons (Figs 2E,F, 3F and 6).

With their adjacent tendons, individual muscles separate from muscle masses between st 28 and 35 in a stereotypic temporal sequence and spatial pattern (summarized in Fig. 5). The sequence and spatial pattern of splitting events agrees with previous studies of the thigh (Schroeter and Tosney, 1991) and shank (Pautou et al., 1982). In general, segregation of muscle masses proceeds in a proximal to distal progression, and individuation of dorsal muscles in the thigh, shank and foot occurs before individuation of comparable ventral muscles. An examination of splitting events within the thigh, shank and foot muscle masses reveals no obvious overall organization to their sequence. However, the segregation of the ventral shank muscles inserting into the phalanges appears to be constrained by the future topography of their distal insertion tendons (Fig. 6C). In the adult, the tendons of flexor digitorum longus are deep (adjacent to bone) and extend to the distal-most phalanges; the tendons of flexor perforatus et perforans 2 and 3 are intermediate and extend to the penultimate phalanges; and the tendons of flexor perforatus 2, 3 and 4 are superficial and extend to more proximal phalanges. During cleavage of the ventral shank muscle mass, flexor digitorum longus individuates first, succeeded by flexor perforans et perforatus 2 and 3 and finally followed by flexor perforatus 2, 3 and 4.

**Distal tendons inserting into the phalanges are derived from multiple sources**

The long distal tendons of the shank and foot muscles inserting into the phalanges are derived from multiple parts (Fig. 6). The proximal parts of these tendons are derived from the intermediate and/or distal tendon primordia. The distal parts are from dorsal and ventral tissue extensions from the metatarsal/phalangeal or interphalangeal joints. These dorsal and ventral extensions are tenascin-positive and appear concurrently with the formation of

their associated joints and joint capsules (Fig. 6B). For foot muscles, the insertion tendons are derived from the distal tendon primordia and the dorsal or ventral extensions from the joints. For shank muscles inserting into the phalanges (extensor digitorum longus, flexor digitorum longus, flexor hallucis longus, flexor perforatus et perforans 2 and 3 and flexor perforatus 2, 3 and 4), the insertion tendons are derived from the intermediate and distal tendon primordia as well as the dorsal or ventral extensions from the joints (Fig. 6B). Initially, these three segments of each tendon



**Fig. 5.** Summary of muscle, tendon and cartilage assembly in the chick hind limb. In all stages, identifiable, but unsegregated muscles (light green) are distinguished by fibers whose angles are distinct from and discontinuous with neighboring fibers; segregated muscles (dark green) are separated from neighboring muscles and are physically separable by microdissection. The presence of identifiable, but unsegregated muscles indicates that a prepattern of anatomical muscles is present in muscle masses before actual muscle mass segregation. In general, segregated muscles separate from their muscle masses in contact and in tandem with their origin and insertion tendons (pink). The timing of tendon attachment to cartilage (blue) relative to muscle individuation varies in different regions of the limb. Attachment of tendons to the phalanges is a late event. Limb lengths are measured in fixed limbs (approximately 75% of fresh limb length).

are separate from one another. Gradually, the tendon segments from the intermediate tendon primordia join with the segments from the distal primordia in the region of the tibial cartilage and the segments from the distal primordia elongate and attach to the dorsal and ventral extensions.

#### Attachment of tendons to their cartilage origin and insertion sites differs between thigh, shank and foot muscles

The last step in the assembly of muscle and tendon is the attachment of tendons to their cartilage origin and insertion sites during st 26 to 35 (Fig. 5). The timing and method of tendon attachment to cartilage differs between the thigh, shank and foot muscles. The proximal tendon primordia that give rise to the insertion tendons of thigh muscle and origin tendons of shank muscles extends to the underlying femur, tibia and fibula from the earliest stages that they can be visualized (Fig. 4A). During the process of tendon splitting and muscle cleavage, tendon contact with cartilages is maintained and refined into attachment sites spatially appropriate to their associated muscles.

Shank muscle insertion tendons and foot muscle origin and insertion tendons are not associated with cartilage initially. The distal tendons of shank muscles inserting into the tarsometatarsus (tibialis cranialis, fibularis longus and brevis, plantaris and gastrocnemial muscles), are derived from the intermediate tendon primordia and initially lie subjacent to the ectodermal basement membrane. After individuation of their muscle partners, they extend deeply to the appropriate cartilages. For shank and foot muscles inserting into the phalanges, tendon attachment is delayed in association with the late appearance of distal cartilages. Attachment of these insertion tendons to cartilage results from the connection of two or three initially separate tendon segments, the distal-most of which is attached to cartilage (Fig. 6).

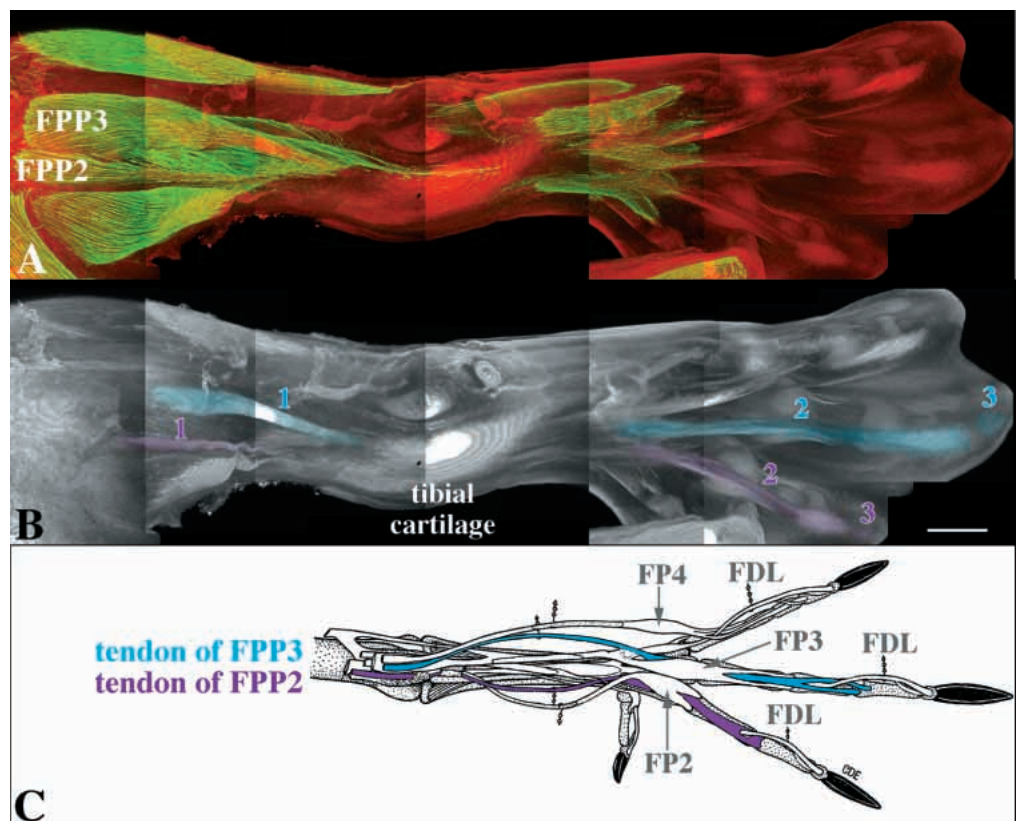
#### Tendon development in muscleless limbs

During normal development, three pairs of tendon primordia form and subdivide in close association with the developing thigh, shank and foot musculature. The myoblasts or differentiating myotubes of the muscle masses may be critical

for establishing the tendon primordia and/or directing the segregation of tendon primordia into individual tendons. To test these hypotheses, tendon development was examined in muscleless limbs. These limbs were produced by grafting young limb buds into the coelom before myoblasts had migrated into them.

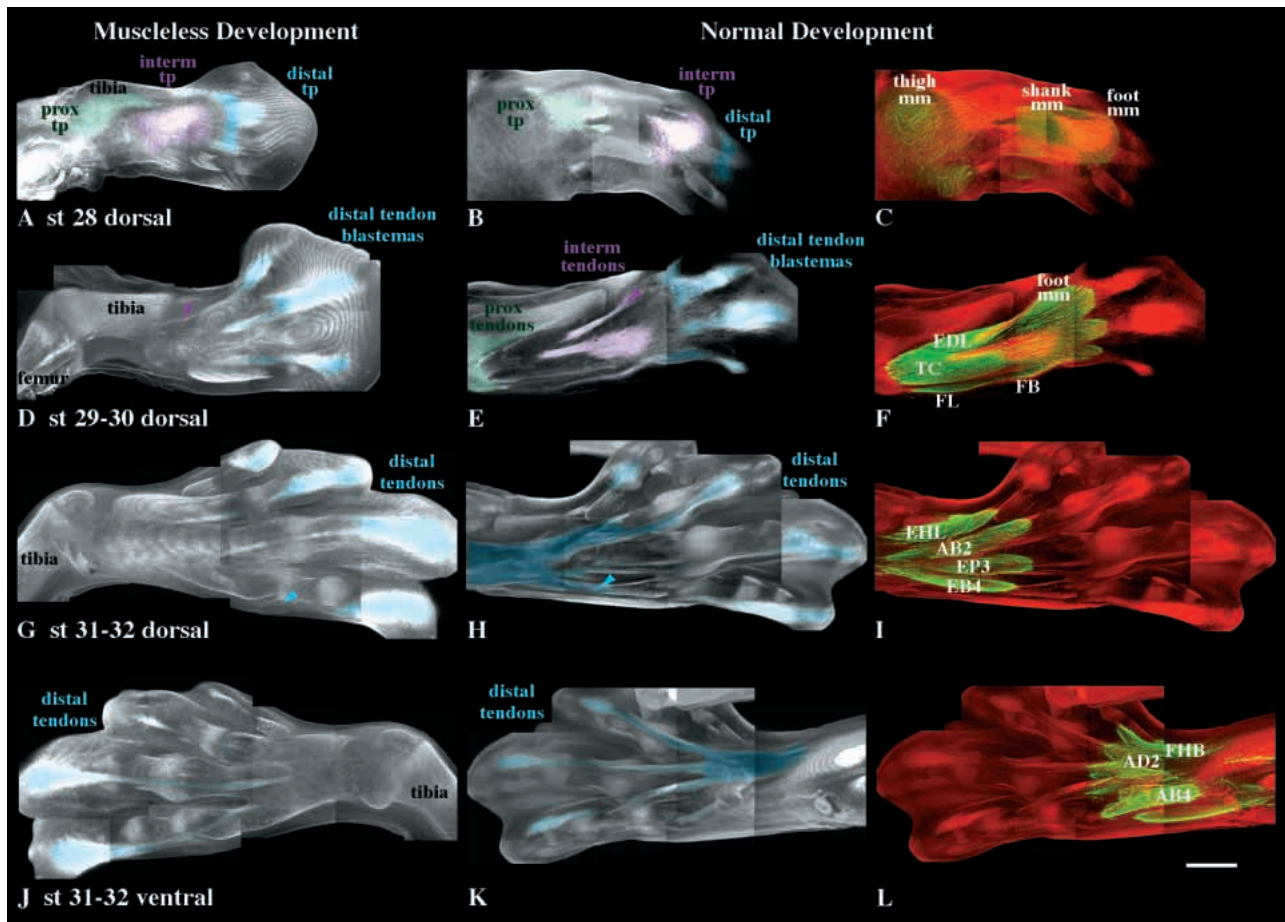
Tendon primordia form autonomously with respect to muscle, but aspects of their subsequent morphogenesis require interactions with muscle

In the absence of muscle, the three dorsal and ventral pairs of tendon primordia appear autonomously in a normal temporal sequence and spatial pattern (Fig. 7A). The tendon primordia appear in a proximal to distal sequence in association with the appropriate joints. In addition, they maintain their characteristic spatial relationship with the underlying cartilage and superficial ectoderm: the proximal tendon primordia extend from the ectodermal basement membrane to the cartilages of the knee, while the intermediate and distal primordia lie subjacent to the ectodermal basement membrane. On a fine scale, the arrangement of tenascin in a



**Fig. 6.** Distal tendons of shank muscles inserting into the phalanges are derived from multiple, disparate sources. (A,B) Ventral projection of shank and foot of st 32 limb. Anterior at bottom and distal at right of each panel. Scale bar, 400  $\mu$ m. (A) The muscles for digits 2 and 3, FPP2 and FPP3, are stained with F59 antibody to fast MHC (green) and their insertion tendons with HB1 antibody to tenascin (red). B shows only HB1 staining and so just shows tendon (tibial cartilage is also heavily labeled). Tendons of FPP2 (pink) and FPP3 (blue), are derived from three parts (labeled 1, 2, and 3). (1) Intermediate tendon primordium, (2) Distal tendon primordium and (3) ventral extension (barely visible) at the base of the second phalange of digits 2 or 3. At this stage parts 1 and 2 of FPP2 are just beginning to connect just distal to the tibial cartilage, while parts 1 and 2 of FPP3 are not yet connected. (C) Ventral tendons of the adult galliform foot (tibial cartilage has been removed; modified after Hudson et al., 1959).





**Fig. 7.** Dorsal (A,D,G) and ventral (J) views of tendon development in the absence of muscle in comparison with normal dorsal (B,C,E,F and H,I) and ventral (K,L) tendon and muscle development. Each panel is a projection of a stack(s) of optical sections showing dorsal or ventral views of limb. Anterior is at the top of all panels while distal is at the right in A-I and at the left in J-L. (A,B,D,E,G,H,J,K) show only HB1 antibody to tenascin. C,F,I and L show HB1 in red and F59 antibody to fast MHC in green. Scale bar = 500  $\mu$ m. (A-C) Dorsal proximal (green), intermediate (pink), and distal (blue) tendon primordia form in a normal temporal sequence and spatial pattern in both limbs with (B,C) and without (A) muscles. (D-F) The dorsal proximal (green), intermediate (pink), and distal tendon (blue) primordia segregate into individual tendons at st 29-30 during normal development (E,F). In the absence of muscle, dorsal proximal and intermediate tendon primordia do not individuate, but instead degenerate (D). Possible insertion tendon of EDL muscle has developed in the absence of muscle (pink arrowhead in D, compare with pink arrowhead in E). However, the distal tendon primordium begins the process of segregation as individual tendon blastemas (blue) arise in association with each digit. (G-L) Dorsal (G) and ventral (J) distal tendons in muscleless limbs individuate from distal tendon blastemas in a normal temporal sequence and spatial pattern as compared with limbs with muscle (H,I,K,L). However, the earliest formed tendon, a dorsal tendon associated with digit 4, has already begun to degenerate proximally in the absence of muscle (blue arrowhead in G, compare with blue arrowhead in H).

meshwork of radial and concentric fibers in the distal tendon primordia is maintained in the absence of muscle (data not shown).

The proximal and intermediate tendon primordia do not segregate into individual tendons, but instead degenerate without muscle (Fig. 7D). In 4 st 30-31 limbs, one or two distinct regions of tenascin staining are visible, which may correspond to shank insertion tendons (Fig. 7D). However, these tendons are much less robust than their normal counterparts (Fig. 7E) and their identity as anatomically distinct tendons is uncertain. Unlike in normal development, these tendons are never found at later stages. In general, these results indicate that both the maintenance and subsequent segregation of the proximal and intermediate tendon primordia require muscle.

In contrast, both the dorsal and ventral distal tendon primordia

subdivide autonomously into individual tendons in a generally normal temporal sequence and spatial pattern in muscleless limbs (Fig. 7D,G and J). The distal tendon primordia initially form superficial to all four metatarsals (Fig. 7A). From each primordium, four tendinous blastemas emerge superficial to each digit (Fig. 7D). Subsequently, individual tendons emerge from the tendon blastemas and connect with tendinous extensions arising from the metatarsal-phalangeal and interphalangeal joints (Fig. 7G,J). However, maintenance of distal tendons does require interactions with muscle because in the absence of muscle these tendons gradually degenerate (Fig. 7G).

#### Muscle development in the absence of tendon primordia

The differentiation of myotubes within three particular regions

of the limb bounded by tendon primordia suggests that tendon may be critical for establishing the basic division of muscle into thigh, shank and foot musculature. To test this hypothesis, the dorsal proximal tendon primordium was surgically removed from young limb buds (Fig. 8A-B). During normal development, the dorsal proximal tendon primordium defines the non-muscle region between the thigh and shank muscle masses and later forms the insertion tendons (primarily the patellar tendon) of the thigh muscles and the origin tendons of the shank muscles.

#### Tendon primordia restrict the location of muscle differentiation within the limb

Two days after removal of the tendon primordium (st 28-29), the ectoderm generally healed over the surgery site, but much of the underlying mesoderm stained weakly for tenascin (as compared with the normally high levels of tenascin found here). Three days after surgery (st 30) tendons start to appear in the surgery region, either because the tendon primordium was not completely removed or because of subsequent regulation by the developing limb. One day after surgery (st 27), dorsal thigh and shank muscles are often (4 of 5 limbs, not shown) truncated in the region adjacent to the surgery site, but by st 28 the number, arrangement and fiber orientation of these muscles is normal (16 of 16 limbs; Fig. 8C,E). However, in 9 of 11 st 28-29 limbs, myotubes are also visible in the surgery region between the thigh and shank muscles and superficial to the knee (Fig. 8C as compared with D). No myotubes are normally found in this region. Similar ectopic muscles are present in st 30 embryos (5 of 5 limbs; Fig. 8E as compared with F). Close examination of the distribution of ectopic myotubes and tenascin reveals that ectopic muscle appears in regions with low concentrations of tenascin (Fig. 8G). Ectopic myotubes were not oriented randomly, but instead appeared to be either distal extensions of the thigh femorotibialis externus muscle (Fig. 8C) or a proximal extension of the shank muscle, tibialis cranialis (not shown).

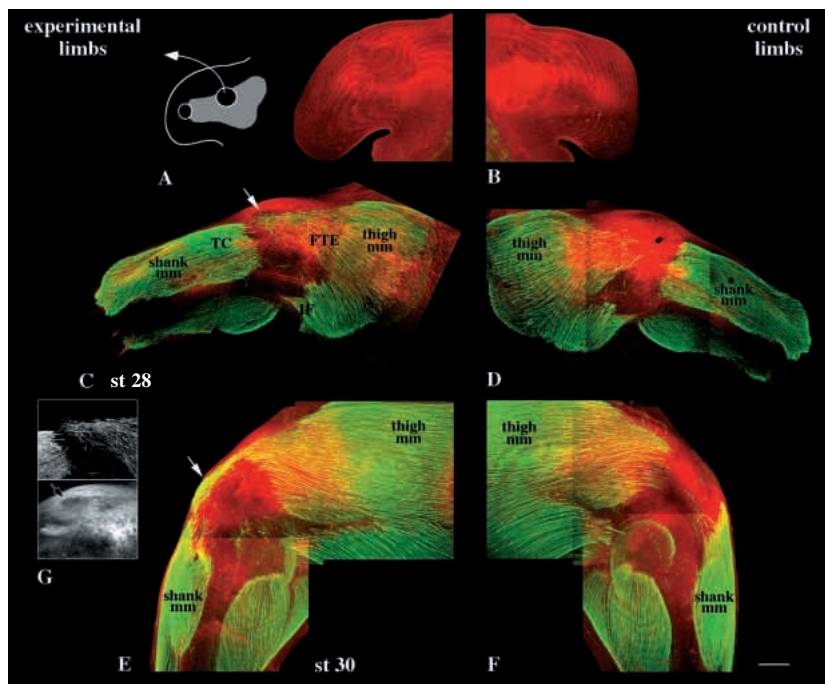
To control for unintended effects due to surgery and from removal of the ectoderm overlying the tendon primordium, two sets of control experiments were performed. Sham removals of the dorsal proximal tendon primordia did not alter muscle pattern (in particular, no ectopic muscles formed) in 8 out of 13 cases. In the remaining 5 cases, some ectopic myotubes were found in between the thigh and shank muscle masses, but these myotubes were generally found in regions where small amounts of mesoderm had been removed accidentally during surgery. These observations suggest that the formation of ectopic muscle is not due to wounding the ectoderm and mesoderm. Removal of only the ectoderm overlying the dorsal tendon primordium also does not alter muscle pattern (in 10 out of 11 cases), eliminating a possible inhibitory effect of ectoderm on muscle differentiation. In both control experiments, tendon pattern is unaffected.

The appearance in experimental, but not in control, limbs of ectopic muscle in the knee region suggests that during normal development the tenascin-rich proximal tendon primordium locally excludes muscle differentiation.

## DISCUSSION

### Formation of anatomically distinct tendons

Analysis of whole mount tenascin-stained limbs indicates that tendon initially appears in the limb as three pairs of primordia located dorsally and ventrally superficial to the knee, intertarsal and metatarsal/phalangeal and interphalangeal joints. These primordia subdivide into individual tendons associated with each of the joints; proximal tendon primordia give rise to the insertion tendons of thigh muscles and the origin tendons of shank muscles, intermediate primordia give rise to the insertion tendons of shank muscles and the origin tendons of foot muscles and distal primordia give rise to the insertion tendons of foot



**Fig. 8.** Dorsal view of muscle development in limbs with the dorsal proximal tendon primordium removed. (A-F) Projections of a stack(s) of optical sections through limbs labeled with HB1 antibody to tenascin in red and F59 to fast MHC in green. A, C and E are experimental limbs; B, D, and F are their contralateral control limbs. (A-B) Limbs at time of surgery. Removal of the proximal tendon primordium is shown diagrammatically (tendon primordia are shown as open circles, myogenic precursors in grey) and on experimental limb (A) as compared with control limb (B). (C-D) Two days after surgery ectopic muscle (arrow) differentiates between the thigh and shank muscle masses, superficial to the knee. This ectopic muscle appears to be a distal extension of FTE muscle. Note that tendons, such as the distal tendon of IF muscle, derived from the dorsal proximal primordium reappear, either because the tendon primordium was not completely removed or because of subsequent regulation by the developing limb. (E-F) Ectopic muscle (arrow) between the thigh and shank muscle masses persists 3 days after surgery. (G) Comparison of ectopic muscle and tenascin levels in experimental limb shown in C. Upper panel shows F59 staining and lower panel HB1 staining. Ectopic muscle appears in a region of low tenascin staining. Scale bar, 250  $\mu$ m (for all images).

muscles and some shank muscles. From the proximal and intermediate tendon primordia, individual tendons appear to emerge directly as tenascin-rich condensations of mesenchymal cells. The distal tendon primordium initially appears as a wide swath (probably equivalent to the mesenchyme lamina of Hurlle et al., 1989, 1990; Ros et al., 1995) of tenascin meshwork superficial to all four metatarsals. From this swath four tendinous blastemas are found (in agreement with Hurlle et al., 1989, 1990; Ros et al., 1995) to emerge superficial to each metatarsal, and subsequently from these blastemas individual tendons appear.

Most individual tendons arise from only one of the three pairs of tendon primordia. However, tendons of shank and foot muscles inserting into the phalanges are derived from two or three sources. For these shank muscles, insertion tendons are derived from the intermediate and distal tendon primordia, as well as from dorsal or ventral tendon extensions from the cartilage insertion sites. These extensions have also been noted by Hurlle and co-workers (Hurlle et al., 1989, 1990; Ros et al., 1995). These insertion tendons are the only tendons in the limb that cross multiple muscles and joints. For foot muscles, insertion tendons are derived from the distal tendon primordium and the dorsal or ventral extensions. The relatively late connection between these disparate segments is the last step in tendon morphogenesis.

### Formation of anatomically distinct muscles

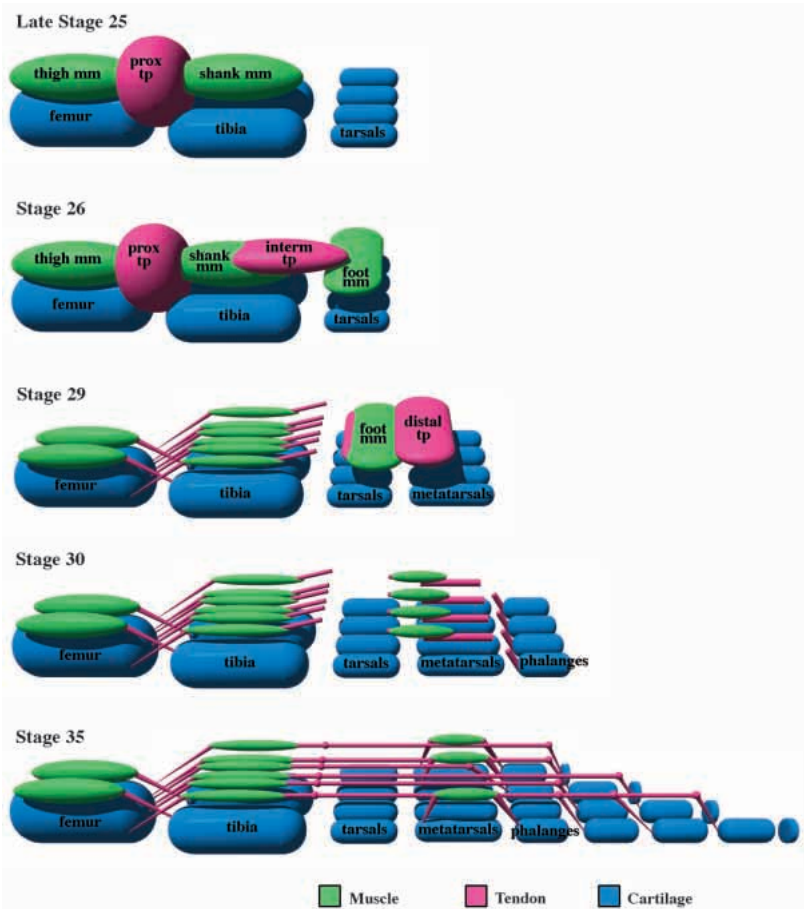
Examination of limbs whole mount antibody stained for differentiated myotubes and tendon reveals two events in muscle morphogenesis not previously recognized from histological studies: (1) in association with tendon primordia, the subdivision of dorsal and ventral muscle masses into thigh, shank and foot muscle masses, and (2) the early alignment of myotubes within the muscle masses.

Muscle patterning begins with the migration and aggregation of myoblasts into the dorsal and ventral regions of the limb. This early division of limb muscle into dorsal and ventral muscle has long been recognized (Romer, 1922). My study reveals that the dorsal and ventral muscle masses are further subdivided as myotubes differentiate. The differentiation of myotubes in between the three dorsal-ventral pairs of tendon primordia results in the formation of thigh, shank and foot muscle masses. This subdivision is a dynamic process; on both dorsal and ventral sides of the limb muscle masses are initially connected, but gradually these connections are lost. Through this subdivision process a dorsal extensor and ventral flexor muscle group become associated with each of the major segments (stylopod, zeugopod, autopod) of the limb.

From their initial appearance within the thigh, shank and foot muscle masses, most myotubes are arranged in a highly structured array and their fiber orientation correctly predicts the fiber orientation of the future individuated muscles of which the

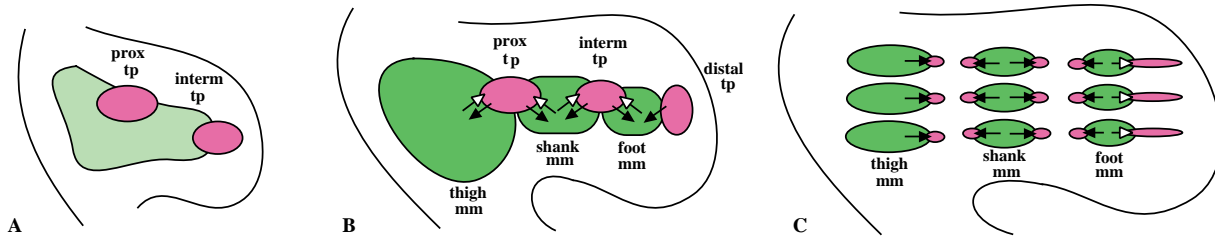
myotubes will be a part. As a result, many individual muscles are distinguishable long before segregation of the muscle masses. Previous studies of limb morphogenesis (Pautou et al., 1982; Romer, 1927; Schroeter and Tosney, 1991; Wortham, 1948) have not reported this early distinctive array of fiber orientations and assumed the muscle masses were homogeneous because fiber orientations were not readily apparent in the transverse histological sections used. However, McClearn and Noden (1988) have also found that in the developing quail head myotubes differentiate in a highly ordered array which presages the future fiber orientation of anatomically distinct muscles.

The final event in the creation of individual, anatomically distinct muscles is the physical segregation of muscle masses. The stereotyped temporal sequence and spatial pattern of segregation of thigh, shank and foot muscle masses (outlined in



**Fig. 9.** Simplified model of muscle, tendon and cartilage assembly in the ventral avian hind limb. Muscle masses and tendon primordia form adjacent to one another (st 25-29) and segregate into individual muscles and tendons in contact and in tandem (st 29-35). While proximal tendons attach to cartilage almost immediately (st 29), distal tendon attachment to cartilage is delayed (st 30-35). The long tendons of shank muscles inserting into the phalanges are derived from three sources: intermediate and distal tendon primordia and extensions from the interphalangeal joints (st 29-35). In the final panel, spheres represent the junction of different tendon sources (in the embryo these junctions are not readily apparent). Note that not all muscles are represented, the proximal tendon primordium appears relatively later during development of the ventral side, and the complexity of tendon attachments to the phalanges is only present on the ventral side.





**Fig. 10.** Model of muscle-tendon interactions during muscle and tendon morphogenesis (dorsal view). Distribution of myogenic precursors is shown in light green, differentiated myotubes in dark green, and tendon primordia and individuated tendons in pink. (A) Tendon primordia form autonomously. (B) Proximal, intermediate and distal tendon primordia establish (black arrows) the boundaries within which myotubes differentiate and thus subdivide the dorsal and ventral (not shown) aggregates of myogenic precursors into thigh, shank and foot masses of myotubes. The muscle masses, in turn, are necessary (white arrows) for maintenance of the proximal and intermediate tendon primordia. (C) Segregation of proximal and intermediate tendon primordia into individual tendons and their subsequent maintenance depends on interactions (black arrows) with muscle. Distal tendon primordium segregates into individual tendons autonomously, but their subsequent maintenance depends on interactions (white arrows) with muscle.

Fig. 5) agrees in general with the previous findings of Schroeter and Tosney (1991) and Pautou and coworkers (1982).

### Muscle, tendon and skeleton assembly in the avian hind limb

This study suggests that the coordination of muscle and tendon morphogenesis generally results from the close spatial and temporal association of these tissues throughout their development (summarized in Fig. 9). Tendon initially appears in the limb as three dorsal and ventral pairs of tendon primordia. Nearly concurrently, muscle masses differentiate in a highly stereotyped spatial pattern in between these tendon primordia. The result is a proximal-distal sequence of alternating tendon primordia and muscle masses. From these muscle masses and adjacent tendon primordia, anatomically distinct muscles and tendons individuate in contact and in tandem. After individuation of muscles and their tendons, mature, functional myotendinous junctions begin to form at st 34 (Tidball, 1989).

For shank and foot muscles inserting into the phalanges, the coordination of muscle and tendon morphogenesis is complicated by the derivation of insertion tendons from both adjacent tendon primordia and distally remote sources. These long insertion tendons are derived from the intermediate and/or distal tendon primordia as well as from dorsal or ventral tendon extensions from the cartilage insertion sites. Development of these muscles with their correct distal tendons not only requires that muscle and adjacent tendon primordia segregate coordinately, but that proximal tendon segments connect appropriately with distal tendon segments.

These observations of the spatial and temporal relationship between muscle and tendon morphogenesis help resolve a controversy in the literature. Histological studies of normal muscle and tendon in the leg and wing, by Wortham (1948) and Sullivan (1962), strongly suggest that muscle and distal tendons individuate in contact and in tandem. However, Milaire's (1963) study of muscle and tendon development in the mouse suggests that certain (distal) tendon blastemas develop in spatial isolation from their muscle bellies. My study finds that the proximal segment of certain distal tendons (tendons of shank muscles inserting into the phalanges) does develop in contact and in tandem with their muscle bellies, while the distal segments develop in spatial isolation from their

partner muscles and only subsequently connect with the muscles via the proximal tendon segments.

In addition to coordination of muscle and tendon morphogenesis, tendon and cartilage development must also be coordinated so that tendons attach to the appropriate cartilage regions. I have found that the timing and mechanism of tendon attachment to cartilage differs between different regions of the limb. Tendons derived from the proximal tendon primordium attach to their cartilage origin and insertion sites nearly concurrently with their formation. This is a consequence of the early connection between the proximal tendon primordium and the underlying cartilages. Subsequent attachment of individual tendons to particular cartilage sites results from refinement of the tendon primordia connection to the cartilages. Tendons derived from the intermediate and distal tendon primordia lie initially subjacent to the ectoderm and only later attach (in many muscles, via connection to distal tendon segments) to the appropriate cartilage sites, many of which form much later in development. This relatively late cartilage attachment of more distal tendons has also been observed by Wortham (1948), Sullivan (1962) and Hurle and coworkers (Hurle et al., 1989, 1990). How tendons attach to the correct cartilage sites is unknown.

### Specification of muscle and tendon pattern in the limb

Both analysis of normal development and experimental manipulations of muscle and tendon provide strong evidence that in the avian hind limb some aspects of muscle and tendon patterning are autonomous with respect to one another, while other steps require reciprocal interactions between the tissues (summarized in Fig. 10).

Beginning at st 24, the tendon primordia appear autonomously with respect to muscle in association with the major joints of the limb. Unlike previous investigations (Brand et al., 1985; Kieny and Chevallier, 1979), this study finds that in muscleless limbs the three dorsal and ventral pairs of tendon primordia develop in a normal proximal-distal temporal sequence in association with the major joints of the limb. In addition, the fine scale structure of the tendon primordia is maintained in the absence of muscle.

After migration into the dorsal and ventral sides of the limb, the subdivision of myogenic precursors into thigh, shank and foot masses of differentiated myotubes appears to be intimately

linked with the tendon primordia. During normal development, masses of myotubes differentiate in between and are bounded by the three pairs of tendon primordia. The generally exclusive pattern of muscle and tendon suggests that the tendon primordia define regions in which myotubes do not differentiate. Experimental manipulations of the tendon primordia provide further support for the role of tendon in regulating the formation of three pairs of muscle masses. Removal of the dorsal proximal tendon primordium results in the formation of ectopic muscle in between the thigh and shank muscle masses. Removal of this tendon primordium presumably allows invasive myogenic precursors to migrate in and differentiate where they normally would not. This ectopic muscle forms despite the likely removal of some resident myogenic precursors. Also, control experiments suggest that the ectopic muscle is not stimulated by tissue wounding or ectodermal removal. Differentiation of ectopic muscle in regions lacking tenascin-labeled tendon primordium suggests that during normal development tendon primordia may locally exclude myotube differentiation.

It is unclear whether exclusionary signals from the tendon primordia are solely responsible for the establishment of proximal and distal muscle mass boundaries. Removal of the dorsal proximal tendon primordium only partially disrupts the segregation of the thigh and shank muscle masses. It is however possible that the surgery may have only partially removed the tendon primordium. Alternatively, the surgery may have completely removed the tendon primordium, but because other factors also inhibit myotube differentiation in this region only a relatively small number of ectopic myotubes differentiate here.

Within the thigh, shank and foot muscle masses, specification of the pattern of individual, anatomically distinct muscles appears to occur early within the muscle masses and does not depend on tendon. Previous researchers (e.g. Pautou et al., 1982; Schroeter and Tosney, 1991; Shellswell and Wolpert, 1977) have suggested that muscle pattern is specified as the muscle masses physically segregate into individual muscles, because segregation of apparently homogenous muscle masses was the first manifestation of the pattern of individual muscles. However, the analysis of normal muscle development suggests that individual muscles are specified earlier within the forming muscle masses. Whole-mount analysis reveals that muscle masses are not homogeneous; within the masses, most myotubes immediately differentiate oriented in a highly structured array which prefigures the array of future individuated muscles. This implies that the patterning information necessary to specify individual muscles is present early in the somtopleural mesoderm. The specific tissue or molecular identity of this patterning information is unknown. Tendon appears unlikely to provide the patterning information because tenascin labeling of the mesoderm reveals no obvious prepatter and individual tendons do not emerge from the tendon primordia until after the initial muscle pattern is established. Potentially, the acquisition of differential *hox* gene expression within the muscle masses may specify muscle pattern. Recently, Yamamoto and colleagues (1998) have found that *hoxa-11* and *hoxa-13* are expressed within specific subregions of the wing muscle masses.

What governs the final segregation of muscle masses into individual, anatomically distinct muscles is unknown, but tendon may play a role. Analysis of normal development shows that muscle masses segregate into individual muscles in contact and in tandem with the segregation from tendon primordia of their

individual origin and insertion tendons. The autonomy with respect to muscle of the segregation of the distal tendon primordia into individual tendons and the close contact of distal tendons with their partner foot muscle indicates that distal tendons, in particular, may guide the segregation of the foot muscle mass.

The importance of muscle interactions for the segregation of tendon primordia into individual tendons differs for different proximodistal regions of the limb. After their formation, the proximal and intermediate tendon primordia, which normally develop in close contact with their partner muscles, require interactions with muscle for their maintenance and subsequent segregation into individual tendons. In the absence of muscle, these tendon primordia do not segregate into individual tendons, but instead degenerate. In contrast, the distal tendon primordia, which develops in spatial isolation from some of their partner muscles (the shank muscles), segregates autonomously into individual tendons, but requires interactions with muscle for subsequent maintenance of these tendons. In the absence of muscle, the distal tendon primordia segregate in a normal temporal sequence and spatial pattern into individual tendons, but these tendons subsequently degenerate. These findings are in agreement with the work of Kieny and Chevallier (1979) on the wing.

### Implications for the neomorphic origin of tetrapod digits

For the past century there has been considerable debate on the evolutionary origin of digits (metacarpals/metatarsals and phalanges) in tetrapod paired appendages (reviewed by Shubin et al., 1997). One group of researchers has argued that the antecedents of digits are present in the fins of sarcopterygian fish, the sister group of tetrapods (Gregory and Raven, 1941; Watson, 1913). In opposition to this hypothesis, others have argued that digits are unique (neomorphic) to tetrapods (Holmgren, 1939, 1933). In recent years, the bulk of developmental, genetic and paleontologic data supports the hypothesis that digits are neomorphic structures (Ahlberg and Milner, 1994; Shubin and Alberch, 1986; Sordino et al., 1995).

The data presented here on muscle and tendon development in the chick hind limb lend support to the hypothesis that digits are evolutionary novelties. Significant differences exist between the morphogenesis of tendons in the thigh and shank and those in the foot. Unlike the more proximal tendons, the distal tendons develop by a two step process; the distal tendon primordia segregate into four tendon blastemas associated with each of the digits and these blastemas in turn subdivide into individual tendons. In addition, unlike the more proximal tendons, distal tendons develop for the most part in spatial isolation from their muscle partners. Furthermore, analysis of muscleless limbs reveals that while the segregation of the proximal and intermediate tendon primordia depends on interactions with muscle, individuation of distal tendons is autonomous with respect to muscle. Finally, distal tendons recently have been shown to differ from more proximal tendons in their molecular identity; distal tendons express the transcription factors *six 1* and *six 2* (Oliver et al., 1995) and the eph-related receptor tyrosine kinase *cek-8* (Patel et al., 1996) while proximal tendons do not. These four observations concur that the morphogenetic processes governing tendon development are quite different in the foot from the rest of the limb. Potentially, this difference reflects the novel evolutionary origin of tetrapod digits.

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