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A *Tcf4*-Positive Mesodermal Population Provides a Prepattern for Vertebrate Limb Muscle Patterning

Gabrielle Kardon,[1,2] Brian D. Harfe,[1,2,3] and Clifford J. Tabin[1,*]
[1]Department of Genetics
Harvard Medical School
200 Longwood Avenue
Boston, Massachusetts 02115

Summary

Naïve myogenic cells migrate from the somites into the developing vertebrate limb, where they simultaneously differentiate into myotubes and form distinct anatomical muscles. Limb signals have been hypothesized to direct the pattern of muscles formed, but the molecular nature of these signals and the identity of the cells that produce them have remained unclear. We have identified a population of lateral plate-derived limb mesodermal cells in both chick and mouse that expresses the transcription factor *Tcf4* in a muscle-specific pattern independently of the muscle cells themselves. Functional experiments in the chick demonstrate that TCF4 and the Wnt-β-catenin pathway in these limb mesodermal cells are critical for muscle patterning. We propose that *Tcf4*-expressing cells establish a prepattern in the limb mesoderm that determines the sites of myogenic differentiation and thus establishes the basic pattern of limb muscles.

Introduction

The vertebrate limb musculature consists of over 40 muscles, with each muscle uniquely identifiable by its position within the limb, size and shape, fiber orientation and type (slow or fast contracting), and points of origin and insertion on bone. The limb muscle derives from migratory precursors originating from the ventrolateral dermomyotome of the somites, while the limb muscle connective tissue, tendons, ligaments, and skeletal elements develop from the lateral plate mesodermal cells of the emerging limb bud (Chevallier et al., 1977; Christ et al., 1977; Ordahl, 1993). Muscle precursors migrate into the limb (st 16 in the chick hindlimb; Hamburger and Hamilton, 1951) and aggregate into dorsal and ventral muscle masses (st 17–24), and these masses subsequently subdivide to form the distinctive pattern of limb muscles (st 25–32; summarized in Kardon, 1998). Concurrently, on a cellular level, muscle precursors undergo a phase of proliferation and then differentiate into mature myotubes (st 25–32). Myoblast differentiation involves the expression of the myogenic regulatory factors (*Myf5* and *MyoD*), withdrawal from the cell cycle, synthesis of muscle-specific proteins (e.g., myosin heavy chain; MHC), and fusion of postmitotic myocytes into multinucleate myotubes (reviewed in Buckingham, 2001). The two processes of muscle pattern formation and cellular differentiation (myogenesis) are thought to be intimately linked because as muscle cells differentiate they are immediately oriented in a highly ordered array that prefigures the pattern of future individuated muscles (Kardon, 1998).

Somites from any axial level retain the ability to give rise to normal limb muscle, indicating that the ventrolateral dermomyotome is undetermined with respect to the pattern of muscles to which it will contribute (Chevallier et al., 1977; Christ et al., 1977). Furthermore, recent single-cell lineage analysis of muscle precursors reveals that individual myogenic cells in the somites or within the proximal limb are not predetermined to form particular anatomical muscles or muscles within specific proximal/distal or dorsal/ventral regions of the limb (Kardon et al., 2002). These studies suggest that myogenic cells are patterned by extrinsic signals after the cells have migrated through the proximal limb.

The muscle patterning information is likely to be provided by other cells within the limb (Jacob and Christ, 1980), although neither the molecular nature nor the specific cellular origin of these signals is clear. On a molecular level, SHH, BMP, FGF, and Notch signaling have been found to regulate muscle differentiation in the limb (reviewed in Christ and Brand-Saberi, 2002; Duprez, 2002; Francis-West et al., 2003). However, their generalized expression patterns in the limb suggest that they cannot straightforwardly determine the elaborate pattern of the individual limb muscles. Instead, this generalized patterning information must be integrated and further refined with other patterning signals to determine muscle pattern. On a tissue level, previous studies have identified that the limb ectoderm, progress zone, and tendons negatively regulate muscle differentiation, thus defining regions in the limb where muscle does not differentiate (Amthor et al., 1998; Kardon, 1998; Robson and Hughes, 1996). Several pieces of evidence have suggested that the muscle connective tissue, which lies adjacent to the muscle, may positively regulate muscle development and play a role in determining where muscle cells differentiate in the limb. First, muscle connective tissue forms normally in the absence of muscle (Grim and Wachtler, 1991; Jacob and Christ, 1980; Lanser and Fallon, 1987), showing that it does not depend on muscle for its patterning and hence could be a source of muscle-patterning information. Second, muscle connective tissue can organize even nonmuscle cells to form muscle-like structures (Grim and Wachtler, 1991). However, the hypothesized role of muscle connective tissue as a source of muscle patterning information has not been tested, and the molecular nature of its potential signaling remains unknown.

To identify signals that might be involved in establishing muscle patterning, we initiated an in situ hybridization screen of known members of various signaling pathways. The Wnt proteins and their downstream effectors were attractive candidates because numerous studies have demonstrated an important role for Wnt signaling

*Correspondence: tabin@genetics.med.harvard.edu
[2]These authors contributed equally to this work.
[3]Present address: Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida 32610.

in axial muscle differentiation (reviewed in Borycki and Emerson, 2000). However, the role of Wnt signaling in limb muscle differentiation and patterning has only recently begun to be studied (Anakwe et al., 2003).

TCF/LEF proteins are the most downstream components of the canonical Wnt-β-catenin signaling pathway. The four members of the TCF/LEF family are transcription factors, each of which contains an 80 amino acid DNA binding high mobility group (HMG) box as well as an N-terminal β-catenin binding domain (reviewed in Hurlstone and Clevers, 2002). In response to the binding of secreted Wnts to Frizzled receptors, β-catenin is stabilized and accumulates in both the cytoplasm and nucleus. β-catenin, in turn, binds TCF/LEF proteins, which activate expression of Wnt-responsive genes.

We have found, in both developing chick and mouse limbs, that *Tcf4* is expressed in the lateral plate-derived limb mesoderm in a muscle-specific pattern and appears to be an early marker of the muscle connective tissue. We show by analysis of muscleless chick and mouse limbs that this *Tcf4* expression in a muscle-specific pattern occurs independently of muscle. Furthermore, experiments in the chick either activating the Wnt-β-catenin pathway or disrupting TCF4 activity demonstrate that TCF4 function in these limb mesodermal cells is important for determining where myogenic precursors differentiate in the limb. Thus, the *Tcf4*-expressing cells set up a prepattern in the limb mesoderm that determines the basic pattern of limb muscles formed.

Results

Lateral Plate-Derived Limb Mesodermal Cells Closely Associated with Muscle Express *Tcf4*

To identify genes that may be involved in limb muscle development, we initially screened the expression of genes implicated in Wnt signaling pathways via in situ hybridization of chick hindlimbs at st 32, when the basic muscle pattern is complete (Kardon, 1998). We found that *cTcf4* is expressed in close association with all limb muscles (Figures 1G–1I). Direct comparison of *cTcf4* expression to myotubes labeled with an antibody against MHC reveals that *cTcf4* is not expressed within myotubes themselves, but only within the lateral plate-derived limb mesoderm directly surrounding myotubes (Figures 1J–1L). Examination of other stages of limb development reveals that *cTcf4* is first expressed in the limb buds at st 19-20 when myogenic precursors expressing Pax7 are entering the limb from the somites (Figures 1A–1C). Between st 24 (Figures 1D–1F) and 32, *cTcf4* is expressed in the limb mesoderm closely associated with the dorsal and ventral muscle masses and remains associated with them as these subdivide into individual muscles, but is not found in the myogenic precursors or differentiated myotubes themselves. By st 34, the muscle connective tissue starts to form, marked by the expression of collagen I (Kieny and Mauger, 1984; Shellswell et al., 1980). At this stage, the cells expressing collagen I appear to be the same population as the *Tcf4*-positive cells associated with muscle (Figures 1N and 1O). In addition, section in situ hybridization analysis of developing mouse limbs reveals that, as in chick, *mTcf4* is similarly expressed in limb mesodermal regions closely associated with muscle, but is not found within the muscle cells themselves (Figures 2C and 2D, and data not shown). Furthermore, comparison of mouse TCF4 protein (labeled with an antibody) with muscle markers demonstrates that cells intimately associated with muscle, but not the myotubes, contain TCF4-positive nuclei (Figure 1M). Thus, we have found in both chick and mouse that *Tcf4* is expressed in the lateral plate-derived mesoderm closely associated with muscle during the process of muscle differentiation and pattern formation.

Tcf4 Is Expressed in Limb Mesoderm in a Muscle-Specific Pattern Independently of Muscle

The intimate association of *Tcf4*-expressing cells with muscle suggests that*Tcf4* expression could be induced in limb mesodermal cells by neighboring myogenic cells, or alternatively could be established independently of muscle. To determine whether *Tcf4* expression is dependent on muscle, we examined *Tcf4* expression in muscleless limbs. In the chick, muscleless limbs were created surgically by removing young limb buds, before migration of myogenic precursors into the limbs, and growing these in isolation from myoblasts within the coelomic cavity of host embryos. Section in situ analysis of *cMyoD* and *cTcf4* expression of st 28 control and coelomic graft limbs shows that the grafted limbs contain no muscle, but maintain a relatively normal *cTcf4* expression pattern, with expression in regions where limb muscle would have been present (Figures 2E and 2F versus Figures 2A and 2B). This result was verified in mouse embryos by taking advantage of the mouse mutant *Splotch-delayed* (Sp^d). Sp^d mice have a point mutation in Pax3 that results in the inability of myogenic precursors to migrate into the limb and the consequent loss of all limb muscles (Franz, 1993; Vogan et al., 1993). Section in situ analysis of *mMyoD* of embryonic day 13.5 Sp^d mouse limbs confirms that they contain no muscle (although body wall muscles remain intact). Comparison of Sp^d limbs with wild-type reveals that *mTcf4* is expressed in a pattern similar to the wild-type expression in limb muscle-associated mesoderm (Figures 2G and 2H versus Figures 2C and 2D). Therefore, in both chick and mouse, *Tcf4* is expressed in the limb mesoderm in a muscle-specific pattern independently of muscle.

The Wnt-β-Catenin-TCF4 Pathway in Limb Mesoderm Is Critical for Determining the Limb Muscle Pattern

The muscle-specific, but muscle-independent, pattern of *Tcf4* expression suggested that *Tcf4* expression may serve as a limb mesodermal prepattern for the limb musculature. To test the role of TCF4 in determining limb muscle pattern, we activated or disrupted the Wnt-β-catenin-TCF4 pathway in the developing chick limb using retroviral or adenoviral vectors. Because *Tcf4* is normally expressed specifically in the nonmuscle cells, we confined misexpression to the lateral plate-derived limb mesodermal cells. To accomplish this, we injected replication-defective vectors, either retroviral RISAP or adenoviral ADENO, which contained constructs either

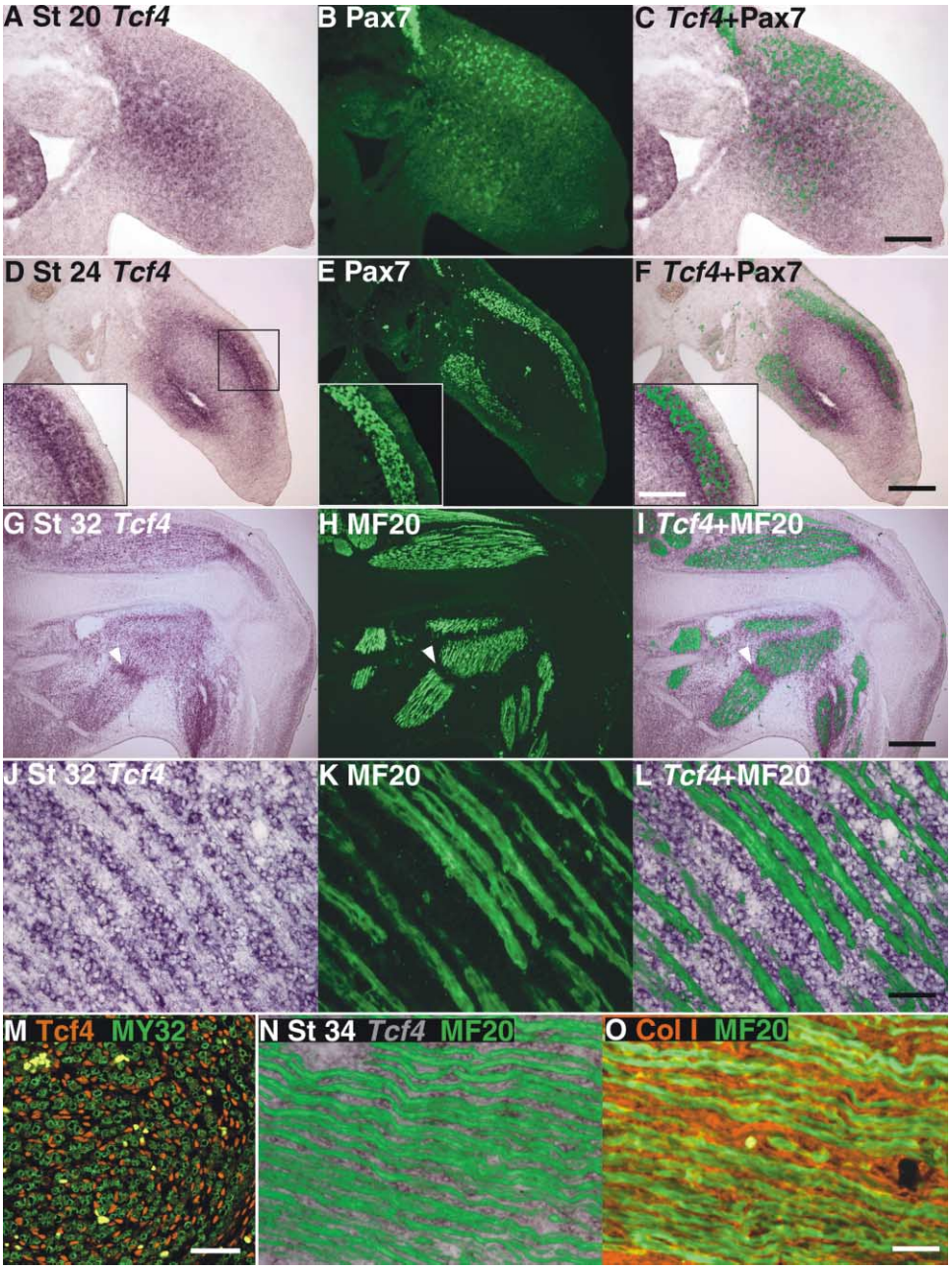

Figure 1. *Tcf4* Is Expressed in the Limb Mesoderm Associated with All Developing Chick and Mouse Limb Muscles, but Not in Myogenic Precursors or Differentiated Myotubes

(A–C, D–F, G–I, and J–L) Single parasagittal sections (dorsal at top) through different stage chick hindlimbs.

(A, D, G, and J) DIC images of *cTcf4* RNA expression (purple) by in situ hybridization.

(B and E) Fluorescent images of Pax7 protein (green) showing myogenic precursors.

(H and K) MHC protein (green) showing all primary myotubes by MF20 antibody labeling.

(C), (F), (I), and (L) are merged views of (A) and (B), (D) and (E), (G) and (H), and (J) and (K), respectively. The box in (D) depicts the area of enlargement shown in (D)–(F). Arrowheads in (G)–(I) show limb muscle connective tissue raphe, which expresses *Tcf4* (and *Scleraxis*; data not shown) but contains no myotubes.

(J–L) Enlargement of flexor cruris lateralis muscle.

(M) Cross-section through day 14.5 mouse quadriceps muscle showing myotubes, labeled by My32 antibody (green), surrounded by TCF4 antibody-labeled nuclei (red). Yellow cells are red blood cells.

(N and O) Adjacent sections showing *cTcf4* expression (purple) and MF20 antibody (green; [N]) or collagen I protein (red) and MF20 antibody (green; [O]). The scale bars in (A)–(C), inserts of (D)–(F), and (M) represent 0.1 mm. The scale bars in (D)–(F) represent 0.2 mm. The scale bars in (G)–(I) represent 0.5 mm. The scale bars in (J)–(L) and (N) and (O) represent 0.05 mm.

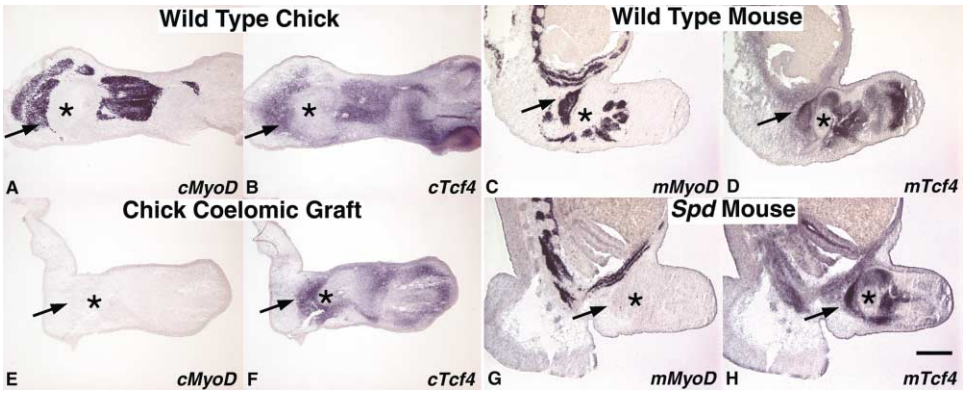

Figure 2. *Tcf4* Is Expressed in the Mesoderm of Chick and Mouse Limbs in a Muscle-Specific Pattern Independently of Muscle

(A, B, E, and F) Adjacent parasagittal sections (anterior at top) through the shank and foot of st 28 control, wild-type (A and B), and muscleless (E and F) chick hindlimbs. *cMyoD* (A and E) and *cTcf4* (B and F) RNA expression by in situ hybridization.

(C, D, G, and H) Adjacent parasagittal sections (anterior at top) through the posterior region of E13.5 day wild-type (C and D) and Sp^d (G and H) mouse embryos. Sections show primarily the shank of the hindlimbs. *mMyod* (C and D) and *mTcf4* (G and H) RNA expression by in situ hybridization. Sp^d mice in (G) and (H) have body wall but no limb muscles, and have an open posterior neural tube. Asterisks in all panels mark the femur. Arrows show the location of femorotibialis muscle in chick (A, B, E, and F) and homologous quadriceps muscle in mouse (C, D, G, and H). The scale bar represents 0.5 mm.

activating or disrupting the Wnt-β-catenin-TCF4 pathway into hindlimb bud mesoderm at st 16 before migration of myogenic precursors into the limb. Because neither virus can spread, misexpression is limited to the lateral plate cells originally infected and their progeny. Using the histochemical tag alkaline phosphatase (AP) contained within the vectors, analysis of the distribution of AP-positive cells at st 32 confirmed that only lateral plate-derived limb mesodermal cells were infected by these viral injections (data not shown). Injection of the RISAP or ADENO vectors alone had no effect on muscle patterning (data not shown).

To test whether TCF4 and the Wnt-β-catenin pathway in the lateral plate-derived limb mesoderm is sufficient to direct muscle formation, we ectopically activated the pathway by infecting the limb mesoderm with RISAP expressing an N-terminal deleted, constitutively activated (act) form of β-catenin. This constitutively activated form of β-catenin both binds and activates TCF4. Cells infected with the RISAP act β-catenin virus were either identified by AP staining or by staining with an antibody (3C2) that recognizes an antigen encoded by the vector. In seven of eight embryos examined with high levels of infection, local regions with high concentrations of AP or 3C2-positive mesodermal cells contained ectopic patches of differentiated myotubes in regions where myotubes normally do not differentiate (Figures 3C and 3D versus Figures 3A and 3B). Such ectopic muscles were found in the dorsal and ventral thigh and shank regions (the foot was not examined), and the density of ectopic myotubes in these regions was roughly equivalent to that in adjacent normal muscle. The boundary of ectopic muscles formed generally corresponded remarkably precisely to the boundaries of limb mesodermal cells infected with virus expressing act β-catenin (Figures 3D and 3F). We also observed (Figure 3G and data not shown) that *Tcf4* expression was upregulated in the limb mesodermal cells infected with the act β-catenin virus (but was not expressed in the neighboring myotubes). We tested whether ectopic activation of β-catenin within muscle precursors and differentiated muscle themselves would affect the pattern of muscle formed. To specifically express act β-catenin in muscle cells, we infected limb level presomitic mesoderm and somites at st 16. Despite high levels of virus in the myotubes, no obvious muscle phenotypes in the hindlimbs ($n = 3/3$) were detected (data not shown). In total, these results indicate that limb mesodermal cells in which *Tcf4* is ectopically upregulated and activated by β-catenin are capable of directing myotube differentiation and the pattern of muscles formed.

To test whether endogenous *Tcf4* activity in lateral plate-derived cells is required for adjacent muscle differentiation and patterning, we misexpressed in these cells an adenovirus containing a dominant-negative (dn) form of *Tcf4* in which just the DNA binding domain is fused to the *engrailed* transcriptional repressor domain. In two of eight embryos examined with high levels of infection with ADENO dn*Tcf4en*, a loss of differentiated myotubes was seen associated with regions of high concentrations of AP-positive lateral plate-derived cells (Figures 3J and 3K versus Figures 3H and 3I). This loss of myotubes resulted in truncated muscles (arrows in Figure 3J versus Figure 3H). In addition, the local lack of myotube formation in small regions with high amounts of virus caused muscles to be misshapen, with myotubes seeming to avoid pockets of infected cells (asterisk in Figure 3J versus Figure 3H). The relatively low efficiency of muscle disruption with the replication-defective adenovirus was expected, as noninfected cells adjacent to infected cells continue to express full-length *Tcf4*. We therefore confirmed this result with replication competent retroviral, RCAS(BP) A and B, versions of this virus, which would lead to widespread limb infection, although not confined to lateral plate derivatives. In seven of nine limbs with high levels of RCAS(BP) A and B dn*Tcf4en*, there was loss of muscle. Strikingly, in six limbs examined (Figure 3M versus Figure 3L), one particular ventral thigh muscle (caudofemoralis pars caudalis) was completely absent. In two limbs, the ventral thigh and shank

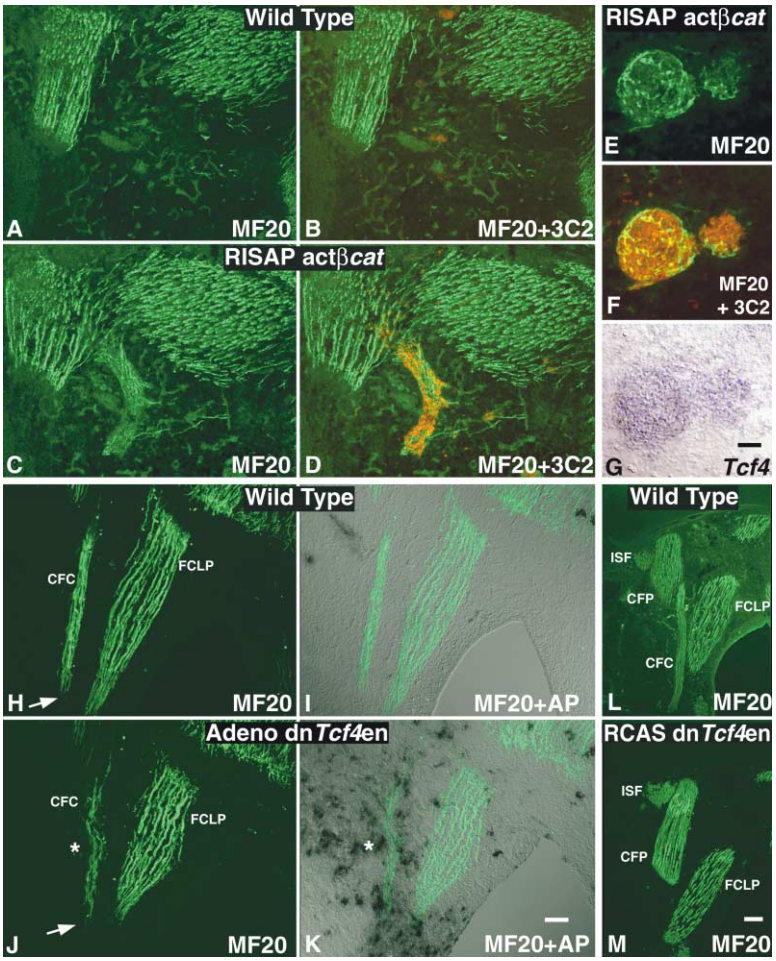

Figure 3. The β-Catenin-TCF4 Signaling Pathway in Limb Mesodermal Cells Is Critical for Myotube Differentiation and Muscle Patterning in the Limb

(A–D) Parasagittal sections through the ventral thigh of st 32 control, contralateral limb (A and B) or through limb with RISAP act β-catenin misexpressed in nonmuscle mesoderm (C and D). In (C) and (D), ectopic muscle is shown, not present in control limb (A and B), induced between two muscles (caudofemoralis pars pelvica, left, and puboischiofemoralis, right).

(E–G) Another ectopic muscle (E) in the pelvis in a region with virally infected cells (F) and *Tcf4* upregulation (G).

(H–K) Parasagittal sections through the ventral thigh of st 32 control, contralateral limb (H and I) or through limb with ADENO dn*Tcf4*en misexpressed in nonmuscle mesoderm (J and K). In (J) and (K), CFC muscle is smaller and misshapen (arrows in [J] versus [H]), with myotubes absent in regions of virally infected cells (asterisk in [J] and [K]).

(L and M) Parasagittal sections through ventral thigh of st 32 control, contralateral limb (L) or through limb with RCAS(BP) A and B dn*Tcf4*en misexpressed in all cells of the limb (M). In (M), CFC muscle is absent.

(A, C, E, H, J, L, and M) Fluorescent images showing MF20 antibody (green), which labels all primary myotubes.

(B, D, and F) Fluorescent images showing MF20 antibody (green) and 3C2 antibody (red), which labels virally infected limb mesodermal cells.

(G) In situ hybridization with *cTcf4* probe (purple).

(I and K) Merged fluorescent and DIC image showing MF20 antibody (green) and AP histochemical staining (black), which indicates virally infected cells. The scale bar in (K) represents 0.1 mm and applies to (A)–(D) and (H)–(K). The scale bar in (E)–(G) represents 0.05 mm. The scale bar in (L) and (M) represents 0.2 mm. In (A)–(D) and (H)–(M), anterior is at top, proximal at left. CFC, caudofemoralis pars caudalis; FCLP, flexor cruris lateralis pars pelvica; ISF, ischiofemoralis; CFP, caudofemoralis pars pelvica.

muscles were smaller and slightly disorganized. It is likely that the ventral muscles are more highly affected than the dorsal muscles because they, in general, form later (Kardon, 1998), and so are more likely to be differentiating in the presence of limb mesodermal cells fully infected by virus. Similarly, the caudofemoralis pars caudalis muscle is probably the most severely affected because it is the last muscle to form in the thigh (Kardon, 1998; Schroeter and Tosney, 1991). To test whether misexpression of the dn*Tcf4en* adenovirus within just myotubes might alter muscle patterning, we also infected limb level presomitic mesoderm and somites with the virus at st 16. Examination of embryos with high levels of viral infection within muscle cells (n = 3/3) revealed no obvious muscle phenotypes (data not shown). Thus, these experiments indicate that *Tcf4* function, specifically in the lateral plate-derived limb mesoderm, is important for establishing the correct location for myotube differentiation.

Discussion

We have identified in both developing chick and mouse a population of lateral plate-derived mesodermal cells that expresses the transcription factor *Tcf4* and lies in close association with all limb muscles. At later stages in the chick, *Tcf4*-expressing cells remain adjacent to muscle and appear to express collagen I, an early marker of muscle connective tissue (Shellswell et al., 1980). These data suggest that the *Tcf4*-expressing population gives rise to the muscle connective tissue of the limb muscles. In the adult, the muscle connective tissue ensheaths muscle fibers (endomysium), binds groups of fibers into fascicles (perimysium), and surrounds whole muscles (epimysium), and is thought to be important for transmitting muscle contractile force (Sanes, 1994). All of these connective tissue sheaths are composed primarily of extracellular matrix consisting of a fibrous collagen network embedded in an amorphous matrix (Light and Champion, 1984). Previous studies analyzing muscle connective tissue development have focused on the expression of extracellular matrix components (Fernandez et al., 1991; Kieny and Mauger, 1984; Shellswell et al., 1980). Because these components are not expressed in the developing connective tissue until late (collagen I is first expressed in chick limb muscle connective tissue at st 35; Shellswell et al., 1980), it has not been possible to study the early development of this tissue. To our knowledge, *Tcf4* is the earliest marker of developing muscle connective tissue and should greatly facilitate

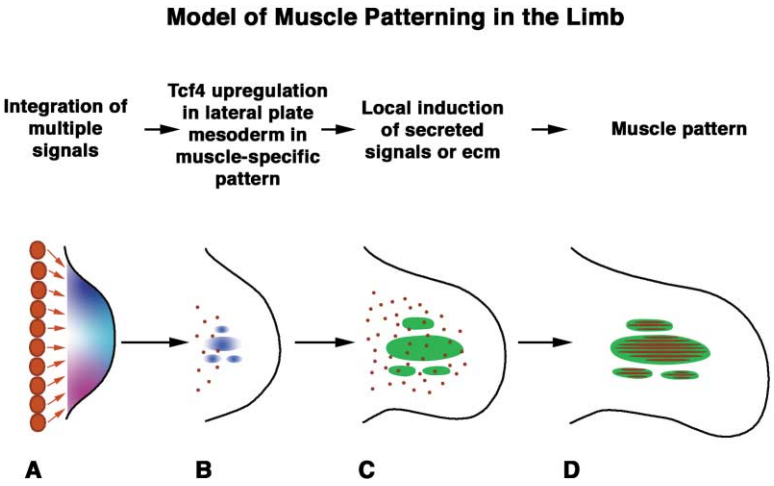

Figure 4. Model of Muscle Patterning in the Vertebrate Limb

(A) Multiple Wnt, Wnt antagonists, and other signals (in pink and blue) are integrated in the limb mesoderm as myogenic precursors (in red) migrate from the somites.

(B) *Tcf4* is upregulated in lateral plate mesoderm in a muscle-specific pattern (in blue).

(C) Secreted signals or extracellular matrix (in green) are locally induced in lateral plate mesoderm in regions expressing Tcf4.

(D) Myotubes (in red) differentiate in the regions expressing high levels of *Tcf4*.

study of the development of this tissue and the musculoskeletal system in general. Future studies will explicitly test whether these *Tcf4*-expressing cells are indeed muscle connective tissue precursors.

Our study strongly suggests that the *Tcf4*-expressing population is important for patterning limb muscles. Consistent with prior studies suggesting a potential patterning function for muscle connective tissue (Grim and Wachtler, 1991; Jacob and Christ, 1980; Lanser and Fallon, 1987), *Tcf4* expression in a muscle-specific pattern in the lateral plate-derived mesoderm is independent of muscle. Moreover, our misexpression studies in the chick demonstrate that TCF4 activity is necessary and β-catenin activity is sufficient in lateral plate-derived cells for specifying where myogenic precursors differentiate and thus the pattern of limb muscle formed. This strongly implicates an endogenous β-catenin-TCF4 pathway in this process. *Tcf4* is also expressed in a muscle-specific pattern, independent of muscle in mouse. An initial analysis of *Tcf4*$^{-/-}$ mice (in collaboration with H. Clevers) has not revealed any obvious limb muscle defects. However, because other members of the *Tcf/Lef* family are expressed in the limb (*Tcf1* and *Lef1*; Oosterwegel et al. 1993 and data not shown), we (in collaboration with H. Clevers) are now in the process of analyzing double mutants of *Tcf4* with other *Tcf/Lef* members.

While TCF4 in the lateral plate mesoderm is critical for specifying muscle pattern, TCF4 alone is not sufficient to determine where myotubes differentiate in the limb. Analysis of normal *Tcf4* expression indicates that some regions expressing *Tcf4* do not locally induce muscle differentiation. Notably, *Tcf4* is also expressed in the tendons, and in regions where *Tcf4* and *Scleraxis*, a tendon marker (Schweitzer et al., 2001), are coexpressed, no muscles are ever found (arrowheads in Figures 1G–1I and data not shown). This likely reflects the fact that TCF4 alone cannot activate transcription, but requires cofactors (reviewed in Hurlstone and Clevers, 2002).

From our study, a preliminary model of muscle patterning in the vertebrate limb emerges. The early limb bud is initially organized by signals defining the dorsal/ventral, proximal/distal, and anterior/posterior axes. Downstream of these early signals, secondary signals (Figure 4A) are integrated to lead to the upregulation of *Tcf4* in discrete regions of the limb mesoderm (Figure 4B). These secondary signals could be Wnts and/or Wnt antagonists, although at present we have found no individual *Wnts* or *Wnt* antagonists expressed in a pattern corresponding to the *Tcf4* expression pattern (unpublished data). Alternatively, *Tcf4* could be upregulated in a Wnt-independent manner. The *Tcf4*-expressing cells signal locally, potentially through secreted inductive factors or via deposition of an instructive extracellular matrix (Figure 4C), to induce myogenic precursors to differentiate into myotubes within these particular regions of the limb (Figure 4D). Each of these local regions of myotube differentiation serves as the nucleus for a future anatomical muscle. Therefore, the pattern of *Tcf4* expression within the limb mesoderm serves as a template that determines the pattern of muscles formed in the limb.

Experimental Procedures

In Situ Hybridization and Immunohistochemistry

Harvested embryos were fixed in 4% paraformaldehyde, washed in PBS, and embedded in OCT or paraffin. In situ hybridization was performed on 20 μm sections as described (Murtaugh et al., 1999), but embryos were not subjected to acid hydrolysis and 1 μg/ml of proteinase K for 10 min was used. Chick *Tcf4* and *MyoD* RNA probes have been described previously (Hartmann and Tabin, 2000; Schweitzer et al., 2001). Mouse *MyoD* and *Tcf4* probes were gifts of M.A. Rudnicki and G. Dressler, respectively (Sassoon et al. 1989; Cho and Dressler, 1998). After section in situ hybridization, antibody labeling was performed as described (Kardon et al., 2002). TCF4 antibody labeling was performed according to Barker et al. (1999). The following antibodies were used: Pax7 and MF20 supernatants at 1:20 (Developmental Studies Hybridoma Bank; DSHB), collagen I at 1:10 (Southern Biotechnology), TCF4 at 1:100 (6H5-3; Upstate Biotechnology), and My32 at 1:125 (Sigma). For virally infected limbs, the extent of viral infection was assessed either by antibody labeling with AMV-3C2 (DSHB; supernatant used at 1:5), which recognizes the viral MA antigen (Logan and Tabin, 1998) or, for the RISAP and ADENO vectors, by AP histochemistry (Kardon et al., 2002).

Chick Surgeries

Muscleless limbs were created by removing st 16 limbs, prior to migration of myogenic precursors into the limbs, and transplanting them into the coelomic cavity of st 16-17 host embryos. In most cases, the transplanted limbs developed with a full complement of bones and were completely vascularized, but contained no muscle (as confirmed by *cMyoD* in situ hybridization).

Misexpression via Retroviral and Adenoviral Vectors

RISAP act β-catenin virus contains an N-terminal deleted *Xenopus* β-catenin that acts as a stable constitutively active variant and is described in Kengaku et al. (1998). RISAP is a replication incompetent viral vector in which the *env* gene is replaced by a human placental alkaline phosphatase expression cassette (Chen et al., 1999). We cloned act β-catenin into pSlires11, an IRES-containing shuttle vector. The IRES transgene inserts were cloned into RISAP downstream of AP, resulting in the expression of both AP and the transgene in the same infected cell. Virus was produced by cotransfecting RISAP vectors with a VSV-G-encoding plasmid into a chicken DF1 cell line as described previously (Chen et al., 1999). RCAS dn*Tcf4en* virus was constructed using the mouse *Tcf4B* splice variant (gift of G. Dressler; Cho and Dressler, 1998), as the chick *Tcf4B* splice variant is expressed in the developing limbs (Figure 1). The dominant-negative (dn) form was created by deleting amino acids 2–50, resulting in the production of a putative dominant-negative protein unable to bind and be activated by β-catenin, but able to bind DNA via its C-terminal HMG box. The repressor domain from the *Drosophila engrailed* gene was fused in-frame to the 3′ end of the dn*Tcf4* construct in the shuttle vector pSlax13 and then subcloned into retroviral vector RCAS(BP) A or B (Logan and Tabin, 1998). The ADENO dn*Tcf4*en was created by subcloning the dn*Tcf4*en insert from pSlax13 into the shuttle vector VQ AD5 RSV K-NpA for targeting into the E1 locus. hPLAP (Fields-Berry et al., 1992) was initially cloned into VQ AD5 RSV, from which a fragment containing RSV hPLAP was digested with BglII and then cloned into E3 2.6delta for targeting into the E3 locus. Adenovirus was produced by Viraquest, Inc., according to Anderson et al. (2000) using VQ AD5RSV dn *Tcf4*en and the new viral backbone containing the RSVhPLAP expression cassette. The adenovirus contained E1 RSV dn*Tcf4*en and E3 RSV hPLAP, so that cells infected with ADENO dn*Tcf4*en expressed both AP and dn*Tcf4*en.

For viral injections, eggs were obtained from SPAFAS and incubated at 37°C to the desired stage. RISAP act β-catenin was injected into the right prospective hindlimb lateral plate or into the hindlimb level presomitic mesoderm and somites at st 16. ADENO dn Tcf4en was injected into the right prospective hindlimb lateral plate successively at st 10 and 16 or was injected into the hindlimb level presomitic mesoderm and somites at st 16. RCASBP (A) and (B) dnTcf4en viruses were coinjected into the right prospective hindlimb lateral plate at st 10. All injected embryos were harvested at st 32.

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