Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development

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Summary

Proper formation of the musculoskeletal system requires the coordinated development of the muscle, cartilage and tendon lineages arising from the somitic mesoderm. During early somite development, muscle and cartilage emerge from two distinct compartments, the myotome and sclerotome, in response to signals secreted from surrounding tissues. As the somite matures, the tendon lineage is established within the dorsolateral sclerotome, adjacent to and beneath the myotome. We examine interactions between the three lineages by observing tendon development in mouse mutants with genetically disrupted muscle or cartilage development. Through analysis of embryos carrying null mutations in *Myf5* and *Myod1*, hence lacking both muscle progenitors and differentiated muscle, we identify an essential role for the specified myotome in axial tendon development, and suggest that absence of tendon formation in *Myf5/Myod1* mutants results from loss of the myotomal FGF proteins, which depend upon *Myf5* and *Myod1* for their expression, and are required, in turn, for induction of the tendon progenitor markers. Our analysis of *Sox5/Sox6* double mutants, in which the chondroprogenitors are unable to differentiate into cartilage, reveals that the two cell fates arising from the sclerotome, axial tendon and cartilage are alternative lineages, and that cartilage differentiation is required to actively repress tendon development in the dorsolateral sclerotome.

Key words: Somite, Syndetome, Sclerotome, Myotome, Tendon, Scleraxis, *Myf5*, *Myod1* (*MyoD*) *Sox5*, *Sox6*, FGF, Mouse

Introduction

The vertebrate axial musculoskeletal system emerges from somites – segmentally arranged, paired blocks of mesoderm that prefigure the metamerism of the spinal column. Lying along either side of the neural tube and notochord, somites bud off as epithelial balls from the anterior end of the presomitic mesoderm, and are patterned, in response to signals from surrounding tissues, into distinct compartments giving rise to different cell lineages. Sonic hedgehog (*Shh*), which is secreted from the notochord and floorplate, instructs the ventral somite to de-epithelialize and form the mesenchymal sclerotome, and to express the transcription factor *Pax1*. The dorsal somite, or dermomyotome, remains an epithelial sheet, dependent for its formation on the secretion of Wnts from the dorsal neural tube and surface ectoderm. Slightly later, cells delaminate from the dermomyotome edges and migrate underneath to form a third compartment, the myotome, located between the dermomyotome and sclerotome. Myotome formation requires the function of the myogenic regulatory factors (MRFs), a conserved family of bHLH transcription factors, the two earliest of which, *Myf5* and *Myod1* (previously known as *MyoD*) are activated by ventral midline *Shh* and dorsal Wnt signaling. As the somite matures, the cells of each compartment differentiate into their respective lineages: from the sclerotome emerges the axial skeleton; from the myotome, the skeletal muscle precursors; and from the dermomyotome, the dorsal dermis and skeletal muscle (Brand-Saberi and Christ, 2000; Brent and Tabin, 2002).

Fate maps, gene expression analyses and generation of mouse mutants reveal that the somitic compartments are further divided into subdomains with unique fates. At the dorsomedial edge or lip (DML) of the dermoyotome, cells migrate underneath to generate the epaxial myotome, which then differentiates rapidly into back muscle. Central dermomyotome cells de-epithelialize to form the dorsal dermis, and at limb bud levels, cells delaminate from the ventrolateral lip (VLL) of the dermomyotome to migrate into the lateral plate mesoderm, where they develop into limb and limb girdle muscle. At interlimb levels, the cells from the VLL of the dermomyotome translocate underneath, producing the hypaxial myotome. The ventrolateral dermomyotome and hypaxial myotome invade the lateral plate mesoderm together as a somitic bud, from which the body wall and abdominal muscle emerge. Finally, within the sclerotome, the ventromedial cells give rise to the vertebral bodies, intervertebral discs and proximal ribs; the lateral cells, to the neural arches and distal ribs; and the dorsomedial cells, to the spinous processes (Brand-Saberi and Christ, 2000; Brent and Tabin, 2002).

In mouse and chick, analysis of the expression of scleraxis (*Scx*), a tendon-specific bHLH transcription factor, has

revealed the presence of a fourth somitic compartment, termed the syndetome, from which the axial tendons emerge (Brent et al., 2003; Schweitzer et al., 2001). Chick-quail chimeras show the tendon progenitors arising within the anterior and posterior dorsolateral sclerotome, in response to fibroblast growth factors (FGFs) secreted from the center of the adjacent myotome (Brent et al., 2003). The FGF signal is received directly, and the response of the sclerotome to it is mediated by the Ets transcription factors *Pea3* (*Etv4* – Mouse Genome Informatics) and *Erm* (*Etv5* – Mouse Genome Informatics) (Brent and Tabin, 2004). Thus, interactions between the somitic muscle and cartilage cell lineages not only lead to establishment of the tendon lineage, but also place the tendon progenitors at the precise junction between the two tissues they must eventually join.

In this study, we sought to determine if axial tendon formation proceeds in mouse by the same mechanisms we observed in chick, making use of previously generated targeted mutations that disrupt development of the different somitic lineages. Our examination of tendon development in mice unable to generate normal muscle or cartilage resulted in novel insights into axial tendon formation that were not evident using gain-of-function approaches in chick. Particularly striking in mouse was the observation that in the absence of cartilage differentiation, there is a progenitor fate switch from cartilage to tendon.

The transcription factors responsible for the specification of muscle and cartilage have been extensively studied. Skeletal muscle development depends upon the activity of the MRFs *Myod1*, *Myf5*, myogenin and *MRF4* (*Myf6* – Mouse Genome Informatics), which are expressed in the myoblasts and function to regulate muscle progenitor specification (*Myf5* and *Myod1*) and differentiation (myogenin and *Myf6*) (Pownall et al., 2002). During somite development, *Myf5* and *Myod1* are activated in all muscle progenitors of the epaxial and hypaxial myotomes, and in the migratory muscle progenitors once they begin differentiating into limb and abdominal muscle. In the mouse myotome, *Myf5* is activated first in the epaxial and hypaxial progenitors, and *Myod1*, ~2 days later, in the differentiated myotome (Pownall et al., 2002).

Myf5 and *Myod1* appear to play largely redundant roles during specification of the muscle progenitors. While mice carrying targeted mutations in either *Myf5*$^{-/-}$ or *Myod1*$^{-/-}$ are born with essentially normal skeletal muscle (Braun et al., 1992; Kaul et al., 2000; Rudnicki et al., 1992), loss of *Myf5* or *Myod1* results in significantly delayed formation, respectively, of the epaxial and hypaxial muscles (Kablar et al., 1998; Kablar et al., 2003; Kablar et al., 1997). Additionally, as *Myf5* is expressed prior to *Myod1*, myogenesis in *Myf5*$^{-/-}$ embryos occurs only upon activation of *Myod1* (Braun et al., 1994). By contrast, *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutants contain almost no muscle progenitors, hence minimal differentiated skeletal muscle (Kassar-Duchossoy et al., 2004; Kaul et al., 2000; Rudnicki et al., 1993).

Interestingly, it appears that the events of specification of the somitic muscle progenitors and onset of myotomal FGF signaling are closely linked: in *Myf5*$^{-/-}$ mutants, expression of the myotomal FGFs is delayed until induction of *Myod1*, and in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutants, expression is never initiated (Fraidenraich et al., 2000; Fraidenraich et al., 1998; Grass et al., 1996). Moreover, the dependency of FGF expression on induction of *Myf5* and *Myod1* appears to be direct. An *Fgf4* myotomal enhancer element has been identified and found to contain E boxes binding *Myf5* and *Myod1*, and it has been shown that these E boxes are required for *Fgf4* expression in the myotome, and that an *FGF4-lacZ* transgene, driven by the myotome-specific enhancer, is not initiated in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ mutants (Fraidenraich et al., 2000; Fraidenraich et al., 1998).

Specification of the skeletal lineage is also well understood. Within the Sox family of transcription factors, characterized by a high-mobility-group (HMG)-box DNA binding domain, three members, *Sox9*, *Sox5* and *Sox6*, are known to be expressed in all chondroprogenitor cells and chondrocytes, and to play essential roles in chondrocyte differentiation. Analyses of the effect of *Sox9*-null mutations on cartilage elements in mouse chimeras and tissue-specific *Sox9* knockouts show that loss of *Sox9* results in absence of cartilage development, and that *Sox9* is required at the earliest steps of chondrocyte differentiation and mesenchymal condensation formation (Akiyama et al., 2002; Bi et al., 1999; Bi et al., 2001; Healy et al., 1996; Healy et al., 1999; Zhao et al., 1997). Moreover, *Sox9* is required for induction of the two other HMG box transcription factors co-expressed with it, *Sox5* and *Sox6* (Akiyama et al., 2002; Lefebvre et al., 2001; Lefebvre et al., 1998). Analysis of cartilage development in *Sox5* and *Sox6* mutants reveals that these genes play redundant and essential roles: while *Sox5*$^{-/-}$ and *Sox6*$^{-/-}$ mice show mild skeletal abnormalities, *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ double mutants present severe chondrodysplasia and die by E16.5 (Smits et al., 2001). Nonetheless, although there is no overt chondrocyte differentiation, mesenchymal condensations do form, and *Sox9* expression is normal, underscoring the role played by *Sox5* and *Sox6* downstream of *Sox9* (Smits et al., 2001).

Mice carrying mutations in the transcription factors specifying muscle and skeletal development are thus a valuable source of new insight into the course of tendon formation when these tissues are absent. Analyses of targeted mutations in *Myf5* and *Myod1* embryos not only allow us to test the necessity for muscle development, but because activation of the myotomal FGFs in mouse is directly controlled by expression of *Myf5* and *Myod1* in the specified muscle progenitors, the effect of FGF signaling loss on tendon development can be assessed as well. Analysis of *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ mutants further enriches our understanding of tendon progenitor formation and differentiation by allowing us to visualize the effect on tendon development when cartilage development is disrupted.

We show here, through analysis of axial tendon development in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ mutants, that specification of the muscle progenitors is essential for expression as well as differentiation of the earliest markers of the somitic tendon progenitors. We propose that defects in tendon development in the absence of skeletal muscle are probably the result of loss of myotomal FGF signaling, and that the somitic tendon cell lineage thus requires the presence of specified muscle for its induction. Our observations of tendon development in *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ mutant embryos revealed that loss of chondrocyte differentiation results in an expanded somitic tendon progenitor population that, in turn, causes the *Sox9*-expressing mesenchymal condensations to begin expressing tendon markers. The two lineages arising from the sclerotome thus appear to be alternative and mutually exclusive: when differentiation into one cell fate is blocked, the other is adopted.

Materials and methods

Generation of embryos and genotyping

The generation of *Myf5*, *Myod1*, *Sox5* and *Sox6* mutant mice has been described (Kaul et al., 2000; Rudnicki et al., 1992; Smits et al., 2001). We used *Myf5* alleles generated by cre-mediated excision of the *PGK-neo* targeting cassette and exon 1, yielding viable mice with no rib defects (Kaul et al., 2000). Both *Myf5* and *Myod1* mice were re-derived onto a B6 background. To generate homozygous $Myf5^{-/-}$ embryos, $Myf5^{+/-}$ and $Myf5^{-/-}$ mice were interbred; for $Myf5^{-/-}$; $Myod1^{-/-}$ embryos, double heterozygous $Myf5^{+/-}$; $Myod1^{+/-}$ mice were interbred; and for the $Sox5^{-/-}$; $Sox6^{-/-}$ embryos, double heterozygous $Sox5^{+/-}$; $Sox6^{+/-}$ mice were interbred. Embryos were genotyped by southern blot or PCR of yolk sac genomic DNA, as previously described (Kaul et al., 2000; Rudnicki et al., 1992; Smits et al., 2001).

In situ hybridization

Whole-mount and section in situ hybridization were performed as previously described (Brent et al., 2003). For section in situ hybridization, embryos were embedded in paraffin and 10 µm sections were collected. Probes included mouse *Scx* (Schweitzer et al., 2001), mouse *Myod1* (Brent et al., 2003), mouse *Myf5* (full length RT-PCR product: 5′ ACGGGTCTCCCATGGACATGACGGACGGCTGCC-AG and ACGGAATTCTCATAATACGTGATAGATAAGTCTGG), mouse *Fgf4* (a gift from Gail Martin), mouse tendin (image clone 463876), mouse myogenin (a gift from Eric Olson), mouse *Sox9* (a gift from Véronique Lefebvre), mouse *mSox5* (a gift from Véronique Lefebvre), mouse *Sox6* (a gift from Véronique Lefebvre), *lacZ* (a gift from Connie Cepko), mouse collagen XII (gift of Ronen Schweitzer) and mouse *Pea3* (full-length RT-PCR product: 5′ ACGGGTCTC-CCATGGAGCGGAGGATGAAAG and 5′ ACGGAATTCCTAGT-AAGAATATCCACCTCTG).

Immunohistochemistry, Alcian Blue staining and TUNEL labeling

For myosin detection, following in situ hybridization, sections were incubated overnight with AP-conjugated MY32 (1:150; Sigma) and detected with INT/BCIP. Phosphorylated MAPK/ERK was detected with Phospho-p44/42 Map Kinase (Thr202/Tyr204) antibody (diluted 1:500; Cell Signaling Technology #9101), followed by a Cy3-conjugated secondary antibody (Jackson ImmunoResearch). For Alcian Blue staining, paraffin sections were rehydrated, incubated in 3% acetic acid/water for 3 minutes and stained in 3 mg/ml Alcian Blue for 30 minutes. TUNEL labeling was performed on sections using a fluorescein in situ cell death detection kit (Roche) according to manufacturer's specifications.

Trunk cultures

Trunk cultures performed as previously described (Zuniga et al., 1999). For inhibition of FGF signaling, 20 µM SU5402 or an equivalent amount of DMSO was added to culture media and E10 cultures were incubated for 24 hours. For FGF4 bead implants, heparin beads (Sigma) were washed in PBS and soaked on ice for 1 hour in FGF4 protein (Peprotech) (1 mg/ml). Beads were implanted into somites of E10 wild-type or $Myf5^{-/-}$; $Myod1^{-/-}$ embryos. Trunks were placed in culture for 12 hours.

Results

Muscle progenitor specification in the myotome occurs prior to expression of *Scx* in the sclerotome

To place the formation of the syndetome within the context of myogenic specification, we first compared the onset of *Scx* expression to that of *Myf5* and *Myod1*. As previously shown (Brent et al., 2003), axial *Scx* expression can be detected by E10.5 in the anterior thoracic and cervical somites (Fig. 1A), and in the tendon progenitors of the forelimb (Fig. 1A, blue arrow), hindlimb (Fig. 1A, purple arrow) and branchial arches (Fig. 1A, green arrow). A frontal section through the thoracic somites shows *Scx* localized to the sclerotome between adjacent myotomes (Fig. 1I). By comparison, *Myf5*, the first MRF to be activated, is initially activated in the dorsomedial epithelial somite at E8.0, when somitogenesis commences (Ott et al., 1991) – and we observed *Myf5* expression throughout the myotome in all somites by E10.5 (Fig. 1B,J). Because *Myf5* is expressed in the newly formed somite, and *Scx* later, as the somite matures, the appearance of *Myf5* and the concomitant specification of the myotome clearly occur prior to onset of *Scx*. At E10.5, *Myf5* expression was also seen in the muscle progenitors of the branchial arches (Fig. 1B) and in the forelimb (Fig. 1B, blue arrow); however, in contrast to *Scx*, *Myf5* was not yet detectable in the hindlimbs (Fig. 1B, purple arrow), suggesting that the limb tendon progenitors initiate *Scx* prior to specification of their muscle counterparts. At E10.5, over 2 days after induction of *Myf5* (Tajbakhsh et al., 1997), *Myod1* expression was observed throughout the myotomes of the cervical somites (Fig. 1C), and at high levels in the dorsomedial and ventrolateral myotomes of the interlimb somites (Fig. 1C,K). By contrast, at E10.5, *Myod1* was not yet seen in the limb muscle progenitors (Fig. 1C, blue and purple arrows).

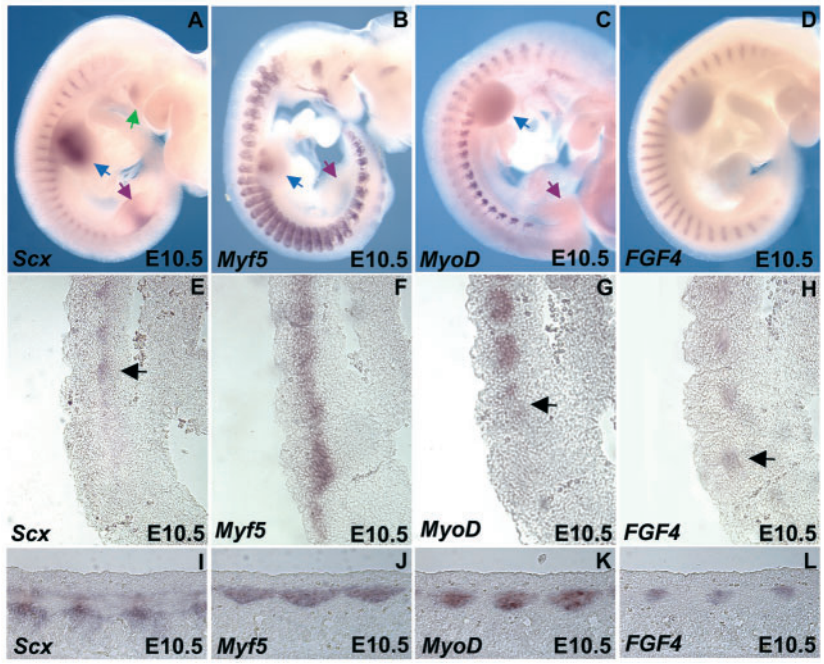

Fig. 1. Somitic muscle progenitor specification occurs prior to induction of *Scx* in the sclerotome. Whole-mount in situ hybridization at E10.5 (A-D) for *Scx* (A), *Myf5* (B), *Myod1* (C) and *Fgf4* (D). (E-L) Section in situ hybridization for *Scx* (E,I), *Myf5* (F,J), *Myod1* (G,K) and *Fgf4* (H, L), on alternate sagittal (E-H) or frontal (I-L) sections, through somites of E10.5 embryos. Blue arrows indicate forelimb buds; purple arrows, hindlimb buds; green arrows, branchial arches. Black arrows in E-H indicate onset of expression.

It has been previously shown that *Myf5* and *Myod1* are required for FGF expression in the mouse myotome (Fraidenraich et al., 2000; Grass et al., 1996), and that FGF signaling is essential for *Scx* induction in chick (Brent et al., 2003). Building on these findings, we decided to compare expression of *Scx* and the myotomal FGFs in mouse. At E10.5, *Fgf4* was observed in the myotomes of all somites anterior to the hindlimb (Fig. 1D). Importantly, onset of both *Scx* and *Fgf4* expression occurred after activation of *Myf5* – hence after myotome formation. To better determine the temporal relationships among the first appearances of *Scx*, *Fgf4*, *Myf5* and *Myod1* in the somites, we compared their expression domains on alternate sagittal sections at E10.5 (Fig. 1E-H). *Myf5* was visible in the myotomes of all somites at E10.5 (Fig. 1F). Interestingly, the onset of *Fgf4* expression in the myotome (Fig. 1H, black arrow) occurred at an earlier somite stage than that of *Scx* in the sclerotome (Fig. 1E, black arrow), consistent with our hypothesized role for the myotomal FGFs in induction of *Scx*. *Myod1* expression in the myotome appeared at the same somite stage as *Scx*, but later than activation of *Fgf4* (Fig. 1G, black arrow), suggesting that *Myod1* may not be required for *Fgf4* induction. A comparison of *Fgf4* expression with that of *Myf5* and *Myod1* on alternate frontal sections of an E10.5 embryo additionally revealed that, like *Fgf8* and *Fgf4* expression in chick, mouse *Fgf4* transcripts were restricted to the center of the myotome, where the myofiber nuclei reside (Fig. 1J-L). A similar expression pattern was noted for *Fgf6* (data not shown).

In summary, our comparisons of *Scx*, *Myf5*, *Myod1* and *Fgf4* confirmed that initiation of the FGFs in the myotome, and of *Scx* in the sclerotome, occur only after *Myf5*-dependent specification of the myotome muscle progenitors takes place. By contrast, *Scx* in the limb is induced prior to expression of the MRFs in the limb muscle progenitors.

In *Myf5* mutant embryos there is a delay in the induction of FGFs in the myotome and *Scx* in the sclerotome

Having determined that *Scx* induction in the somites is initiated after myotome formation and expression of *Myf5*, we asked next whether *Scx* would be expressed normally in the somites of mice carrying null mutations for *Myf5*. *Scx* expression in the somites, limb buds and branchial arches of *Myf5*$^{+/-}$ embryos looked wild type at E10.5 (Fig. 2A), with increased levels at E11.0 (Fig. 2G). By contrast, in E10.5 *Myf5*$^{-/-}$ embryos, while *Scx* expression in the limb and branchial arches still appeared normal (Fig. 2D; blue and green arrows, respectively), it was drastically reduced in the somites, with expression only in the ventrolateral region of the thoracic somites (Fig. 2D, red arrow). At E11.0, while the cervical somites still showed no *Scx*, levels within the interlimb somites, relative to E10.5, appeared to have increased both dorsomedially and ventrolaterally, but less so medially (Fig. 2J). Finally, by E13.5, the pattern and levels of *Scx* expression looked normal (data not shown). Our results thus indicated that *Myf5* is required for timely activation of *Scx* in the somites, but not in the limbs or branchial arches.

As previously shown, *Myf5* is also required during somite development for normal expression of the myotomal FGFs (Fraidenraich et al., 2000; Grass et al., 1996). To determine whether the dynamics of FGF expression would be similar to those of *Scx* in the absence of *Myf5*, we looked at expression of *Fgf4* in *Myf5*$^{-/-}$ embryos. At E10.5, *Myf5*$^{+/-}$ embryos looked identical to wild type (Fig. 2B). By contrast, in *Myf5*$^{-/-}$ embryos, while *Fgf4* expression in the apical ectodermal ridge (AER) of the limb buds appeared normal (Fig. 2E, blue arrow), it was remarkably reduced to only the ventrolateral myotomes of the thoracic somites (Fig. 2E, red arrow) – a domain exactly paralleling that of *Scx* in the *Myf5*$^{-/-}$ sclerotomes. By E11.0, while expression of *Fgf4* in littermate controls persisted at the center of the myotome in all somites (Fig. 2H), in *Myf5*$^{-/-}$ embryos, *Fgf4*, like *Scx*, was still absent in the cervical somites (Fig. 2K). Our comparison of *Scx* and *Fgf4* in *Myf5* mutant embryos thus demonstrated strikingly similar patterns of delay in induction followed by rescue of expression over time. Identical results were seen for *Fgf6* (data not shown).

Because *Myf5* and *Myod1* compensate for one another during muscle development, we wanted to examine *Myod1* expression in *Myf5*$^{-/-}$ embryos in order to determine whether gradual rescue of *Scx* and *Fgf4* expression would correlate with

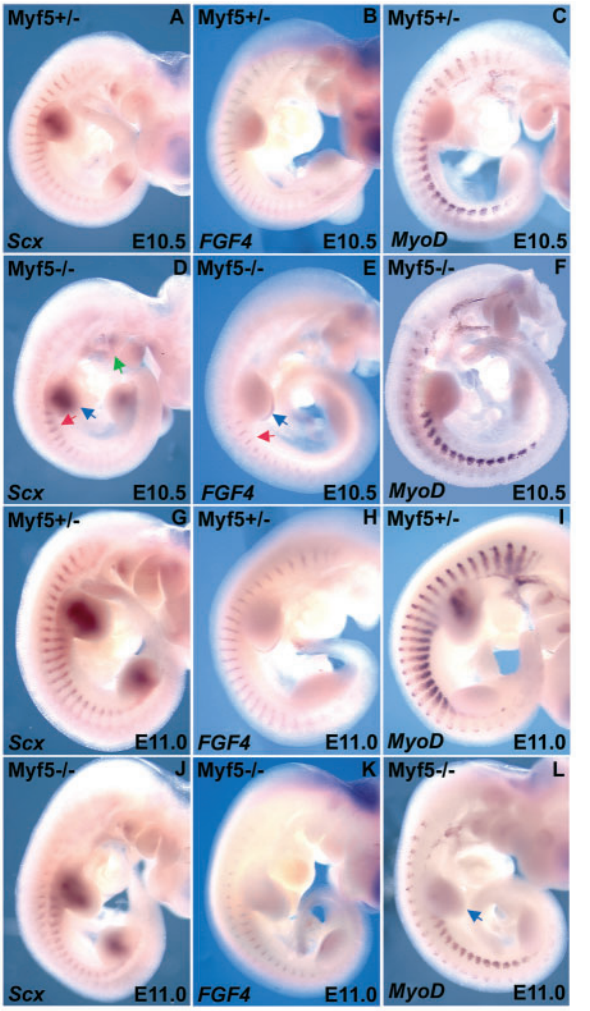

Fig. 2. Induction of *Scx* in the somite is delayed in *Myf5*$^{-/-}$ mutant embryos. Whole-mount in situ hybridization for *Scx* (A,D,G,J), *Fgf4* (B,E,H,K) and *Myod1* (C,F,I,L) at E10.5 (A-F) and E11.0 (G-L) on *Myf5*$^{+/-}$ (A-C,G-I) or *Myf5*$^{-/-}$ (D-F,J-L) embryos. Blue arrows indicate forelimb buds; green arrows, branchial arches; red arrows, interlimb somites.

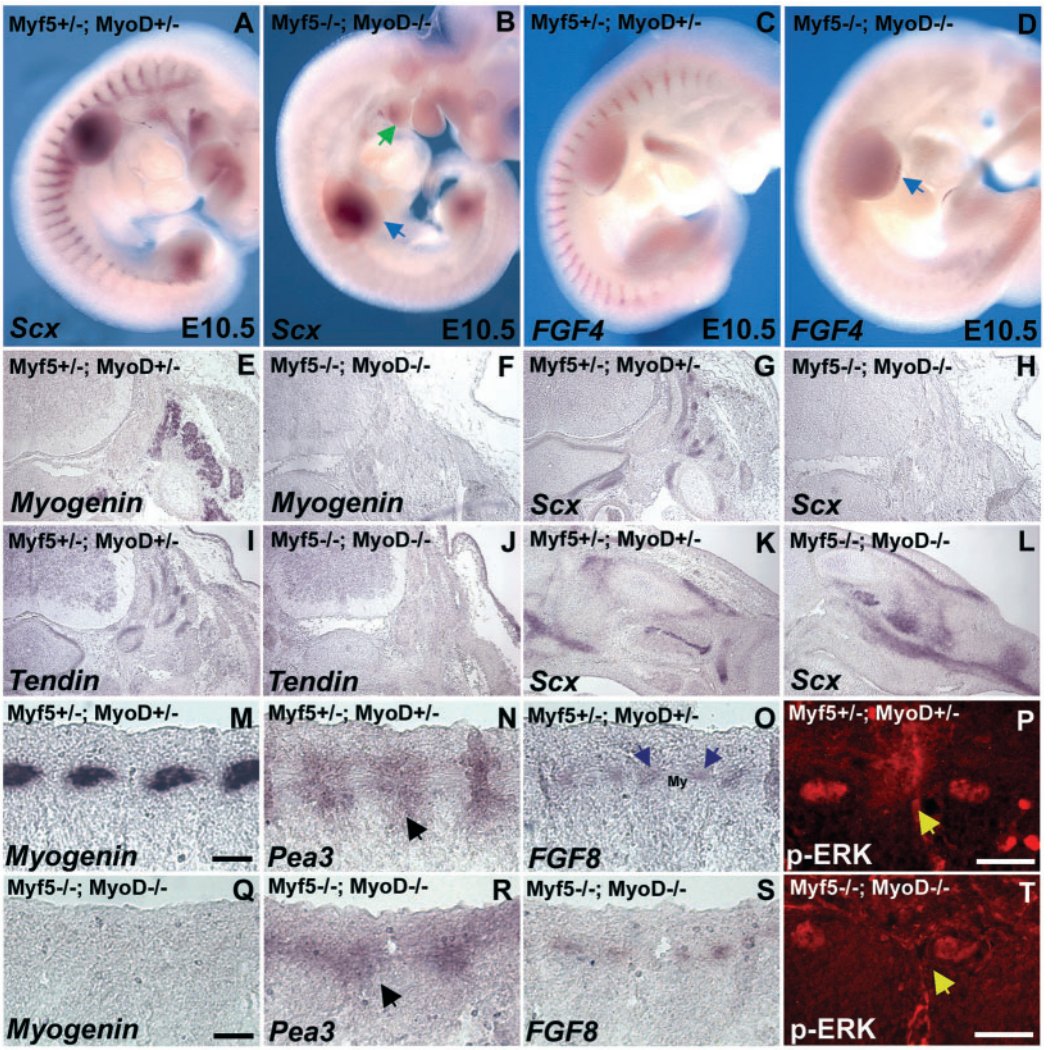

Fig. 3. Axial tendon progenitor specification and differentiation do not occur in the absence of myotome formation. Whole-mount in situ hybridization for *Scx* (A,B) and *Fgf4* (C,D) at E10.5 on *Myf5*$^{+/-}$; *Myod1*$^{+/-}$ (A,C) or *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ (B,D) embryos. Blue arrows indicate forelimb buds; green arrows, branchial arches. Section in situ hybridization for myogenin (E,F), *Scx* (G,H,K,L) and tendin (I,J) on transverse sections of E13.5 *Myf5*$^{+/-}$; *Myod1*$^{+/-}$ (E,G,I,K) or *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ (F,H,J,L) embryos. (E-J) Transverse sections through vertebrae and epaxial muscles; (K,L) sections through forelimbs. Section in situ hybridization for myogenin (M,Q), *Pea3* (N,R) or *Fgf8* (O,S) at E10.5 on *Myf5*$^{+/-}$; *Myod1*$^{+/-}$ (M-O) or *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ (Q-S) embryos. (P,T) Detection of phosphorylated ERK/MAPK on frontal sections at E10.5 on *Myf5*$^{+/-}$; *Myod1*$^{+/-}$ (P) or *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ (T) embryos. Black and yellow arrows in N,P,R,T indicate anterior and posterior sclerotome; blue arrows in O indicate anterior and posterior dermomyotome. Scale bars: 50 μm.

induction of *Myod1*. At E10.5 in the *Myf5*$^{+/-}$ embryos, *Myod1* was expressed throughout the myotome of somites anterior to the forelimb (Fig. 2C). In the interlimb somites, expression was visible throughout the myotome as well, but with strongest levels at the ventrolateral edge (Fig. 2C). It is known that in the absence of *Myf5*, myotome fails to properly form: the *Myod1*-expressing muscle progenitor cells remain trapped along the medial edge of all somites, and along the lateral edge of the interlimb somites. *Myod1* expression remains disrupted in *Myf5* mutants for several days, after which it compensates for *Myf5* loss, and muscle development resumes (Tajbakhsh et al., 1997). Accordingly, at E10.5 in the *Myf5*$^{-/-}$ embryos, we observed *Myod1* expression limited to the medial and lateral edges of the somite, rather than throughout the myotome (Fig. 2F); and while, at E11.0, *Myf5*$^{+/-}$ embryos showed increased *Myod1* expression in the myotome and forelimb (Fig. 2I), *Myf5*$^{-/-}$ embryos at this same stage showed none at the center of the myotome (Fig. 2L) and delayed *Myod1* expression in the forelimb (Fig. 2L, blue arrow). Interestingly, the expression pattern of *Myod1* in *Myf5*$^{-/-}$ embryos closely resembled that of *Scx* and *Fgf4* at both E10.5 and E11.0, lending weight to the likelihood that, in the absence of *Myf5,* induction of *Scx* in the sclerotome and *Fgf4* in the myotome is dependent upon *Myod1* function. By contrast, expression of both *Scx* and *Fgf4* appeared normal in *Myod1*$^{-/-}$ embryos (data not shown), indicating that the later-expressed *Myod1* is dispensable for *Scx* expression as long as *Myf5* is present. It is also worth noting that in mouse, unlike chick, *Fgf8* is not expressed at the center of the myotome but is instead localized to the anterior and posterior edges of the dermomyotome (Fig. 3O, blue arrows) (Crossley and Martin, 1995). Although this domain is spatially consistent with the proposed role of *Fgf8* in the induction of *Scx* within the adjacent sclerotome, *Fgf8* does not appear to depend on *Myf5* or *Myod1* for its expression (Fig. 3S).

Axial tendon progenitor formation and differentiation does not occur in the absence of myotome specification

To determine whether myotome specification is absolutely required for axial tendon progenitor formation, we analyzed tendon development in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos, which form no muscle. At E10.5, *Scx* expression in the somites, limbs and branchial arches of the *Myf5*$^{+/-}$; *Myod1*$^{+/-}$ group was normal (Fig. 3A). In *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos, however, while limb and branchial arch expression looked identical to that of littermate controls (Fig. 3B, blue and green arrows respectively), *Scx* expression in the somites was undetectable (Fig. 3B). As noted for the *Myf5* mutants, loss of somitic *Scx*

paralleled loss of the myotomal FGFs. Thus, while *Fgf4* and *Fgf6* expression was normal in the myotomes of the double heterozygous embryos (Fig. 3C, data not shown), both were virtually absent in the *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutants (Fig. 3D, data not shown), with the exception of some very occasional, faint FGF expression in the ventrolateral myotomes of the anteriormost thoracic somites in one or two embryos (data not shown) – probably a reflection of the residual *Myf6*-dependent muscle development that occurs in *Myf5/Myod1* double mutants (Kassar-Duchossoy et al., 2004). By contrast, expression of *Fgf4* in the AER was unaffected by *Myf5* and *Myod1* loss (Fig. 3D, blue arrow).

We next queried if loss of the *Scx*-expressing tendon progenitors in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ somites would translate into failed tendon differentiation during later development. In the double heterozygous embryos, *Scx* expression at E13.5 was seen marking the maturing tendons attaching the myogenin-expressing epaxial muscle to the vertebrae (Fig. 3E,G). By contrast, in the *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutants, neither myogenin in the epaxial muscle (Fig. 3F) nor, strikingly, *Scx* in the epaxial tendons (Fig. 3H) was detected, indicating failure of both muscle and tendon differentiation in these regions. Interestingly, some muscle development was recently observed (Kassar-Duchossoy et al., 2004) in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutants; we too found myogenin expressed in the intercostal region of E13.5 embryos, along with associated expression of *Scx* in the intercostal tendons (data not shown). We attribute the expression of *Scx* here to the faint and occasional myotomal FGF signaling we observed in the ventrolateral myotomes. In the epaxial region, however, our findings confirmed that where there was no muscle development, there was no expression of *Scx*, We were able to further verify loss of the differentiated epaxial tendons in the *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ mutants by observing the behavior of tendin, a type II transmembrane protein that is normally highly expressed in the maturing tendons and ligaments (Brandau et al., 2001). In double heterozygous embryos, tendin was found in the axial tendons associated with the epaxial muscle (Fig. 3I); however, in the *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos, as with *Scx,* no tendin was detected (Fig. 3J), corroborating lack of differentiated tendons in the absence of specified or differentiated muscle. In the limb, *Scx* expression in E13.5 *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos was similar to that of wild type (Fig. 3K,L), hence, as opposed to the somites, unaffected by loss of *Myf5* and *Myod1* (Fig. 3B, blue arrow). Limb tendon progenitor formation, unlike its somitic counterpart, thus does not appear, at least as late as E13.5, to depend on the presence of specified or differentiated muscle.

In chick, we showed that FGF-dependent induction of *Scx* is mediated by the Ets transcription factors *Pea3* and *Erm*, and that transcriptional activation by *Pea3* and *Erm* is necessary for *Scx* expression to occur (Brent and Tabin, 2004). To determine whether loss of *Scx* in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutant embryos also correlates with changes in *Pea3* activity, we analyzed expression of *Pea3* at E10.5. As in chick, *Pea3* was seen in wild-type embryos in the anterior and posterior sclerotome, in a broader domain than that of *Scx* (Fig. 3M, black arrow). In *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutant embryos, absence of myotome differentiation was confirmed by lack of myogenin expression (Fig. 3M,Q); however, *Pea3* expression in the anterior and posterior sclerotome was still discernable (Fig. 3R, black arrow), although the domain was not as well defined as in wild-type (Fig. 3M). As *Pea3* is not only a transcriptional effector of FGF signaling, but also initially dependent upon FGF signaling for its expression, it is likely that the *Pea3* expression domain observed in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos persists, despite loss of the myotomal FGFs, because *Fgf8* expression is still present in the anterior and posterior dermomyotome (Fig. 3O,S). Nonetheless, as we showed in chick, clear refinement of the *Pea3* expression domain to the anterior and posterior sclerotome correlates distinctly with restriction of FGF signaling to the center of the myotome (Brent and Tabin, 2004); thus, the more diffuse *Pea3* expression domain seen in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ double mutants (Fig. 3R) is probably attributable to the absence of myotomal FGFs. To confirm that loss of *Scx* expression in double mutant embryos correlated with loss of FGF signaling from the anterior and posterior sclerotome, we utilized phosphorylated ERK/MAPK, which identifies when and where signaling is active (Corson et al., 2003). Employing an antibody specific to phosphorylated ERK1 and ERK2, we detected phosphorylated ERK/MAPK in the anterior and posterior sclerotome of *Myf5*$^{+/-}$; *Myod1*$^{+/-}$ embryos (Fig. 3P, yellow arrow). By contrast, in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos, this active FGF signaling site was absent (Fig. 3T, yellow arrow), while expression of phosphorylated ERK/MAPK in the dorsal root ganglia appeared wild type (Fig. 3P,T). It thus appears that loss of somitic *Scx* expression in *Myf5/Myod1* double mutant embryos correlates with the absence of active FGF signaling.

FGF signaling is required for *Scx* induction in mouse

In chick, a role was identified for the myotomal FGFs in the induction of *Scx* within the anterior and posterior sclerotome (Brent et al., 2003), consistent with the striking correlation observed between the losses of *Scx* and myotomal FGF expression in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ mutant mice. To verify that FGFs are required for *Scx* induction in mouse, we took advantage of the FGF receptor inhibitor SU5402 to block FGF signaling in a trunk culture system. E10.0 trunks were placed in culture for 24 hours, in either the presence or absence of SU5402. We observed normal induction of *Scx* in the somites and limbs of control embryos (Fig. 4A); however, in the presence of SU5402, *Scx* was lost in both the somites and limbs (Fig. 4B), indicating a requirement for FGF signaling in the induction of *Scx* in mouse, and strengthening our hypothesis that absence of somitic *Scx* expression in *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos reflects a loss of myotomal FGF signaling.

To determine whether FGFs are also sufficient to induce *Scx* expression in mouse, we implanted beads soaked in recombinant FGF4 protein into E10 somites. After 12 hours in culture, control embryos showed normal *Scx* expression (Fig. 4C); however, when *Fgf4*-soaked beads were implanted, strong upregulation of *Scx* was observed (Fig. 4D), indicating that FGFs are indeed sufficient to induce somitic *Scx* expression in mouse. If loss of *Scx* expression in the absence of *Myf5* and *Myod1* is ultimately attributable to failure of FGF signaling, we reasoned that exogenously applied FGFs should rescue *Scx* expression in the double mutants. We thus implanted *Fgf4* beads, at E10.0, into the somites of *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ embryos, and cultured them for 12 hours. As expected, with the PBS beads, no *Scx* expression was observed (Fig. 4E); however, when *Fgf4* beads were implanted, although *Scx* was still absent

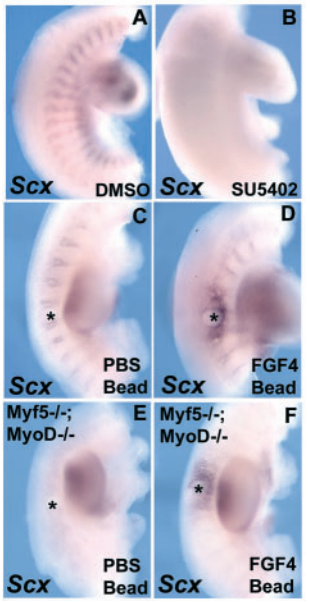

Fig. 4. FGF signaling is necessary and sufficient for induction of *Scx* in mouse. (A,B) Whole-mount in situ hybridization for *Scx* on trunks cultured with DMSO (A) or SU5402 (B). (C-F) Whole-mount in situ hybridization for *Scx* on either wild type (C,D) or *Myf5*$^{-/-}$; *Myod1*$^{-/-}$ (E,F) trunks with either a PBS (C,E) or FGF4 (D,F) bead implanted in somites. (C-F) Asterisks indicate location of beads.

from its normal domain, it was strongly induced surrounding the bead (Fig. 4F), suggesting that, in the absence of *Myf5* and *Myod1*, the FGFs are nonetheless able to induce somitic *Scx* expression when exogenously applied.

The dorsolateral sclerotome co-expresses markers of tendon and cartilage lineages

Having established a crucial role for myotome specification and myotomal FGF signaling in the induction of *Scx* within the somite, we wanted to learn more about the relationship between the two sclerotome-derived lineages: the axial cartilage and tendons. To do so, we compared the somitic expression patterns of some markers of cartilage and tendon progenitors as well as their differentiated derivatives in mouse. Because *Scx* is expressed continuously, it can be used to identify tendon development from the earliest to most mature stages (Brent et al., 2003; Schweitzer et al., 2001). For cartilage markers, we selected *Sox9*, expressed in and required for specification of all chondroprogenitors (Bi et al., 1999; Healy et al., 1996), and *Sox5*, which is required for and a marker of chondrocyte differentiation (Smits et al., 2001). Following differentiation, the cartilage-producing cells generate an extracellular matrix, recognizable by its ability to stain with Alcian Blue. In an E10.5 frontal section, expression of *Scx* was seen in the anterior and posterior sclerotome between adjacent myotomes (Fig. 5A). *Sox9* was observed throughout the sclerotome, particularly in the posterior somite (Fig. 5B), and more restricted in the anterior dorsolateral region, which also contains the dorsal root ganglia (Fig. 5B, asterisks). Interestingly, both *Scx* and *Sox9* appeared to be expressed within the anterior and posterior dorsolateral sclerotome (Fig. 5A,B). *Sox5* was also observed in the sclerotome, in a domain overlapping with that of *Sox9* throughout the sclerotome, and with that of *Scx* at the anterior and posterior margins (Fig. 5C). At this stage, no detectable Alcian Blue staining was visible (Fig. 5D,H), indicating that although the sclerotomal cartilage progenitors had begun to differentiate into chondrocytes, as evidenced by expression of *Sox5*, they were not yet producing extracellular matrix. Transverse sections at E10.5 allowed for clearer visualization of the overlapping expression domains of *Scx*, *Sox9* and *Sox5*. Although *Scx* was restricted to the dorsolateral-most sclerotome just beneath the myotome (Fig. 5E), *Sox9* was strongly expressed throughout the sclerotome, including the dorsolateral, ventromedial and dorsomedial regions (Fig. 5F). *Sox5*, although expressed, like *Scx,* in the dorsolateral sclerotome, extended further into the ventromedial area, where it partially overlapped with *Sox9* (Fig. 5G). That *Scx*, *Sox9* and *Sox5* were expressed in the dorsolateral sclerotome at E10.5 suggests that this domain contains either a mixture of cartilage and tendon progenitors, or a multipotent progenitor population co-expressing early markers of both cartilage and tendon. In situ hybridization did not provide enough resolution to distinguish between these possibilities.

At E11.5, the components of the axial skeleton begin to take shape within their respective sclerotome subdomains. Expression of *Sox9* and *Sox5* was observed in the forming vertebral bodies and neural arches (Fig. 5J,K) and in the rib primordia as they extended ventrally (Fig. 5N,O). But unlike the overlapping domains seen in the dorsolateral sclerotome at E10.5, by E11.5 *Scx* expression was clearly distinct, marking both the incipient axial tendons associated with the vertebral bodies, neural arches and epaxial muscle (Fig. 5I), and those surrounding the rib primordia (Fig. 5M). Also at E11.5, Alcian Blue staining showed the vertebral bodies and neural arches beginning to differentiate into chondrocytes (Fig. 5L), although the rib primordia remained Alcian Blue negative (Fig. 5P). Thus, by E11.5, the cartilage and tendon progenitors arising from the sclerotome had clearly sorted out their respective lineages, and by E13.5, both the vertebrae (Fig. 5R-T) and ribs (Fig. 5V-X) had differentiated into cartilage, were Alcian Blue positive, and were expressing *Sox9* and *Sox5*. Moreover, at E13.5, *Scx* expression was also clearly visualized in the differentiating axial tendons associated with the vertebrae (Fig. 5Q) and ribs (Fig. 5U).

Our comparison of specification and differentiation of the cartilage and tendon progenitors within the sclerotome revealed that although both populations initially occupied overlapping dorsolateral domains, they became non-overlapping and distinct as they began to differentiate. Interestingly, we found *Scx*, *Sox9* and *Sox5* co-expressed at additional sites in mouse. As early as E10.5, *Sox9* and *Sox5* were detected in a population of cells in the neural tube (Fig. 5F,G), perhaps reflecting their role in the development of glia (Stolt et al., 2003), and by E13.5 that domain had narrowed to surround the lumen of the neural tube (Fig. 5R,S). Also at E13.5, we saw *Scx* expressed in the dorsal neural tube, although more laterally so than *Sox9* and *Sox5* (Fig. 5Q), and also co-expressed, together with *Sox9* and *Sox5*, in the developing lung (Fig. 5U-W, arrows).

Scx expression is slightly upregulated in the dorsolateral sclerotome of *Sox5/Sox6* mutant embryos

In *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ embryos, the chondroprogenitors are unable

to differentiate into chondrocytes – and as a result, no cartilage elements form. *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ double mutants thus allow for examination of the effect of blocked chondrogenesis on both establishment of the tendon progenitor pool and formation of properly patterned tendons. We looked at the effect of *Sox5* and *Sox6* loss on E10.5 tendon progenitors in the dorsolateral sclerotome. In the anterior and posterior dorsolateral sclerotome of double heterozygous embryos, we found *Scx* expression resembling wild type in frontal sections (Fig. 6A). *Sox9* expression mirrored that of *Scx* in this domain, but was additionally present in the rest of the dorsomedial sclerotome, and in the ventromedial sclerotome (Fig. 6B). Because the null alleles of *Sox5* and *Sox6* were generated by targeting *lacZ* to each locus (Smits et al., 2001), we used *lacZ* expression to identify cells expressing the mutant *Sox5* and *Sox6* alleles. At E10.5, detection of *lacZ* transcripts by in situ hybridization revealed that the targeted alleles were expressed within the sclerotome – in the same domain as wild-type *Sox5* and *Sox6* (Fig. 6C).

In the *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ double mutants, *Scx* expression appeared somewhat upregulated in the anterior and posterior dorsolateral sclerotome (Fig. 6D, red arrow). As expected, *Sox9* expression was unaffected in the absence of *Sox5* and *Sox6*, consistent with its role acting upstream of both genes (Fig. 6E) (Akiyama et al., 2002; Smits et al., 2001). The presence of *lacZ* within the dorsolateral sclerotome of the double mutants (Fig. 6F) suggested either that in the absence of *Sox5* and *Sox6,* cells that normally express *Sox5* and *Sox6*, but not *Scx*, develop the capability to express *Scx*, or that cells normally co-expressing all three transcription factors express higher levels of *Scx* in mutant embryos.

Because FGF signaling is necessary and sufficient for somitic *Scx* expression in both mouse and chick, we wondered if the upregulation of *Scx* we had observed in *Sox5/Sox6* double mutant embryos was the result of increased FGF signaling. However, neither *Pea3* (Fig. 3G,J) nor phosphorylated ERK/MAPK (Fig. 3I,L, yellow arrows) appeared altered in the anterior and posterior sclerotome, indicating that FGF signaling had not increased. Alternatively, we considered that perhaps the extra *Scx*-expressing cells in the anterior and posterior sclerotome of the *Sox5/Sox6* double mutant embryos were not undergoing normal programmed cell death. To determine this, we performed TUNEL assays at E10.5. In *Sox5*$^{+/-}$; *Sox6*$^{+/-}$ embryos, the majority of cells undergoing programmed cell death appeared to be restricted to the dermomyotome (Fig. 3H); and as we observed a similar pattern of cell death in *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ double mutant embryos (Fig. 3K), we were not able to associate the increase in *Scx* expression in the anterior and posterior sclerotome with any change in cell death in the somite.

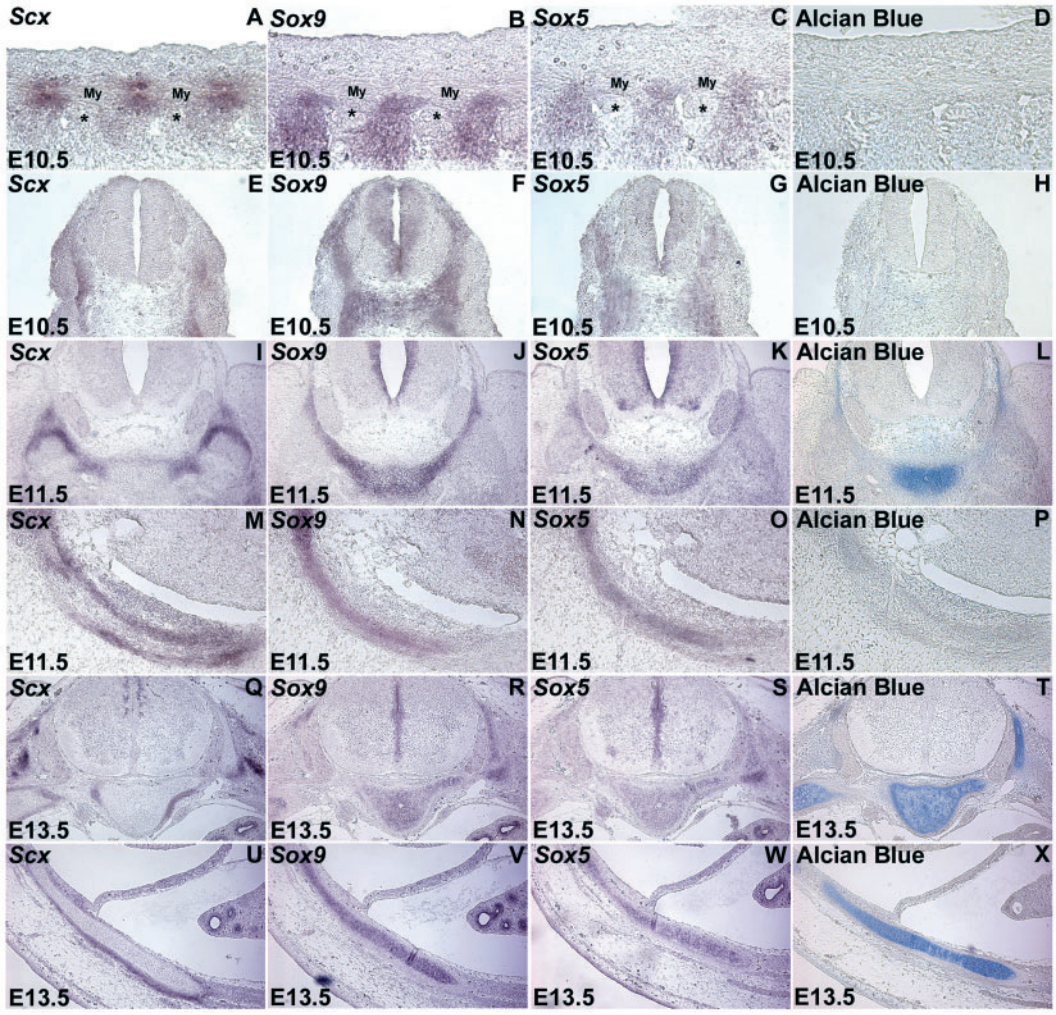

Fig. 5. Spatial and temporal comparison of axial tendon and cartilage progenitor specification and differentiation. Section in situ hybridization for *Scx* (A,E,I,M,Q,U), *Sox9* (B,F,J,N,R,V), and *Sox5* (C,G,K,O,S,W). (D,H,L,P,T,X) Alcian Blue staining. (A-D) Alternate frontal sections through thoracic somites at E10.5. Asterisks indicate dorsal root ganglia. (E-H) Alternate transverse sections through thoracic somites at E10.5. (I-L) Alternate transverse sections through vertebrae at E11.5. (M-P) Alternate transverse sections through ribs at E11.5. (Q-T) Alternate transverse sections through vertebrae at E13.5. (U-X) Alternate transverse sections through ribs at E13.5. My, myotome.

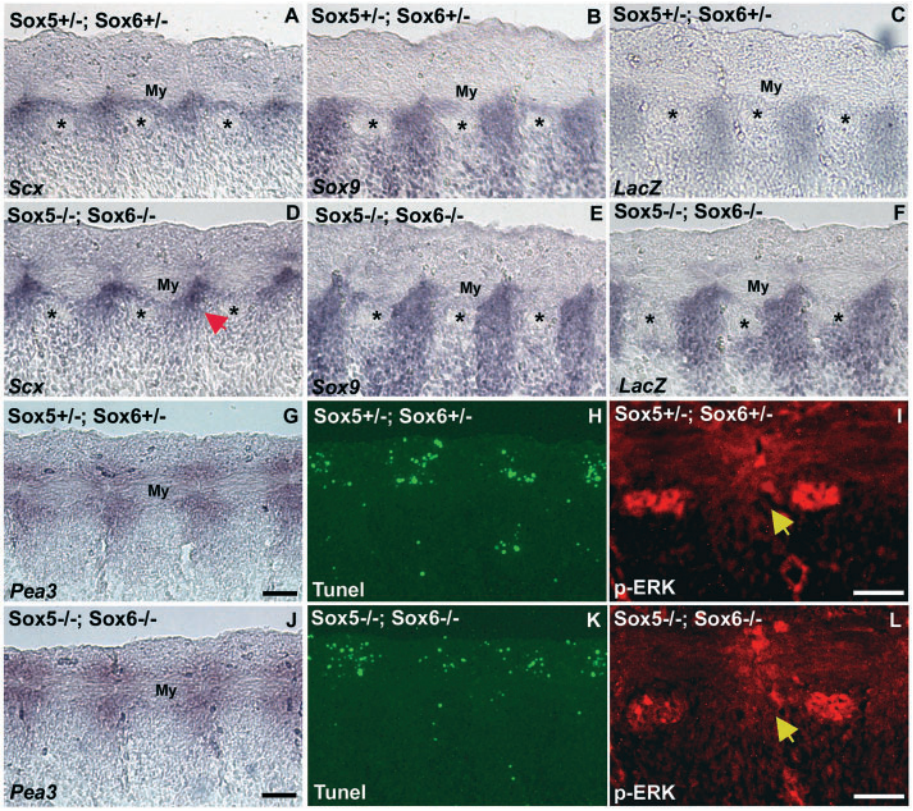

Fig. 6. *Scx* expression is upregulated in the dorsolateral sclerotome of *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ embryos. Section in situ hybridization for *Scx* (A,D), *Sox9* (B,E), *lacZ* (C,F) or *Pea3* (G,J) on frontal sections through thoracic somites of *Sox5*$^{+/-}$; *Sox6*$^{+/-}$ (A-C,G) or *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ (D-F,J) embryos. Red arrow in D indicates upregulation and expansion of *Scx* expression. (H,K) Tunnel assays at E10.5 on frontal sections of *Sox5*$^{+/-}$; *Sox6*$^{+/-}$ (H) or *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ (K) embryos. (I,L) Detection of phosphorylated ERK/MAPK on frontal sections at E10.5 on *Sox5*$^{+/-}$; *Sox6*$^{+/-}$ (I) or *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ (L) embryos. Yellow arrows indicate anterior and posterior sclerotome; asterisks indicate dorsal root ganglia. My, myotome. Scale bars: 50 μm.

In the absence of cartilage differentiation, the chondroprogenitors adopt a tendon cell fate

Analysis of *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ embryos at E10.5 revealed that when the *Sox9*-expressing chondroprogenitors were unable to express *Sox5* and *Sox6*, *Scx* was either upregulated or initiated de novo. As the dorsolateral sclerotome appeared to express *Scx*, *Sox9*, *Sox5* and *Sox6*, reflecting either a mixture of tendon and cartilage progenitors, or a single cell population co-expressing markers of both fates, our observation that *Scx* levels increase in this region when the chondroprogenitors are unable to differentiate, might indicate that without *Sox5* and *Sox6*, more dorsolateral sclerotome cells are able to respond to tendon inducing signals, such as myotomal FGFs, at early stages; yet, this increase may not be indicative of a fate change. To resolve this issue, we needed to determine, using double mutants, what happens to the developing tendons at later developmental stages, when chondrocyte differentiation should be occurring but cannot. In wild type, as shown above, by E11.5 the cartilage and tendon lineages become spatially distinct and nonoverlapping as the somitic derivatives began to differentiate. To assess tendon development after this transition, we decided to look at E14.5 embryos. In double heterozygous mice, *Scx* could be seen in the mature axial tendons associated with the vertebrae and ribs (Fig. 7A,G). The axial skeletal elements were differentiated at E14.5 as well, indicated by Alcian Blue staining (Fig. 7B,H), and were expressing *lacZ* (Fig. 7C,I). Importantly, in the double mutants at E14.5, the axial cartilage elements were also identifiable using expression of *lacZ* because, as previously shown, in the absence of *Sox5* and *Sox6*, the *lacZ*-expressing chondroprogenitors still form mesenchymal condensations at the appropriate sites for axial skeleton assembly (Fig. 7F,L) (Smits et al., 2001). But although *lacZ* expression was seen in the *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ vertebral bodies (Fig. 7F, green arrow), neural arches (Fig. 7F, red arrow) and ribs (Fig. 7L), these mutant skeletal elements failed to undergo normal chondrocyte differentiation: Alcian Blue staining revealed only slight differentiation in the vertebral bodies (Fig. 7E, green arrow), and none in the neural arches (Fig. 7E, red arrow) or ribs (Fig. 7K).

We next looked at formation of the mature axial tendons in *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ double mutants. *Scx* expression associated with the partially differentiated vertebral bodies looked similar to that of wild type (Fig. 7D, green arrow); however, the neural arches (Fig. 7D, red arrow) and ribs (Fig. 7J) showed a striking phenotype: in both cases, the *lacZ*-expressing but Alcian Blue-negative mutant skeletal elements expressed high levels of *Scx* throughout (Fig. 7D,J). As at earlier stages, no decrease in programmed cell death was observed at E14.5 (data not shown). It thus appears that when the chondroprogenitors making up the axial mesenchymal condensations were unable to differentiate into chondrocytes, they expressed *Scx*, and that the upregulation of *Scx* we saw in the dorsolateral sclerotome of double mutants at E10.5 had translated into undifferentiated cartilage elements that were continuing, at E14.5, to express tendon markers.

Because *Sox9* is expressed in specified chondroprogenitors both prior to and during differentiation, we next asked whether those *Sox5*$^{-/-}$; *Sox6*$^{-/-}$, *Scx*-expressing cartilage elements were also *Sox9* positive. In double heterozygous E14.5 embryos, *Scx* and *Sox9* appeared clearly mutually exclusive, with *Scx* marking the tendons surrounding the ribs (Fig. 7M) and *Sox9* marking the differentiated chondrocytes (Fig. 7N). Interestingly, *Sox9* was also found throughout the *Scx*-expressing double mutant ribs (Fig. 7P,Q). As these mutant undifferentiated *Scx*-expressing condensations were nonetheless able to form in appropriate locations, and to express *Sox9*, we wondered if they were capable of making proper muscle attachments. Simultaneous detection of tendon and muscle revealed that while the intercostal muscles did appear to attach to the *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ ribs (Fig. 7R), the muscles looked mispatterned compared with littermate controls (Fig. 7O).

Our observation that the undifferentiated axial cartilage elements expressed *Scx* in the absence of *Sox5* and *Sox6* suggests that the cartilage derivatives of the dorsolateral sclerotome had indeed undergone a fate change. To determine if the chondroprogenitors had actually differentiated into tendon, we looked at expression of two tendon markers, tendin (Brandau et al., 2001) and collagen XII (Dublet and van der Rest, 1987; Oh et al., 1993) that, unlike *Scx*, are found not in the progenitors but in the differentiating tendons. Strikingly, although in the E14.5 double heterozygous embryos, tendin (Fig. 8B) and collagen XII (Fig. 8C) expression, like that of *Scx* (Fig. 8A), was observed surrounding the ribs, in the double mutants, again mimicking *Scx* (Fig. 8D), tendin (Fig. 8E) and Collagen XII (Fig. 8F) were seen expressed throughout the undifferentiated rib primordia. We thus conclude that in the absence of cartilage differentiation, the chondroprogenitors switched to a genuine tendon cell fate.

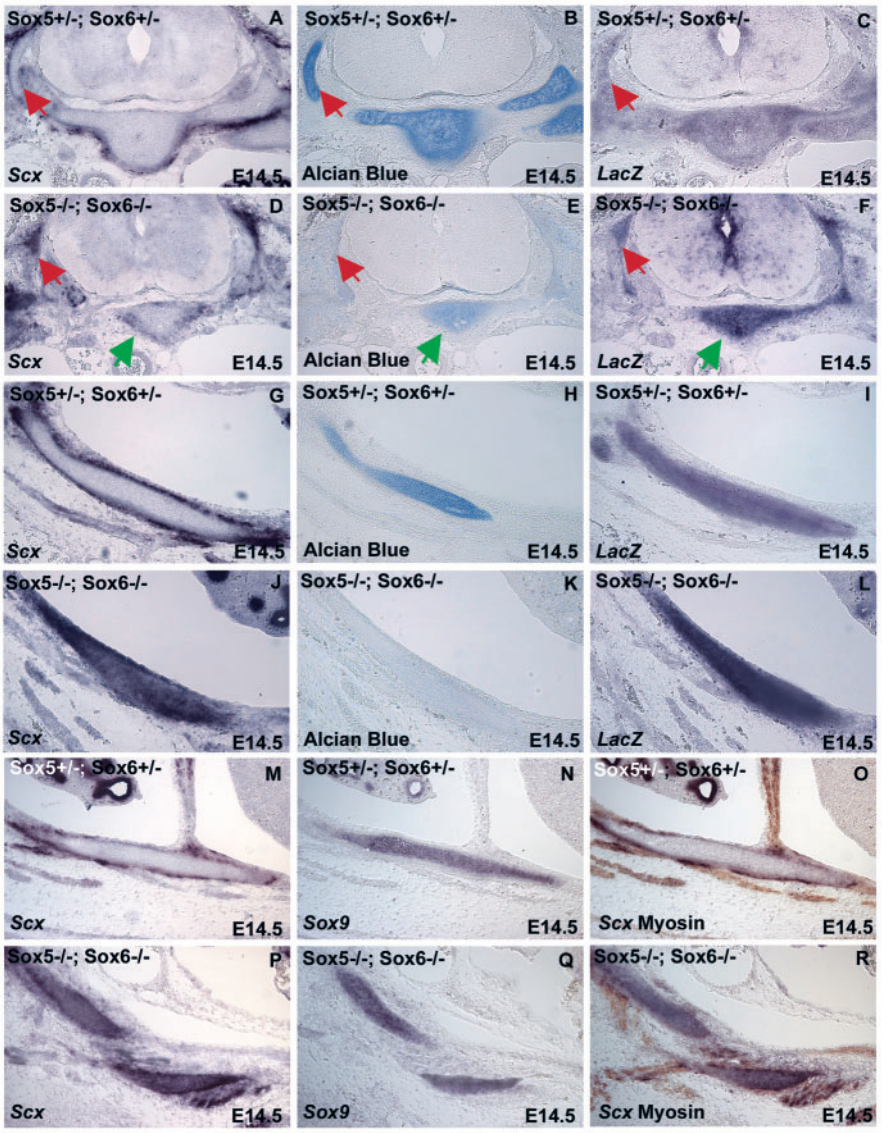

Fig. 7. *Scx* is expressed throughout the undifferentiated *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ skeletal elements. Section in situ hybridization for *Scx* (A,D,G,J,M,P,O,R), *lacZ* (C,F,I,L), and *Sox9* (N,Q) on sections through E14.5 *Sox5*$^{+/-}$; *Sox6*$^{+/-}$ (A-C,G-I,M-O) or *Sox5*$^{-/-}$; *Sox6*$^{-/-}$ (D-F,J-L,P-R) embryos. (B,E,H,K) Alcian Blue staining. (O,R) Combined section in situ hybridization and immunohistochemistry for *Scx* (purple) and myosin heavy chain (brown), respectively. (A-F) Transverse sections through vertebrae. Green arrows indicate vertebral bodies; red arrows, neural arches. (G-R) Transverse sections through ribs.

Discussion

Interactions between the somitic lineages during assembly of the axial musculoskeletal system

Through analysis of mouse mutants with disrupted muscle and cartilage development, we were able to genetically dissect interactions between the somitic muscle, cartilage and tendon lineages during assembly of the axial musculoskeletal system. Shortly after somite formation, *Myf5* and *Myod1* are expressed in the muscle progenitors of the myotome. In addition to controlling muscle specification, *Myf5* and *Myod1* are also required for activation of FGFs at the center of the myotome, where the myofiber nuclei reside (Fig. 9). It is striking that the myotomal FGFs are not expressed throughout the Myf5/Myod1-expressing myotome, but instead restricted to the center of the myotome. The reason for the restriction remains unclear. In chick, we previously showed that the myotomal FGFs are secreted from the myotome, and then signal to the underlying mesenchymal sclerotome to induce expression of *Scx* and tendon progenitor formation within the dorsolateral anterior and posterior sclerotome (Brent et al., 2003). Here, we demonstrate that this same FGF signaling pathway is both necessary and sufficient for induction of *Scx* in the dorsolateral sclerotome of mouse. Importantly, we illustrate that because *Myf5* and *Myod1* are required for expression of the myotomal FGFs, they are likewise necessary for formation of the axial tendon progenitors. The tendon progenitors are thus dependent on the presence of a specified myotome (Fig. 9), and their failure to form in *Myf5/Myod1* double mutants underscores the idea that muscle specification must both take place and precede the crucial intrasomitic signaling events that establish the tendon lineage. Interestingly, although *Fgf8* expression in *Myf5/Myod1* double mutant somites is normal, when the myotomal FGFs, *Fgf4* and *Fgf6*, are absent, *Scx* is not induced; there thus appears to be a specific dependence on the FGFs expressed at the center of the myotome for *Scx* induction. *Fgf4* and *Fgf6* probably act redundantly – hence the presence of axial tendons in mice carrying targeted mutations for *Fgf6* (Fiore et al., 1997; Floss et al., 1997).

We also demonstrate that the dorsolateral sclerotome initially houses two populations, tendon progenitor and chondroprogenitor, and thus has the capability to differentiate along either an axial tendon or cartilage

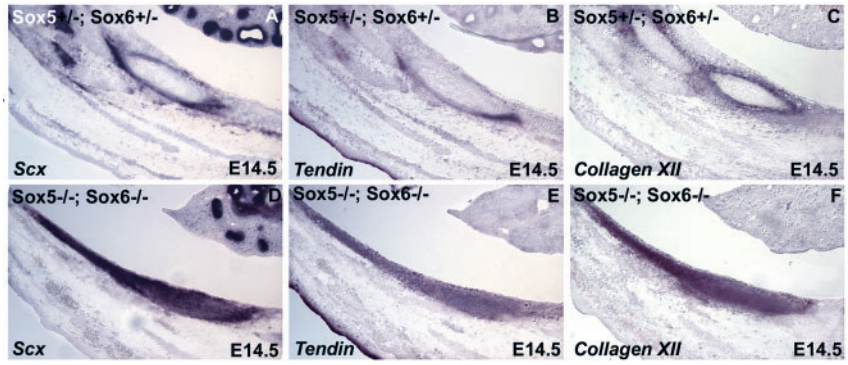

Fig. 8. In the absence of *Sox5* and *Sox6*, the chondroprogenitors express markers of differentiated tendon. Section in situ hybridization for *Scx* (A,D), tendin (B,E) and collagen XII (C,F) on alternate transverse sections through ribs of E14.5 $Sox5^{+/-}$; $Sox6^{+/-}$ (A-C) or $Sox5^{-/-}$; $Sox6^{-/-}$ (D-F) embryos.

pathway (Fig. 9). Comparisons of *Scx* with markers of specified chondroprogenitors and differentiated chondrocytes showed that *Scx*, *Sox9* and *Sox5/Sox6* were all expressed in this region, but that by E11.5, the tendon and cartilage lineages had become clearly distinguishable. Thus, at early stages of mouse somite differentiation, it appears that the syndetome, located in the dorsolateral sclerotome, contains a mixed cell population. It is at present unclear if the two populations are intermingled, the one expressing *Scx* and the other *Sox9* and *Sox5/Sox6*, or if there is one multipotent group co-expressing markers of each lineage. Our analyses of tendon progenitor formation in embryos carrying null mutations for *Sox5* and *Sox6* revealed that the two potential fates of the dorsolateral sclerotome are alternative, and that differentiation along one pathway actually blocks differentiation along the other. We know that expression of *Sox9* is required for specification of the chondroprogenitors, and for the subsequent activation of *Sox5* and *Sox6*, which, in turn, are required for chondrocyte differentiation (Akiyama et al., 2002; Smits et al., 2001) (Fig. 9). Thus, in the absence of *Sox5* and *Sox6*, cartilage differentiation cannot occur. We found that preventing cartilage differentiation in the sclerotome had a striking effect on cell fate choice. In *Sox5/Sox6* mutants, although *Sox9* expression was normal, *Scx* expression and/or the number of axial tendon progenitors increased and expanded within the dorsolateral sclerotome. As development proceeded, this increase translated into more somitic cells adopting a tendon cell fate – culminating in the appearance of *Sox9*-positive undifferentiated cartilage elements expressing markers of differentiated tendon throughout. Our studies thus suggest that *Sox5/Sox6*-dependent cartilage differentiation is required to actively repress tendon development in the dorsolateral sclerotome, that the cartilage and tendon lineages are alternative, and that a crucial and fine-tuned balance must be negotiated between them in order for proper development and assembly of the axial musculoskeletal system to proceed.

Secreted factors control the balance of sclerotome lineage formation

Initial patterning of the somite into sclerotome, dermomyotome and myotome depends upon signals secreted from surrounding tissues (Brent and Tabin, 2002). Formation of the syndetome, which gives rise to the tendon lineage, also requires the activity of secreted factors, but in this case they arise from within the somite itself. Our analyses show that these external and internal signaling pathways must be carefully regulated in order for the different somitic lineages to form properly. Ventral midline *Shh* signaling has been found to play an essential role in both the induction and/or maintenance of several of these fates (Brent and Tabin, 2002). During sclerotome formation, *Shh* signaling is required for normal expression of a number of genes that function during development of the axial skeleton, among them *Pax1*, *Pax9*, *Nkx3.2* and *Sox9* (Buttitta et al., 2003; Murtaugh et al., 1999; Murtaugh et al., 2001; Zeng et al., 2002; Zhang et al., 2001).

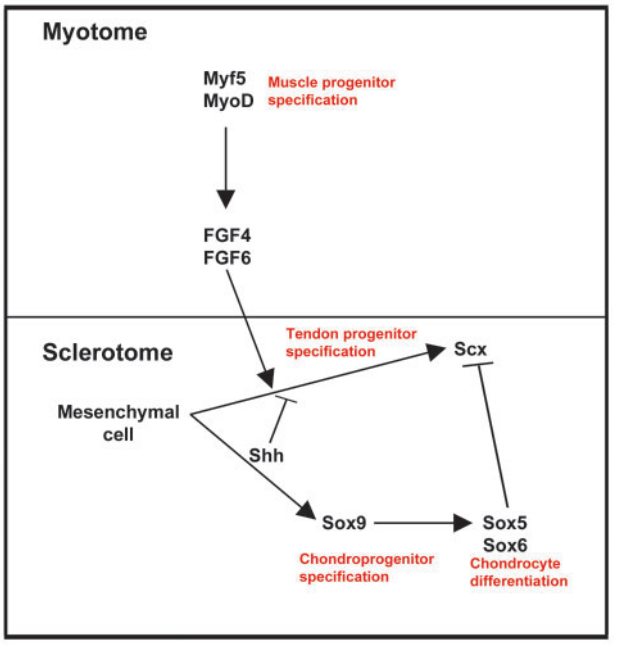

Fig. 9. Summary of interactions between the somitic lineages. Shortly after somite formation, expression of *Myf5* and *Myod1* appears in the muscle progenitors of the myotome. Following myotome specification, FGFs are activated at the center of the myotome. Myotomal FGFs, secreted from the myotome, signal to the underlying mesenchymal sclerotome, where they induce expression of *Scx* and tendon progenitor formation within the dorsolateral anterior and posterior sclerotome. The dorsolateral sclerotome also contains chondroprogenitors that are induced to express *Sox9* in response to patterning signals, including *Shh* (and this same *Shh* signal negatively regulates *Scx* expression). The *Sox9*-expressing chondroprogenitors then activate expression of *Sox5* and *Sox6*, which, in turn, are required for chondrocyte differentiation. The dorsolateral mesenchymal sclerotome can thus follow one of two differentiation pathways: axial tendon or cartilage. *Sox5* and *Sox6* inhibit expression of *Scx* such that those sclerotome cells undergoing differentiation into cartilage are blocked from adopting a tendon fate. However, in the absence of *Sox5* and *Sox6*, when these same cells are prevented from differentiating into chondrocytes, they switch their fate to tendon and begin expressing markers of both tendon progenitors and differentiated tendons, suggesting that cartilage differentiation is required to actively repress tendon development.

Additionally, we have observed that overexpression of *Shh* during chick somite development leads to ectopic expression of not only *Sox9*, but also *Sox5* and *Sox6* (A.E.B. and C.J.T., unpublished). It is likely that varied levels of *Shh* signaling are important for patterning the sclerotome into its different subdomains: highest levels regulating ventromedial expression of *Pax1*, *Pax9* and *Nkx3.2* and formation of the vertebral bodies; and lower levels controlling both expression of genes in the dorsolateral sclerotome, such as *Zic1* and *Uncx4.1*, and development of the neural arches and ribs (Aruga et al., 1999; Bussen et al., 2004; Leitges et al., 2000; Mansouri et al., 2000; Nagai et al., 1997). Interestingly, we found, in chick, that overexpression of *Shh* negatively regulates formation of the other dorsolateral sclerotome lineage, the tendon progenitors (Brent et al., 2003) – an effect probably due to a concomitant upregulation, within the same cells, of *Pax1* which, in turn, inhibits expression of *Scx* (Brent et al., 2003). It is too simplistic, however, to conclude that *Shh* signaling promotes sclerotome to adopt a cartilage over a tendon fate. Rather, different levels probably pattern the sclerotome into dorsolateral and ventromedial domains (Kos et al., 1998). In addition, like *Myf5* and *Myod1*, *Shh* has been shown to be required for expression of FGFs in the myotome (Fraidenraich et al., 2000): in *Shh* mutants, expression of the myotomal FGFs is reduced (Fraidenraich et al., 2000), and similar disruption of somitic *Scx* expression has also been observed (A.E.B., C.J.T. and A. P. McMahon, unpublished). It is possible that *Shh* regulates FGF induction by controlling the myotomal expression of *Myf5* and *Myod1*; and in support of this conjecture, there is evidence that *Shh* is required to induce or maintain expression of myogenic factors – in particular *Shh* functions via the Gli proteins to regulate *Myf5* (Gustafsson et al., 2002; Kruger et al., 2001; Teboul et al., 2003). Alternatively, *Shh* signaling might directly activate expression of the myotomal FGFs.

It is in any case clear that *Shh* signaling arising from the ventral midline plays several pivotal roles in patterning the somite and sclerotome, and regulating development of both the axial cartilage and tendons through activation of the myotomal FGFs. Building on our previous observation that FGFs negatively regulate expression of the ventromedial sclerotome marker, *Pax1* (Brent et al., 2003) – an observation probably relevant to the mediolateral patterning of the sclerotome – we can speculate on how these tendon-promoting, myotomal FGF signals might affect development of the chondroprogenitors. Although *Pax1* is initially expressed throughout the sclerotome, it is eventually downregulated everywhere except the ventromedial-most domain, where it then functions in development of the vertebral bodies. It is thus possible that myotomal FGF signaling plays a normal role in the downregulation of *Pax1* in the dorsolateral sclerotome; and in support of this hypothesis, it has been previously reported that in *Myf5* mutant embryos, prior to onset of *Myod1* and subsequent rescue of myotomal FGF expression, *Pax1* expression is seen reaching further dorsolaterally into the somite than it does in wild type (Grass et al., 1996).

But does a role for the myotomal FGFs in promoting *Scx* and inhibiting *Pax1* expression mean that the FGFs negatively regulate chondroprogenitor formation in the sclerotome? Analysis of *Myf5/Myod1* double mutant embryos indicates that this is not the case: despite the fact that *Myf5/Myod1* double mutants show no myotomal FGF signaling and do not develop axial tendons, they nonetheless form a normal skeleton, implying that loss of FGF signaling does not impact axial skeleton development from the sclerotome (Kaul et al., 2000). Additionally, whereas FGF overexpression negatively regulates *Pax1* expression in the chick somite (Brent et al., 2003), *Sox9*, *Sox5* and *Sox6* remain unaffected (A.E.B. and C.J.T., unpublished) – an unsurprising result given our observation that *Scx*, *Sox9* and *Sox5/6* are all normally co-expressed in the dorsolateral sclerotome. Furthermore, it has been shown in chick that *Fgf8* actually promotes formation of rib cartilage (Huang et al., 2003), a requirement that might be masked in *Myf5/Myod1* mutant embryos because of the persisting expression of *Fgf8*. We propose that the myotomal FGFs function to induce *Scx* in the dorsolateral sclerotome at the same time that ventral midline *Shh* signaling induces *Sox9* throughout the sclerotome, and that *Sox9*, in turn, activates expression of *Sox5* and *Sox6* – after which some sclerotome cells differentiate into cartilage, and the tendon fate is inhibited. The actual mechanism by which, within a uniform or intermingled population, some dorsolateral sclerotome cells choose the tendon or cartilage fate remains unknown, but does not appear to involve either an increase in FGF signaling or a decrease in cell death.

The balance between the activities of the FGF and *Shh* signaling pathways not only determines the fate each sclerotomal subdomain adopts, but also provides insight into the tendon phenotype observed in *Sox5/Sox6* mutant embryos. In the absence of *Sox5* and *Sox6*, the derivatives of the dorsolateral sclerotome, the ribs and neural arches, undergo a fate change, from cartilage to tendon, while the ventromedial derivatives, the vertebral bodies, do not. Thus, the capacity of the sclerotome to switch fates could be specific to the dorsolateral region which, in fact, expresses both tendon and chondroprogenitor markers. This crucial regional difference is probably attributable, at least in part, to the spatial relationship each of the two subdomains maintains to FGF and *Shh* signaling. Higher *Shh* levels in the ventromedial sclerotome may simultaneously allow for the slight cartilage differentiation that does take place in $Sox5^{-/-}$; $Sox6^{-/-}$ embryos, and prevent adoption of the tendon fate. By contrast, *Shh* signaling in the dorsolateral sclerotome, which is located further from the source of the signal, is lower, whereas FGF levels in this region are robust; it is probably just because the dorsolateral chondroprogenitors, unable to differentiate in the absence of *Sox5* and *Sox6*, are in range of the myotomal FGFs that they are able to switch fates and differentiate into tendon. Conversely, as the myotomal FGFs would most probably be unable to reach the ventromedial sclerotome, no fate change would take place there. Additionally, because the notochord cells of $Sox5^{-/-}$; $Sox6^{-/-}$ embryos undergo massive cell death between E11.5 and E14.5 (Smits and Lefebvre, 2003), *Shh* signaling probably becomes reduced, from E11.5, in the developing double mutant axial skeleton. Thus, because the ribs and neural arches undergo overt cartilage differentiation slightly later than do the vertebral bodies, these lateral sclerotome derivatives may be more affected by *Shh* loss, not only because they are located further from the notochord, but also because the notochord itself is vanishing.

Tissue interactions during somite versus limb development

Our analyses of tendon development in *Myf5/Myod1* and *Sox5/Sox6* mutant embryos also revealed some interesting differences in formation of the somite versus limb tendon progenitors. Both arise from a common mesenchymal origin, and in mouse (this study) as well as chick (A.E.B. and C.J.T., unpublished) (Brent et al., 2003), both lose *Scx* expression when FGF signaling is inhibited. Thus, some patterning mechanisms during axial and limb tendon development appear to be shared. We know that *Myf5*- and *Myod1*-dependent specification of the myotomal muscle progenitors is required for induction of somitic *Scx,* and for subsequent axial tendon differentiation. By contrast, the limb tendon progenitors form and differentiate normally in the absence of *Myf5* and *Myod1*, demonstrating, in line with observations in chick, that muscle need not be present for the limb tendons to form (Kardon, 1998). Similarly, branchial arch expression of *Scx* is normal in *Myf5/Myod1* mutants.

As in the mesenchymal sclerotome, the segregation of limb bud mesenchyme into both cartilage and tendon lineages must also be accomplished. However, unlike the mechanisms employed within the somite to complete this task, different processes function during specification of the limb cartilage and tendon progenitors (Brent et al., 2003; Murtaugh et al., 1999; Schweitzer et al., 2001). Our observation that *Sox5/Sox6*-dependent cartilage differentiation actively precludes sclerotome from adopting a tendon fate does not appear to apply to limb development. Although defects in tendon patterning were seen at later stages in the absence of *Sox5* and *Sox6*, we observed no increase in the number of tendon progenitors, nor any fate change from cartilage to tendon (A.E.B. and C.J.T., unpublished), suggesting that in the limb, unlike the somite, cartilage differentiation does not actively repress tendon development.

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