FGF acts directly on the somitic tendon progenitors through the Ets transcription factors *Pea3* and *Erm* to regulate scleraxis expression

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Summary

During somite development, a fibroblast growth factor (FGF) signal secreted from the myotome induces formation of a scleraxis (*Scx*)-expressing tendon progenitor population in the sclerotome, at the juncture between the future lineages of muscle and cartilage. While overexpression studies show that the entire sclerotome is competent to express *Scx* in response to FGF signaling, the normal *Scx* expression domain includes only the anterior and posterior dorsal sclerotome. To understand the molecular basis for this restriction, we examined the expression of a set of genes involved in FGF signaling and found that several members of the *Fgf8* synexpression group are co-expressed with *Scx* in the dorsal sclerotome. Of particular interest were the Ets transcription factors *Pea3* and *Erm*, which function as transcriptional effectors of FGF signaling. We show here that transcriptional activation by *Pea3* and *Erm* in response to FGF signaling is both necessary and sufficient for *Scx* expression in the somite, and propose that the domain of the somitic tendon progenitors is regulated both by the restricted expression of *Pea3* and *Erm*, and by the precise spatial relationship between these Ets transcription factors and the FGF signal originating in the myotome.

Key words: Somite, Syndetome, Sclerotome, Tendon, Scleraxis, FGF, Ets, *Pea3*, *Erm*

Introduction

The vertebrate axial musculoskeletal system arises from somites – transient, segmented, epithelial blocks of mesoderm that bud off from the anterior end of the unsegmented presomitic mesoderm (psm). Once formed, the somite subdivides into compartments that give rise to distinct cell lineages. In response to signals from surrounding tissues, the ventral somite de-epithelializes to form the mesenchymal sclerotome, while the dorsal region, the dermomyotome, remains an epithelial sheet. As the somite matures, cells delaminate from and migrate underneath the edges of the dermomyotome to form a third compartment, the myotome, located between the dermomyotome and sclerotome. The development of the axial musculoskeletal system from these three somitic compartments is well understood (Brand-Saberi and Christ, 2000; Brent and Tabin, 2002): the axial skeleton arises from sclerotome, the skeletal muscle from myotome, and the dorsal dermis from dermomyotome. Yet, although a functional musculoskeletal system is entirely dependent upon the transmission of force from muscle to bone, until recently little was known about the origin of the axial tendons – those mediating attachment between the epaxial muscles and vertebrae, and the intercostal muscles and ribs.

It has now been shown, however, through analysis of the expression pattern of the tendon-specific bHLH transcription factor scleraxis (*Scx*) (Brent et al., 2003; Cserjesi et al., 1995; Schweitzer et al., 2001), that the tendon progenitors arise from a fourth somitic compartment, termed the syndetome (Brent et al., 2003), which occupies a unique location within that region of the dorsal sclerotome closest to the anterior and posterior edges of the myotome. Molecularly defined by expression of *Scx*, the position of the syndetome is determined when fibroblast growth factors (FGFs) secreted from the center of the myotome induce the anterior and posterior sclerotome abutting the myotome to adopt a tendon cell fate (Brent et al., 2003). Thus, interactions between the somitic muscle and cartilage cell lineages lead to specification of the tendon lineage – placing the tendon progenitors at the interface of the two tissue layers they must ultimately join. Yet, while FGF signaling between myotome and sclerotome has been shown to be both necessary and sufficient for *Scx* expression in the syndetome (Brent et al., 2003), it is unclear whether this signaling acts cell autonomously within the future *Scx*-expressing cells, or indirectly through a secondary signal. The mechanism responsible for restricting *Scx* expression to only that region of the anterior and posterior sclerotome abutting the myotome is also puzzling, particularly in light of experiments showing that overexpression of *Fgf8* during somite development leads to ectopic *Scx* expression throughout the sclerotome (Brent et al., 2003) – hence demonstrating that the entire sclerotome is competent to express *Scx* in response to FGF signaling. In the current study, we set out to understand the molecular basis for this competency, as well as the mechanism by which myotomal FGFs determine the restricted position of the syndetome, by asking if the circumscribed *Scx* domain could be a reflection of localized FGF signal transduction.

To explore our hypothesis, we looked at the expression of several members of the *Fgf8* synexpression group, a set of

genes known to be induced in regions of active *Fgf8* signaling and thought to either transduce or modulate the FGF signaling pathway. During signaling, the secreted FGF ligand binds to the extracellular domain of the *Fgf* receptor (*Fgfr*), a protein with a tyrosine kinase intracellular domain. Ligand-binding causes receptor dimerization, autophosphorylation and activation of the intracellular tyrosine kinase domains. A number of intracellular signaling cascades follow, in particular, the RAS-MAPK/ERK pathway, in which sequential phosphorylation of a series of protein kinases ultimately activates MAPK/ERK to control a variety of downstream responses, including gene transcription. Among the *Fgf8* synexpression group members are the transcription factors *Pea3* and *Erm*, and the inhibitors MAPK phosphatase 3 (*Mkp3*), similar expression to FGF (*Sef*) and sprouty (*Spry*). *Pea3* and *Erm* are defined by the presence of an evolutionarily conserved Ets domain that mediates DNA binding (Sharrocks et al., 1997). FGF signaling is both necessary and sufficient for their expression (Firnberg and Neubuser, 2002; Kawakami et al., 2003; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), and as both have been shown to be present at regions of FGF signaling in several developmental contexts, they are thought to be general transcriptional targets of FGF signaling (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Moreover, it has been demonstrated in vitro that DNA binding of *Pea3* and *Erm* to their targets is activated following phosphorylation by MAPK/ERKs (Janknecht et al., 1996; Munchberg and Steinbeisser, 1999; O'Hagan et al., 1996). Thus, in addition to being potential transcriptional targets of FGF signaling, *Pea3* and *Erm* function as transcriptional effectors within cells to transduce FGF signals. By contrast, *Mkp3*, *Sef* and *Spry* act within cells as negative feedback inhibitors, modulating and restricting the levels and extent of FGF signaling. FGFs are both necessary and sufficient to control their expression, and the three are known to be present at sites of *Fgf8* signaling (Chambers and Mason, 2000; Dickinson et al., 2002; Eblaghie et al., 2003; Furthauer et al., 2002; Kawakami et al., 2003; Mailleux et al., 2001; Minowada et al., 1999; Ozaki et al., 2001; Tsang et al., 2002).

We present evidence that the FGF signal responsible for inducing the *Scx* expression domain can be directly received by the anterior and posterior sclerotome, that the *Fgf8* synexpression group members *Pea3*, *Erm*, *Mkp3*, *Sef* and *Spry* are co-expressed with *Scx*, and that the activity of the transcription factors *Pea3* and *Erm* is necessary and sufficient for FGF-dependent induction of *Scx*. Importantly, we found that overexpression of *Pea3* led to ectopic expression of *Scx* in both the sclerotome and dermomyotome – but only in those regions within effective signaling range of the myotomal FGFs. Our results suggest that the domain of *Scx* expression, and hence the unique location of the syndetome, is dependent on the combined conditions of the restricted expression pattern of *Pea3* and *Erm* within the anterior and posterior sclerotome, and the distances that FGFs secreted from the center of the myotome are able to travel. It is thus the interplay of factors that act downstream of the FGFR that defines the boundaries of *Scx* expression.

Materials and methods

In situ hybridization

Single and double whole-mount or section in situ hybridization was performed as previously described (Brent et al., 2003). DIG-labeled probes were detected with NBT/BCIP (Sigma), and FITC-labeled probes with INT/BCIP (Sigma). For section in situ hybridization, chick embryos were embedded in paraffin wax, and 10 μm sections were collected. Probes included chick *Scx* (Schweitzer et al., 2001), chick *Fgf8* (Brent et al., 2003), quail *Frek* (Brent et al., 2003), chick *Fgfr1*, chick *Pea3* (RT-PCR product using primers 5′ ACGTC-TAGAGTGCATAATAACCATAGG 3′ and 5′ ACGGAATTCCT-AGTAGGTGTAGCCTTTGCC 3′), chick *Erm* (RT-PCR product using primers 5′ ACGTCTAGACCGGCCCCAGCC-TGCCCG 3′ and 5′ ACGGAATTCATCAGTAGGCAAAG-CCCTCCG 3′), chick *Mkp3* (ChEST246m1 obtained from MRC geneservice), chick *Sef* (ChEST528k13 obtained from MRC geneservice), chick *Spry2* (gift of Connie Cepko) and chick *Myf5* (gift of Laura Gamer).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Brent et al., 2003). Phosphorylated MAPK/ERK was detected with phospho-p44/42 map kinase (Thr202/Tyr204) antibody (diluted 1:500; Cell Signaling Technology #9101), myosin heavy chain with MF20 [diluted 1:100; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA] and RCAS infection with AMV-3C2 (1:5; DSHB). Primary antibodies were followed by either Cy2- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch).

Cloning of retroviral constructs and viral misexpression

Cloning of retroviral constructs using SLAX13 and transfection and growth of RCAS viruses was performed as previously described (Logan and Tabin, 1998; Morgan and Fekete, 1996). RCASBP (A) constructs included full-length chick *Fgf8* (gift of Connie Cepko), full-length mouse *Pea3* (RT-PCR product using primers 5′ ACGGGTCTCCCATGGAGCGGAGGATGAAAG 3′ and 5′ ACGG-AATTCCTAGTAAGAATATCCACCTCTG 3′), and the mouse *Pea3* Ets DNA binding domain (RT-PCR product using primers 5′ ACG-GTTCTCCCATGCAGCGCCGGGGTGCCTTAC 3′ and 5′ ACGGA-ATTCCGGCTCGCACACAAACTTGTAC 3′). Pea3EnR was made by cloning the *Pea3* Ets DNA-binding domain into the SLAX-EnR vectors. Psm infection was performed as previously described (Brent et al., 2003).

Bead implants

Heparin beads (Sigma) were washed in PBS and incubated on ice for 1 hour in FGF8 protein (Peprotech) (1 mg/ml). Bead implants were performed as previously described (Brent et al., 2003).

Dermomyotome ablation

Psm injections were performed on Hamburger Hamilton (HH) (Hamburger and Hamilton, 1951) stage 12 embryos. Following 9 hours of incubation, dermomyotomes were removed from somite stages V and VI as previously described (Brent et al., 2003).

Trunk cultures

Trunks (including thoracic and limb levels) of HH stage 16 embryos were isolated and cultured on nucleopore filters in chick embryo media (DMEM, 10% chicken serum, 5% fetal calf serum, 1% pen-strep, 1% L-glut) (Palmeirim et al., 1997) with 30 μM SU5402 (Calbiochem, dissolved in DMSO) or an equivalent amount of DMSO. Following 24 hours of incubation, trunks were fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization.

Results

Myotomal FGFs can signal directly to the sclerotome

Our previous analysis revealed that *Scx* is induced in a subpopulation of sclerotome (Fig. 1A) in response to FGF

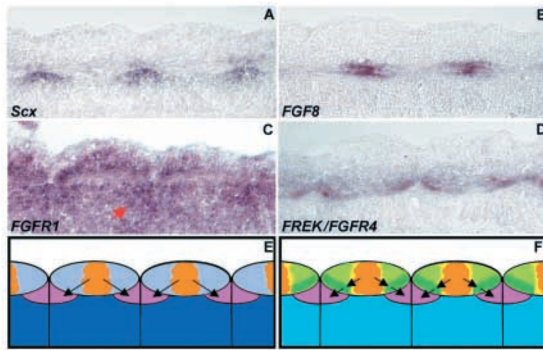

Fig. 1. FGF-dependent induction of *Scx* in the somite may be direct or indirect. Section in situ hybridization on alternate frontal sections comparing expression of *Scx* (A), *Fgf8* (B), *Fgfr1* (C) and *Frek/Fgfr4* (D) in a HH stage 20 embryo. (C) Red arrow indicates slight upregulation of *Fgfr1* in the *Scx*-expressing region. (E,F) Models for direct or indirect induction of *Scx* in the anterior and posterior dorsal sclerotome. Four somites shown in frontal view, with myotomes represented as ovals, sclerotomes as squares. Anterior is towards the left, posterior towards the right. (E) In a model for direct *Scx* induction, FGFs (orange) expressed in the center of the myotome signal directly to *Fgfr1* (blue) in the sclerotome, thereby activating expression of *Scx* (purple) in the sclerotome. Dark blue represents high expression levels of *Fgfr1* in the sclerotome, light blue indicates lower expression levels of *Fgfr1* in the myotome. (F) In a model for indirect *Scx* induction, FGFs (orange) signal through *Frek/Fgfr4* (green), localized to the anterior and posterior myotome, to activate expression of a secondary factor that then signals to the underlying anterior and posterior sclerotome to induce *Scx* expression (purple). Light green indicates low levels of *Frek/Fgfr4* in the myotome, dark green indicates higher levels of *Frek/Fgfr4* in the ventral anterior and posterior myotome. Yellow represents myotome, aqua indicates sclerotome.

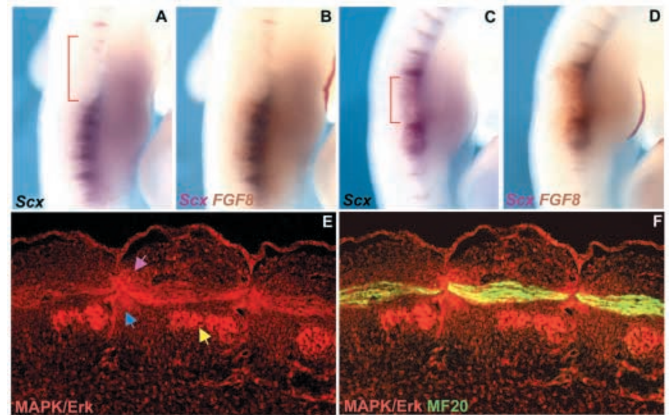

Fig. 2. *Fgf8* can induce ectopic expression of *Scx* in the sclerotome in the absence of the myotome. (A-D) Results of overexpression of *Fgf8* in the psm with RCAS-FGF8, followed by surgical removal of dermomyotomes. (A,C) Whole-mount in situ hybridization for *Scx* following manipulation. Region of dermomyotome removal indicated by red bracket. (B,D) Double whole-mount in situ hybridization for *Fgf8* on embryos shown in A,C. Antibody staining for phosphorylated MAPK/ERK (E,F) and myosin heavy chain (MF20) (F) on frontal sections of HH stage 20 embryos. (E) Phosphorylated MAPK/ERK (red) is seen in dorsal sclerotome, myotome and dermomyotome. Blue arrow indicates expression in the sclerotome, purple arrow in the dermomyotome, yellow arrow in the dorsal root ganglia. (F) Overlay of phosphorylated MAPK/ERK (red) and MF20 (green).

signals secreted from the myotome. Within the myotome, several FGFs, including *Fgf8*, are localized to the center, where the postmitotic myofiber nuclei also reside (Fig. 1B) (Kahane et al., 2001; Stolte et al., 2002). Two out of the four FGF receptors are expressed in the somite at the time of *Scx* induction: *Fgfr1*, which is expressed broadly throughout the somite, slightly reduced in the myotome and slightly increased at the site of *Scx* expression (Fig. 1C, arrow); and *Frek/Fgfr4*, which is restricted to the anterior and posterior myotome borders (Brent et al., 2003; Kahane et al., 2001), with upregulation in the ventral region abutting the underlying sclerotome (Fig. 1D) (Kahane et al., 2001; Marics et al., 2002). These patterns suggested to us two models for *Scx* expression within the somite: the myotomal FGFs could be diffusing directly from myotome to sclerotome and then activating *Scx* through *Fgfr1* (Fig. 1E), or the myotomal FGFs could be regulating a secondary signal, through the *Frek/Ffgr4* receptor, that would then induce *Scx* (Fig. 1F). As the expression pattern alone of *Fgfr1* cannot account for the restricted *Scx* domain, one would additionally have to postulate either that some mechanism was present whose activity ensured that the FGF signal was received only by the anterior and posterior sclerotome, or that only those regions of the sclerotome were competent to respond to it (Brent et al., 2003). We thus considered that activation via *Frek/Fgfr4* might provide a better rationale for the *Scx* expression domain, as long as the existence of the secondary factor, which is produced by the *Frek/Fgfr4*-expressing myotome in response to the FGFs and then signaling to the adjacent underlying sclerotome, was allowed for (Brent et al., 2003).

In our current study, we decided to test these two models by asking if *Scx* could still be induced when *Fgf8* was overexpressed in the absence of myotome – i.e. in the absence of any potential secondary factors. Surgical ablation of the dermomyotome prior to myotome formation results in loss of *Scx* expression when assessed either 1 (data not shown) or 2 days after ablation (Brent et al., 2003), presumably owing to loss of myotomal FGFs. To determine if *Scx* is induced in response to *Fgf8* after removal of the dermomyotome, we misexpressed *Fgf8* throughout the psm, using a retrovirus (RCAS-FGF8), and then surgically ablated the dermomyotomes from somite stages V and VI, 9 hours after infection but, importantly, prior to expression of retrovirally encoded *Fgf8*. Viral infection was detected by in situ hybridization with a probe to chick *Fgf8*, which can detect virally expressed *Fgf8* (Fig. 2B,D). Our results revealed that while operated uninfected somites showed loss of *Scx* expression (Fig. 2A,B), in operated infected somites, ectopic *Scx* expression was observed, even in the absence of myotome formation (Fig. 2C,D). We thus concluded that the sclerotomal cells are indeed capable of responding directly to FGF signaling to activate *Scx*, and that *Fgfr1* is therefore the more likely receptor. Interestingly, however, while overexpression of *Fgf8* throughout the sclerotome led to widespread expression of *Scx*, the more intensely staining normal anteroposterior localization was lost when myotome formation was blocked (Fig. 2C). That the ectopic expression of *Scx* induced in the

sclerotome following overexpression of *Fgf8* was not as strong as the endogenous expression of *Scx* in the syndetome, suggests either that retrovirally encoded *Fgf8* is not as potent as the myotomally expressed FGFs, or that while the entire sclerotome is competent to express *Scx* in response to FGFs, the normal induction of *Scx* in the syndetome is somehow potentiated, resulting in higher levels of *Scx* expression in that region.

To further test the model that *Fgfr1* acts directly within the sclerotome during *Scx* induction, we decided to locate sites of active FGF signaling and determine whether they coincided with *Scx* expression. To do so, we made use of phosphorylated MAPK/ERK, which identifies when and where signaling is active (Corson et al., 2003). Using an antibody specific to phosphorylated MAPK/ERK1 and MAPK/ERK2, we detected phosphorylated MAPK/ERK throughout the dermomyotome, dorsal sclerotome and myotome (Fig. 2E), with higher levels in the anterior and posterior ventral myotome, a domain reminiscent of *Frek/Fgfr4*, and in the adjacent sclerotome, where *Scx* is expressed (Fig. 2E, blue arrow). A comparison of phosphorylated MAPK/ERK with an antibody marker for myotome, myosin heavy chain, shows the clearly delineated dorsal sclerotomal domain of activated MAPK/ERK (Fig. 2F). In addition, there are elevated levels of phosphorylated MAPK/ERK in the anterior and posterior dermomyotome (Fig. 2E, purple arrow) and in the dorsal root ganglia (Fig. 2E, yellow arrow). The spatial pattern of phosphorylated MAPK/ERK during *Scx* induction, particularly within the sclerotome, further supports the model of a myotomal FGF signaling directly to the sclerotome to activate *Scx*.

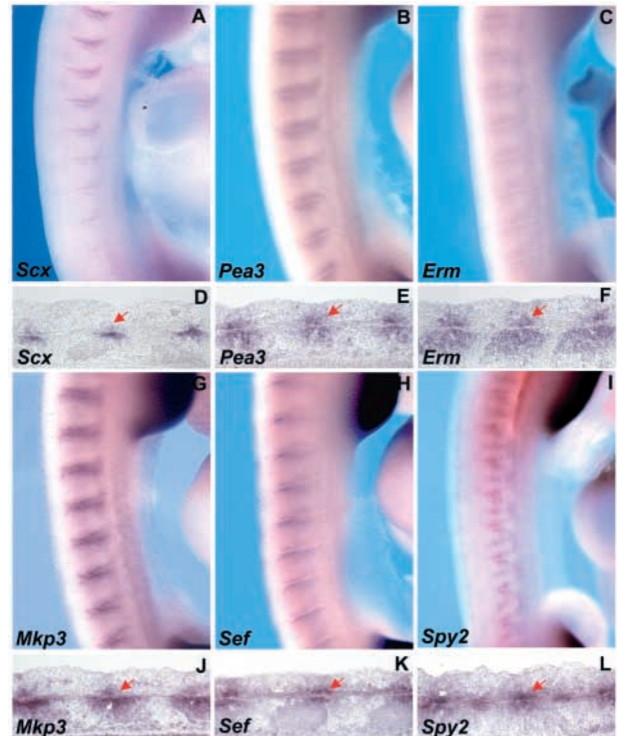

Fig. 3. *Scx* is co-expressed with several members of the *Fgf8* synexpression group. (A-C,G-I) Whole-mount in situ hybridization on HH stage 20 embryos. (D-F,J-L) Section in situ hybridization of frontal sections of HH stage 20 embryos. Comparison of *Scx* expression (A,D) with that of *Pea3* (B,E), *Erm* (C,F), *Mkp3* (G,J), *Sef* (H,K) and *Spry2* (I,L). Red arrows in D-F and J-L indicate expression in the anterior and posterior dermomyotome.

Scx is co-expressed with several members of the *Fgf8* synexpression group

If FGF*s* secreted by the myotome can directly signal to the sclerotome, the receptor most likely to be receiving the signal is *Fgfr1*; yet, as earlier pointed out, the broad expression pattern of *Fgfr1* throughout the somite challenges us to understand why FGF signaling within the sclerotome is nonetheless restricted to only the anterior and posterior regions, and excluded from the middle section abutting the myotome. An expression screen for transcription factors in mouse indicated that two members of the *Fgf8* synexpression group, *Pea3* and *Erm*, are expressed in the anterior and posterior somites (A. P. McMahon, J. Yu and T. Tenzen, unpublished). We thought a closer look at the temporal and spatial expression patterns of these two transcription factors, as well as three FGF-regulated inhibitors, *Mkp3*, *Sef* and *Spry2*, in chick embryos at HH stage 20, might provide further insight into the restricted *Scx* domain. We found that *Pea3* and *Erm* were expressed, like *Scx*, in the anterior and posterior sclerotome (Fig. 3A-C), occupying a domain that overlaps with but is also much larger than that of *Scx* (Fig. 3D-F). As in other regions where *Pea3* and *Erm* are expressed, the two form a nested pattern, with *Erm* the broader of the two – a configuration that perhaps reflects their dependence on different levels of FGF signaling (Fig. 3E,F) (Firnberg and Neubuser, 2002; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Additionally, *Pea3* and *Erm* are expressed in the anterior and posterior ventral dermomyotome (Fig. 3E,F, red arrows). *Mkp3*, *Sef* and *Spry2* are similarly expressed in the anterior and posterior sclerotome and dermomyotome (Fig. 3G-L), and *Spry2* is also expressed at the center of the myotome, where FGFs are found (Fig. 3I,L). The presence of both phosphorylated MAPK/ERK (Fig. 2E) and *Fgf8* synexpression group members within the anterior and posterior dermomyotome (Fig. 3E,F,J-L, red arrows) suggests that this region is a site of active FGF signaling. Moreover, closer investigation of *Scx* expression reveals the presence of a small group of *Scx*-positive cells in the anterior and posterior dermomyotome (Fig. 3D, red arrow). Thus, *Scx* expression closely parallels that of the members of the *Fgf8* synexpression group examined here.

Transcriptional activation by Ets transcription factors is required for induction of *Scx*

As expression of the FGF transcriptional effectors *Pea3* and *Erm* is localized, we reasoned that this pattern could explain the restricted activation of FGF signaling and, as a result, restricted *Scx* expression within the somite. To determine whether *Pea3* and *Erm* function during induction of *Scx,* we looked at *Scx* expression under conditions where their function is blocked. The well-characterized protein domain structure of *Pea3* includes an N-terminal acidic transcription activation domain and a C-terminal Ets DNA-binding domain (Fig. 4D) (Bojovic and Hassell, 2001) – each flanked by two inhibitory domains that keep *Pea3* inactive until it is

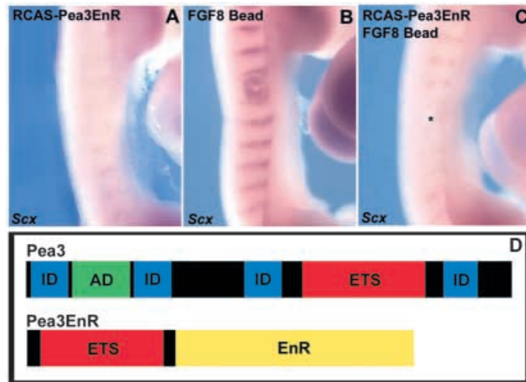

Fig. 4. Dominant-negative version of *Pea3* blocks induction of *Scx*. (A-C) Whole-mount in situ hybridization for *Scx* following infection with either RCAS-Pea3EnR (A), an FGF8 bead implant (B) or RCAS-Pea3EnR combined with an FGF8 bead implant (C, asterisk indicates bead). (D) Domain structure of mouse *Pea3* and Pea3EnR. *Pea3* contains an acidic transcriptional activation domain (green), an Ets DNA-binding domain (red), and four inhibitory domains (blue). Dominant-negative *Pea3* constructed by fusing the Ets DNA-binding domain to Engrailed (yellow). AD, activation domain; ID, inhibitory domain; Ets, Ets DNA-binding domain; EnR, Engrailed repressor.

stimulated by triggers such as MAPK/ERK phosphorylation (Fig. 4D) (Bojovic and Hassell, 2001; O'Hagan et al., 1996; Shepherd et al., 2001). To block transcriptional activation by *Pea3* and *Erm*, we constructed a dominant-negative version of *Pea3*, similar to dominant-negative constructs previously shown to function in vitro (Paratore et al., 2002; Sheperd at al., 2001), by fusing the *Pea3* DNA-binding domain to the *Engrailed* repressor domain, which acts as a strong transcriptional repressor (Fig. 4D). Because Ets DNA-binding domains are highly conserved among family members, we reasoned that our fusion construct should repress transcription of the target genes for *Pea3* as well as *Erm*, thus circumventing the possibility that a phenotype might be obscured by redundancy. Following RCAS retroviral misexpression within the psm of the dominant-negative *Pea3* construct (RCAS-Pea3EnR), we observed loss of *Scx* expression in the somite (Fig. 4A), suggesting that transcriptional activation by *Pea3* and *Erm* is necessary for *Scx* induction, and that *Scx* may be a direct or indirect target of *Pea3*, *Erm*, or both.

To further assess if *Fgf8*-dependent induction of *Scx* can occur in the absence of transcriptional activation by *Pea3* and *Erm*, we tested whether overexpression of *Fgf8* could induce ectopic *Scx* in the presence of RCAS-Pea3EnR. One day after infection of the psm with RCAS-Pea3EnR, we implanted beads soaked in FGF8 protein into infected somites, and looked at *Scx* expression 12 hours later. An *FGF8* bead alone induced strong ectopic *Scx* expression after 12 hours (Fig. 4B); however, when the bead was combined with RCAS-Pea3EnR, no *Scx* expression was observed (Fig. 4C). These results underscore the likelihood that transcriptional activation by the Ets transcription factors is necessary to mediate *FGF8*-dependent induction of *Scx*, and that FGF signal transduction within the somite leads to phosphorylation of *Pea3* and *Erm*, which then activate transcription of their targets, resulting in the induction of *Scx*.

Ectopic expression of *Pea3* is sufficient to induce *Scx* expression within range of an FGF signal

Our observation that transcriptional activation by the Ets transcription factors is required for induction of *Scx* suggests that the restricted *Scx* domain reflects the localization of *Pea3* and *Erm* within the somite. But can the restricted expression of the Ets transcription factors sufficiently account for the restricted expression of *Scx*? To answer this question, we decided to look at the effect on *Scx* when the expression domain of the Ets transcription factors was expanded. Using a retrovirus encoding full-length *Pea3* (RCAS-Pea3), we overexpressed *Pea3* throughout the somites. Upregulation of *Scx* was seen (Fig. 5A); however, strikingly, this ectopic *Scx* expression did not resemble that observed after overexpression of *Fgf8*, when *Scx* was induced throughout the sclerotome (Fig. 5F). By contrast, widespread overexpression of *Pea3* led to expanded *Scx* expression only in the dorsalmost sclerotome abutting the myotome, but not in the more ventral sclerotome [we confirmed that this absence was not due to limited infection by observing extensive viral spread throughout the dermomyotome, myotome and sclerotome (Fig. 5D)]. *Pea3* misexpression also differed from that of *Fgf8* in that *Pea3* misexpression resulted in additional ectopic *Scx* expression within the dermomyotome (Fig. 5C).

But if overexpression of *Fgf8* reveals that the entire sclerotome is competent to express *Scx* when exposed to FGFs, why does overexpression of *Pea3* fail to result in more extensive ectopic *Scx* in the sclerotome? We think the answer probably lies in the observation that the activation of target genes by *Pea3* and *Erm* depends on the conversion of these transcription factors to an active state in response to MAPK phosphorylation (Bojovic and Hassell, 2001; O'Hagan et al., 1996). Thus, despite widespread RCAS-Pea3 infection, myotomal FGFs might only be able to reach virally expressed *Pea3* in those dermomyotome and sclerotome regions abutting the myotome. The domain of *Scx* induction following RCAS-Pea3 infection could therefore be demarcating the effective range within which endogenous myotomal FGFs are able to activate virally encoded *Pea3* to a level sufficient for *Scx* expression. As our results show that most of the dermomyotome and dorsal sclerotome is normally exposed to FGF signaling and is competent to express *Scx*, it is likely that *Scx* is excluded from those regions and localized instead to the anterior and posterior sclerotome and dermomyotome precisely because of the restricted endogenous expression patterns of *Pea3* and *Erm*.

To test whether ectopic expression of *Scx* following RCAS-Pea3 infection indeed requires the presence of FGFs, we blocked FGF signaling in embryos injected with RCAS-Pea3. Sixteen hours after infection, trunks of injected embryos were placed in culture in either the presence or absence of the FGFR inhibitor, SU5402. As expected, *Scx* was expressed normally in uninjected trunks (Fig. 5I) but completely lost in the presence of SU5402 (Fig. 5J). By contrast, however, neither *Fgf8* (Fig. 5O,P) nor any other examined genes expressed during somite development, such as *Myf5* (Fig. 5M,N), were affected, demonstrating that culturing embryos in the presence of SU5402 does not result in general defects in somite development, nor in loss of myotomal FGFs. Embryos injected with RCAS-Pea3 showed upregulation of *Scx* when cultured in DMSO (Fig. 5K), but in embryos exposed to SU5402, RCAS-

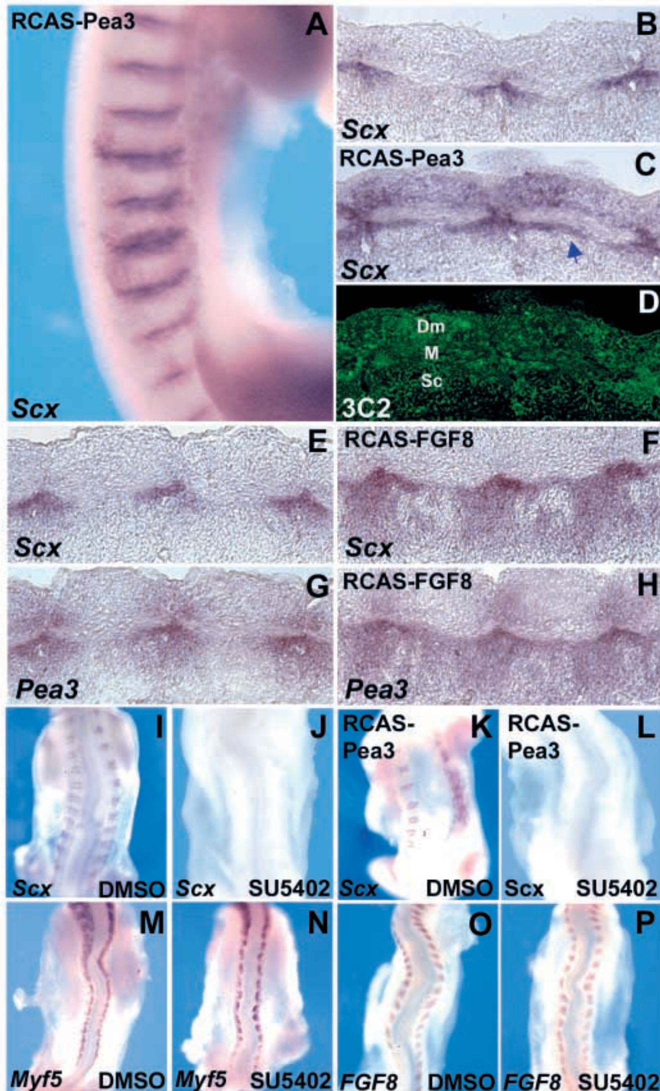

Fig. 5. Overexpression of *Pea3* results in ectopic *Scx* expression in the dermomyotome and dorsal sclerotome. (A) Whole-mount in situ hybridization for *Scx* following infection with RCAS-Pea3. (B,C) Section in situ hybridization for *Scx* on frontal sections of control (B) or RCAS-Pea3-infected embryos (C). (D) Detection of viral infection using 3C2 antibody on section shown in C. (C) Blue arrow indicates ectopic *Scx* in dorsal sclerotome. (E,H) Section in situ hybridization for *Scx* (E,F) or *Pea3* (G,H) on frontal sections of control (E,G) or RCAS-FGF8-infected embryos (F,H). (I-P) Whole-mount in situ hybridization for *Scx* (I-L), *Myf5* (M,N) or *Fgf8* (O,P) on trunks cultured in either DMSO (control) (I,K,M,O) or 30 μM SU5402 (J,L,N,P). Trunks shown in J and L were infected with RCAS-Pea3 on their right sides. Dm, dermomyotome; M, myotome; Sc, sclerotome.

Pea3-mediated ectopic induction of *Scx* was blocked (Fig. 5L), further supporting our conclusion that *Pea3* activity requires exposure to FGF signaling.

If *Pea3* is necessary for induction of *Scx*, we reasoned that we would expect *Pea3* to be present in any instance where overexpression of FGFs resulted in ectopic expression of *Scx*. As several studies have shown that transcription of *Pea3* and *Erm* is induced in response to FGF signaling (Firnberg and Neubuser, 2002; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), we decided to look at the effect of RCAS-FGF8 infection on *Pea3* expression. Following infection, we found *Pea3* expressed throughout the sclerotome (Fig. 5G,H), but not expanded in the dermomyotome – coinciding with and thereby providing a basis for understanding the ectopic expression pattern of *Scx* in response to the same manipulation (Fig. 5E,F). It thus appears that overexpression of *Fgf8* regulates ectopic *Scx* expression on two levels: *Pea3* is activated, and then, within the context of continued FGF signaling, *Pea3* goes on to activate *Scx*.

We previously showed that application of an *Fgf8*-soaked bead can induce ectopic expression of *Scx* in the sclerotome as early as 4 hours after implantation (Brent et al., 2003). If FGF-dependent induction of *Scx* is mediated by the Ets transcription factors, we hypothesized that we would be able to observe their induction, following bead implantation, prior to that of *Scx*. To test our assumption, we implanted *Fgf8*-soaked beads into the somites of HH stage 18 embryos, and then observed expression of *Pea3*, *Erm* and *Scx* at different times. As expected, all three were strongly induced at 12 hours following implantation (Fig. 6A-C). However, after 4 hours, only weak *Scx* expression (Fig. 6F), and stronger expression of *Pea3* and *Erm* (Fig. 6D,E), were observed. Moreover, 3 hours after bead implantation, while *Pea3* and *Erm* were still detectable, *Scx* was not (Fig. 6G-I), indicating that *Pea3* and *Erm* are indeed induced prior to *Scx*. Interestingly, we observed that after bead implantation, the *Erm* expression domain was broader than that of *Pea3*, mirroring the endogenous nested domains of *Pea3* and *Erm* expression in the somite.

Myotomal FGF signaling establishes the expression domains of *Pea3*, *Erm* and *Scx*

Having demonstrated that FGF-dependent induction of *Scx* in the somite requires transcriptional activation by *Pea3* and *Erm*, and that the *Pea3* expression domain, combined with an effective range of FGF signaling, is sufficient to explain restriction of *Scx* expression to the syndetome, we next sought to determine how the *Pea3* expression domain within the somite is initially established. In addition to modulating the activation state of the Ets transcription factors, FGF signaling has been shown to be both necessary and sufficient for expression of *Pea3* and *Erm* in other regions of the embryo (Firnberg and Neubuser, 2002; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). As we had already established that *Fgf8* is capable of inducing *Pea3* and *Erm* within the somite (Fig. 5H; Fig. 6A,D,G), we now asked if FGF signaling is also required. To determine this, we placed trunks of HH stage 16 embryos in culture for 24 hours, in either the presence or absence of the *Fgfr* inhibitor SU5402. Although the control embryos showed normal expression of *Pea3* and *Erm* (Fig. 7A,C), in those treated with SU5402, expression of the transcription factors was never seen (Fig. 7B,D). Our results confirm that FGF signaling is both necessary and sufficient for activation of *Pea3* and *Erm* in the somite.

To establish that FGF signaling occurs simultaneously and consistently with induction of the Ets transcription factors, we compared the expression patterns of *Fgf8* and *Pea3*. It has been shown that *Fgf8* expression is dynamic, beginning in the psm and newly formed somites, then moving ventral to dorsal as the somite develops (Dubrulle et al., 2001; Stolte et al., 2002).

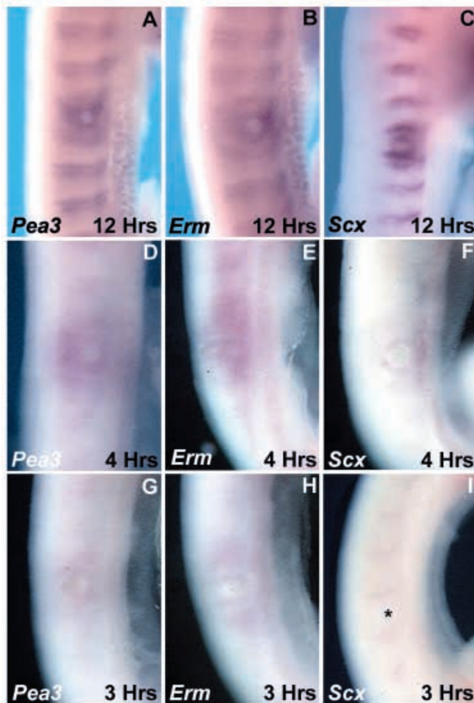

Fig. 6. *Pea3* and *Erm* are induced prior to *Scx* following implantation of an *Fgf8* bead. Whole-mount in situ hybridization for *Pea3* (A,D,G), *Erm* (B,E,H), or *Scx* (C,F,I) following implantation of an *Fgf8*-soaked bead for 12 (A-C), 4 (D-F) or 3 hours (G-I). Asterisk in F indicates location of bead.

By somite stage VII, *Fgf8* is expressed in the anteromedial corner of the forming myotome, and by somite stage IX, expression accumulates at the center of the myotome, where myofiber differentiation also occurs (Fig. 7E,H) (Stolte et al., 2002). Mirroring *Fgf8*, *Pea3* expression is also highly dynamic, commencing in the psm and newly formed somites (data not shown). By somite stage X, shortly after *Fgf8* expression becomes restricted to the myotome, *Pea3* is seen at the anterior and posterior borders of the dermomyotome and sclerotome (Fig. 7F), and by somite stage XVI, this domain has become even more apparent (Fig. 7F). Thus, it is only after localization of *Fgf8* to the myotome that expression of *Pea3* in the anterior and posterior sclerotome and dermomyotome appears.

As previously reported, expression of *Scx* in the anterior and posterior dorsal sclerotome is clearly seen by somite stage XVI (Fig. 7G) (Brent et al., 2003), and persists in this domain as morphogenesis of the axial tendons occurs (Brent et al., 2003). To determine if *Scx* induction is, like that of *Pea3*, also associated with an accumulation of *Fgf8* in the myotome, we decided to look for *Scx* expression at earlier somite stages. Continued staining indeed revealed expression in the more posterior somites, albeit quite weak. By somite stage XII, *Scx* is detected in the anterior and posterior sclerotome (Fig. 7G,J, blue arrow), after *Fgf8* becomes restricted to the myotome (Fig. 7H) and *Pea3* to the sclerotome (Fig. 7I). In addition, by somite stage XII, *Scx* is seen in the anterior and posterior dermomyotome (Fig. 7J, red arrows). Interestingly, the sclerotome domains of *Scx* and *Pea3* in somite stages XII and XIII (Fig. 7I,J) appear much broader than those in and after somite stage XVI (Fig. 3D,E), an observation that possibly reflects the expression patterns of *Fgf8* at these same somite stages. Expression of *Fgf8* thus initially occupies a greater proportion of myotome (Fig. 7H), perhaps allowing the secreted FGFs to reach further ventrally into the sclerotome. But by somite stage XVI, *Fgf8* expression becomes localized to the center of the myotome (Fig. 1B), thereby limiting the distance that the secreted FGFs can travel. There is also some detectable *Scx* expression in the newly formed somites, in a domain coinciding with that of *Fgf8* and *Pea3* during somitogenesis (data not shown); however, because the level of expression in these posterior-most somites is so weak, it is difficult to determine whether *Scx* remains on after somite formation and continues to follow the dynamic expression patterns of *Fgf8* and *Pea3*, or turns off and then on again at a later somite stage. In either case, by somite stage XII *Scx* is detectable within the sclerotomal domain that it will occupy throughout axial tendon development. A comparison of the spatial and temporal dynamics of *Fgf8*, *Pea3* and *Scx* thus

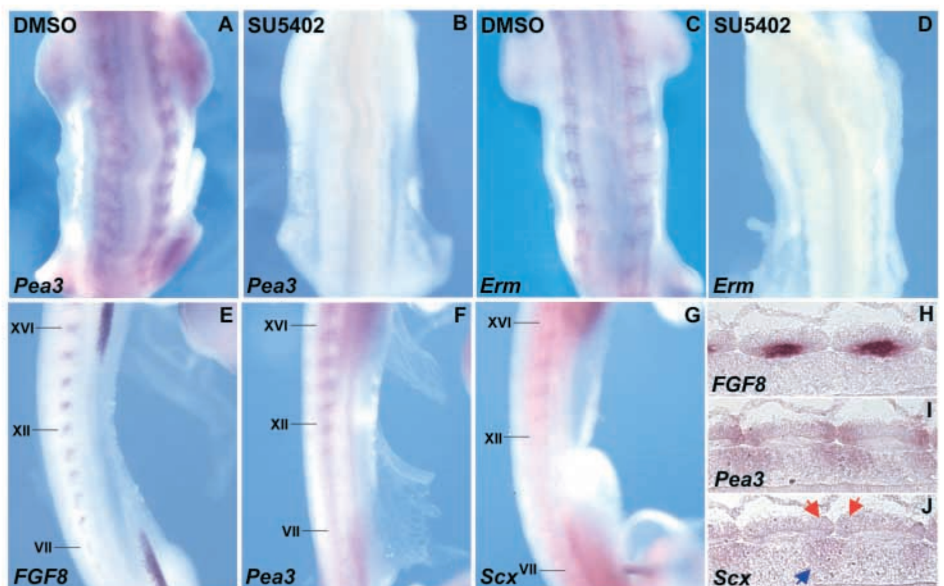

Fig. 7. *Fgf* signaling is required for expression of *Pea3* and *Erm* in the somites. Whole-mount in situ hybridization for *Pea3* (A,B) or *Erm* (C,D) on trunks cultured for 24 hours with either DMSO (control) (A,C) or 30 μM SU5402 (B,D). (E-G) Whole mount in situ hybridization for *Fgf8* (E), *Pea3* (F) or *Scx* (G) in HH stage 17 embryos; somite stages VII, XII and XVI are indicated. (H-J) Section in situ hybridization for *Fgf8* (H), *Pea3* (I) or *Scx* (J) on alternate frontal sections of somite stages XII and XIII in a stage 17 embryo. (J) Red and blue arrows, indicate dermomyotomal and sclerotomal *Scx*, respectively.

suggests that it is the myotomal expression domain of *Fgf8* that plays a role in establishing expression first of *Pea3* in the anterior and posterior dermomyotome and sclerotome, and then of *Scx* in the same domain.

Discussion

Localized expression of Ets transcription factors in the somite defines the domain of *Scx* expression

In this study, we have demonstrated that the Ets transcription factors *Pea3* and *Erm* are co-expressed with *Scx* in the anterior and posterior dorsal sclerotome, and that transcriptional activation of target genes by *Pea3* and *Erm* is both necessary and sufficient for induction of *Scx* in the somite. Moreover, we show that FGF-mediated induction of *Scx* cannot occur without transcriptional activation by the Ets transcription factors, and that *Pea3*-dependent activation of *Scx* requires FGF signaling. Based on these findings, we propose the following model for induction of *Scx* and establishment of the somitic tendon progenitors. Onset of FGF expression in the myotome results in activation of an FGFR, most probably *Fgfr1* (Fig. 8A, green arrow). A cascade of phosphorylation events (Fig. 8A, red arrows) ensues, culminating in transcription, within the anterior and posterior sclerotome and dermomyotome, of both the Ets transcription factors *Pea3* and *Erm*, in a nested pattern (Fig. 8C), and of the inhibitors *Mkp3*, *Sef* and *Spry* (Fig. 8A, black arrow). As the somite matures, FGFs secreted from the center of the myotome diffuse to the surrounding dermomyotome and sclerotome (Fig. 8C). When these signals reach the cells expressing *Pea3* and *Erm* in the anterior and posterior sclerotome and dermomyotome, the FGF signal transduction cascade causes phosphorylation and subsequent activation of the Ets transcription factors (Fig. 8B). In turn, the Ets transcription factors regulate transcription of their target genes, resulting in direct or indirect activation of *Scx* (Fig. 8B). The observation that *Scx*, *Pea3* and *Erm* occupy progressively broader nested domains (Fig. 8C) suggests that each is regulated by particular levels of FGF signaling, with *Scx* requiring the highest and *Erm* the lowest; within this context, the inhibitors *Mkp3*, *Sef* and *Spry* (Fig. 8B) might be the factors responsible for regulating the varied levels of FGF signaling in the somite. The position of the tendon progenitors is thus determined by the combined forces of an effective range of FGF signaling (Fig. 8C) and expression of the Ets transcription factors within that range. However, although it is clear that activation of the target genes is required for *Scx* expression, we do not know if that expression is controlled directly by *Pea3* and *Erm*, or if there are intermediate players. The rapid induction of *Pea3*, *Erm* and *Scx* after implantation of an *Fgf8*-soaked bead suggests that *Scx* may be a direct target of their signaling; consistent with this view, analysis of sequence upstream of the mouse *Scx* start site reveals several potential Ets transcription factor binding sites (data not shown). Nonetheless it has not been determined whether these sites represent actual *Pea3*- or *Erm*-binding sites.

Interestingly, there appears to be a continuous requirement for FGF signaling in the regulation of somitic *Scx* expression. Our trunk culture experiments, in which trunks were cultured in the presence or absence of the FGFR inhibitor SU5402, revealed that when FGF signaling was completely blocked, *Scx* expression was lost at all axial levels, including those somites

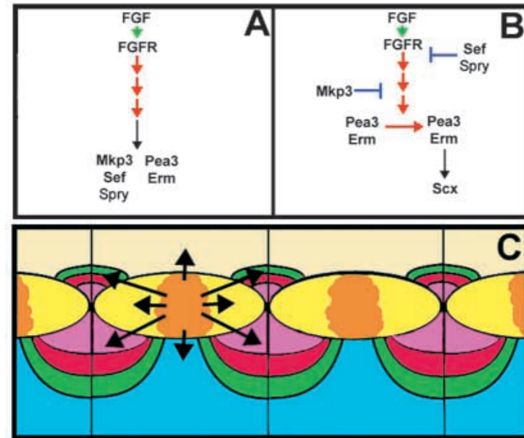

Fig. 8. Model for FGF-dependent activation of *Pea3* and *Erm*, and subsequent induction of *Scx* in the somite. (A) FGF signaling leads to expression of Ets transcription factors *Pea3* and *Erm* and inhibitors *Mkp3*, *Sef* and *Spry* in the anterior and posterior sclerotome and dermomyotome. FGFs secreted by the myotome bind to and activate an FGFR (green arrow). Receptor activation results in series of phosphorylation events (red arrows), culminating in direct or indirect transcriptional activation (black arrow) of target genes such as *Pea3*, *Erm*, *Mkp3*, *Sef* and *Spry*. (B) Once *Pea3* and *Erm* expression domains have been established, further FGF signaling triggers phosphorylation and subsequent activation of *Pea3* and *Erm*, which, in turn, activate transcription of target genes resulting in *Scx* expression. (C) Schematic of four somites, frontal view: dermomyotomes are beige; myotomes are yellow; sclerotomes are aqua. FGFs expressed in center of myotome (orange) can diffuse to surrounding dermomyotome, myotome and dorsal sclerotome (arrows). FGF signaling here results in expression of *Pea3* (red) and *Erm* (green), in a nested pattern, within anterior and posterior dermomyotome and dorsal sclerotome. *Scx* expression (purple) is induced when myotomal FGFs signal to the *Pea3*- and *Erm*-expressing dermomyotome and sclerotome.

already expressing *Scx* when placed in culture. Moreover, implantation of an SU5402-soaked bead into somites in which *Scx* expression was either just assuming its sclerotomal domain (somite stages XI and XII), or already present in the anterior and posterior sclerotome (somite stages XV and XVI), resulted in loss of *Scx* expression within 6 hours (data not shown). *Pea3* showed similar loss of expression following inhibition of FGF signaling. It thus seems likely that continuous FGF signaling from the myotome is essential to the maintenance as well as induction of *Scx* expression in the somite, and that continuous interactions between the muscle and tendon lineages are essential to the formation of the tendonous attachments.

Finally, in addition to the syndetome, we note here that a small population of *Scx*-expressing cells can be seen in the anterior and posterior ventral dermomyotome (Fig. 8C). It is at this point unclear whether these *Scx*-expressing cells represent a dermomyotomal population of tendon progenitors, or whether they are an extension of cells from the syndetome. In either case, these *Scx*-expressing cells overlap with *Pea3* and *Erm* in the anterior and posterior ventral dermomyotome (Fig. 8C), and analysis of phosphorylated MAPK/ERK expression suggests that active FGF signaling is also taking place. It will be interesting to determine which components of the axial tendons, if any, arise from this additional domain.

Overexpression of *Pea3* reveals regional competence for *Scx* expression

Overexpression of *Fgf8* during somite development results in ectopic expression of *Scx* throughout the sclerotome, but not in the dermomyotome or myotome (Brent et al., 2003), demonstrating that, upon exposure to FGF signaling, the entire sclerotome is competent to adopt a tendon cell fate. Our present study builds upon this finding by showing that ectopic induction of *Scx* in the sclerotome following overexpression of *Fgf8* is triggered by FGF-induced expansion of *Pea3* within the sclerotome, combined with the expanded FGF signaling needed to activate *Pea3*. By contrast, overexpression of *Pea3* results in ectopic expression of *Scx* not only in the sclerotome but throughout the dermomyotome – a result never seen after *Fgf8* overexpression. The inability of overexpressed FGFs to upregulate *Pea3* in the dermomyotome may be at least partially responsible for the restriction of ectopic *Scx* induction to the sclerotome. Because the ability of *Pea3* to control expression of target genes requires activation by the FGF signaling pathway, the induction of *Scx* throughout the dorsal sclerotome, as well as in the dermomyotome following overexpression of *Pea3*, provides a readout for the minimal distances to which myotomal FGFs can diffuse within the somite. Thus, although FGFs can reach the entire dermomyotome and dorsalmost sclerotome, *Scx* is not normally expressed throughout those regions, at least in part because *Pea3* and *Erm* are not present (Fig. 8C).

If overexpression of *Pea3* reveals that endogenous myotomal FGFs are able to reach the entire dermomyotome at levels sufficiently high to induce ectopic *Scx* expression, why is the expression of *Pea3* and *Erm*, which appear to require lower levels of FGFs for their induction, not normally found in the dermomyotome? Likewise, if the Ets transcription factors are induced in response to FGF signaling, why does overexpression of *Fgf8* fail to result in ectopic expression of *Pea3* throughout the dermomyotome? Although we do not yet know the molecular mechanisms underlying these observations, they do suggest that there are additional levels of regulation within the dermomyotome that control the ability of its cells to respond to FGFs by activating target genes such as *Pea3* and *Erm*. As the dermomyotome is clearly competent to express *Scx* when *Pea3* is present, that same regulating mechanism which prevents the entire dermomyotome from expressing *Pea3* and *Erm* is also functioning to prevent those dermomyotome cells from expressing markers for a tendon cell fate.

It is particularly striking that, following overexpression of *Pea3*, the ectopic expression of *Scx* in the dermomyotome extends a greater distance from the source of the myotomal FGFs than does the ectopic expression of *Scx* in the dorsal sclerotome. This difference suggests either that the myotomal FGFs are able to diffuse more freely in the dermomyotome, or that the endogenous myotomal FGFs lack the capacity to override the cartilage-inducing signals that direct the ventral somite to adopt its cartilage fate. In either case, overexpression of *Pea3* reveals that, just as the majority of the dermomyotome appears to have mechanisms in place to block the expression of *Pea3*, *Erm* and *Scx* in response to the myotomal FGFs, the sclerotome has mechanisms in place to prevent the myotomal FGFs from extending too far ventrally, thus possibly interfering with cartilage formation. In fact, limiting the number of cells that can adopt a tendon cell fate in the sclerotome may be an important aspect of somite patterning. Previously, we have shown that the two lineages arising from sclerotome, the cartilage and tendons, are mutually exclusive, and that cartilage-inducing signals function to repress tendon-inducing signals and vice versa (Brent et al., 2003). In particular, we have found that FGF signaling negatively regulates expression of *Pax1*, a cartilage marker, underscoring that the extent of exposure of the sclerotome to FGF signaling may be critical.

The possibility that regulation of the levels of FGF signaling plays a role in *Scx* induction is supported by our observation that three intracellular inhibitors of FGF signaling, *Mkp3*, *Sef* and *Spry2*, are co-expressed with *Scx*, thus perhaps functioning to lower the level of signaling in the *Pea3*- and *Erm*-expressing cells. These inhibitors might also act to restrict the extent of *Scx* expression in the anterior and posterior sclerotome. Although all three inhibitors are thought to act intracellularly in cells receiving FGF signals, *Spry* has additionally been shown in *Drosophila* to have indirect non-cell autonomous effects on surrounding regions (Hacohen et al., 1998). Such downstream responses might also function during somite development to control the FGF signaling range.

Interestingly, overexpression of *Pea3* does not appear to result in *Scx* expression within the myotome – where FGF signaling is also active. The inability of the myotome to express *Scx* could be indicative either of its early acquisition of a determined state relative to the sclerotome (Dockter and Ordahl, 1998; Williams and Ordahl, 1997), or of its exposure to higher levels of FGFs. In the case of the latter, high levels of FGFs might be required to control proliferation and differentiation in the myotome (Kahane et al., 2001; Marics et al., 2002), while lower levels in the sclerotome could be necessary for tendon progenitor formation. However, the different outcomes of FGF signaling could be a reflection of the diverse activities of the FGFRs. As both *Frek/Fgfr4* and *Fgfr1* are expressed in the myotome, signaling through both receptors might regulate myotomal functions, whereas, in the sclerotome, signaling solely through *Fgfr1* could result in induction of *Scx*.

Direct versus indirect FGF signaling

Based on our observations that FGFs can activate *Scx* in the absence of myotome, and that transducers of FGF signaling are co-expressed with *Scx*, we believe that FGFs most probably signal directly to the *Scx*-expressing cells; because, to our knowledge, *FGFR1* is the only receptor expressed in the sclerotome, we conclude that *Scx* expression is activated in the sclerotome through this receptor. The broad expression pattern of *Fgfr1*, combined with our observation that, following overexpression of *Pea3*, FGFs are able to extend beyond the *Scx* expression domain in both the sclerotome and dermomyotome, suggest that other components of the FGF signaling cascade undergo localization in order to prevent widespread *Scx* induction. Indeed, if downstream effectors of *Fgf* signaling, such as *Pea3* and *Erm*, were not restricted, FGFs would be able to signal directly through the broadly expressed *FGFR1*, consequently activating target genes, such as *Scx*, in inappropriate regions. Interestingly, there does appear to be an upregulation of *Fgfr1* at the site of *Scx* expression, perhaps reflecting either an additional mechanism for restriction of *Scx*

to that region, or a positive-feedback effect of increased FGF signaling.

Nonetheless, although our findings implicate *Fgfr1* in the regulation of *Scx* expression, a role for *Frek/Fgfr4* cannot be ruled out. Several studies have attempted to sort out the different functions controlled by the individual receptors, using either dominant-negative truncated (Brent et al., 2003; Itoh et al., 1996) or soluble receptors (Marics et al., 2002). Each, however, may have nonspecific effects: truncated receptors can dimerize with and block signaling through the other FGF receptors, and soluble receptors can interfere with the activities of any other FGF receptor binding to the same ligand. Because both *Fgfr1* and *Frek/Fgfr4* are expressed in the somites and are likely to bind to the same FGF ligands, neither approach is capable of distinguishing their individual functions. It thus remains possible that in addition to the likely role of *Fgfr1* in directly receiving the FGF signal within the sclerotome, *Frek/Fgfr4* may also play a part, perhaps regulating the expression or position of *Scx* in the sclerotome, or even preventing *Scx* expression in the myotome in response to FGF signaling.

Myotomal FGF signaling regulates gene expression in the syndetome

As has been demonstrated in several systems, FGF signaling in the somite is both necessary and sufficient to establish the nested expression domains of *Pea3* and *Erm*. Once their domains are in place, *Pea3* and *Erm* continue to depend on FGF signaling to activate expression of their target genes. Thus, FGF signaling makes two important contributions to tendon progenitor formation: controlling expression of *Pea3* and *Erm*, and regulating their activity as transcriptional effectors (Fig. 8A,B). Interestingly, expression of *Pea3*, *Erm*, and *Scx* in the anterior and posterior sclerotome and dermomyotome appears to be a response to myotomal expression of *Fgf8*: while *Fgf8* is expressed throughout somite development, *Pea3*, *Erm* and *Scx* only become restricted to their respective sclerotomal and dermomyotomal domains after *Fgf8* has become localized in the myotome. In mouse, it has been shown that expression of FGFs in the myotome is directly controlled by a myotome-specific enhancer activated by the myogenic determinancy factors *Myf5* and *Myod*; thus, it is only upon differentiation that the myofibers express FGFs (Fraidenraich et al., 2000; Grass et al., 1996). Additionally, there is evidence in both mouse and chick that sonic hedgehog signaling arising from the ventral midline is required for expression of the myotomal FGFs (Fraidenraich et al., 2000; Huang et al., 2003).

It is clear that FGF expression in the myotome is central to the regulation of gene expression in the syndetome, and that the localized activity of factors acting downstream of the activated FGFR, such as *Pea3* and *Erm*, results in restricted expression of genes such as *Scx* within the syndetome, thereby defining the boundaries of the syndetome. We have shown that despite widespread expression of *Fgfr1*, once *Pea3* and *Erm* have become circumscribed to the anterior and posterior dorsal sclerotome encompassing the syndetome, their restricted expression, combined with the presence of continued FGF signaling, restricts *Scx* activation to the syndetome. But although we have identified a role for *Pea3* and *Erm* acting downstream of FGF signaling to produce restricted activation of target genes, it must be emphasized that, in addition to transducing FGF signaling, *Pea3* and *Erm* actually depend on FGFs for their own induction. Thus, a new question is introduced: how does myotomal FGF signaling regulate expression of *Pea3* and *Erm* within the anterior and posterior sclerotome and dermomyotome? The combined expression, only within the anterior and posterior dorsal sclerotome, and ventral dermomyotome, of *Scx*, members of the *Fgf8* synexpression group and phosphorylated MAPK/ERK, suggests that there is a very specific region of localized, active FGF signaling in the somite. But what is striking is that while the focus of this signaling – within the anterior and posterior dorsal sclerotome and ventral dermomyotome – does not correspond with the source of the ligand at the center of the myotome, the secreted FGFs from the center of the myotome nonetheless activate FGF signal transduction only within the anterior and posterior somite to produce restricted expression of *Pea3* and *Erm*. The fact that FGF signaling is most active in and around the syndetome suggests that the induction of *Pea3* and *Erm* within the anterior and posterior sclerotome is not the result of a simple diffusion gradient of FGFs from the center of the myotome. Instead, these observations indicate a good deal of complexity underlying when and where FGFRs are activated in the somites, and suggest that additional mechanisms for regulating FGF signaling must be present to ensure reception specifically in the anterior and posterior somite encompassing the syndetome. One of these mechanisms might involve control of FGF translation and secretion. Although *Fgf8* mRNA is localized to the center of the myotome, it remains possible that FGF8 protein is either specifically expressed in the anterior and posterior myotome, or preferentially secreted from those regions of the myotome, thus increasing the exposure of the anterior and posterior sclerotome and dermomyotome to FGF signals. Other levels of regulation might include either localized expression within the anterior and posterior somite of a co-receptor required for FGFR activation, or localized expression of components of the extracellular matrix, such as heparan sulfate proteoglycans, that could act to restrict or potentiate FGF signaling within those domains. Finally, it remains possible that the upregulation of FGFR1 expression within the syndetome is sufficient to restrict FGF signaling to this region. Although it is as yet uncertain which, if any, of these mechanisms for controlling the spatial distribution of active FGF signaling plays a role during activation of *Pea3*, *Erm* and, later, *Scx* within the syndetome, it is clear that the domains of these three FGF-responsive genes are dependent on more than just the expression patterns of ligand and receptor.

Anterior and posterior localization of somitic tendon progenitors and formation of the vertebral motion segment

The fact that the future muscle lineage signals to the future cartilage lineage to induce the tendon progenitors at the border between the two, ensures that the developing axial tendons will be in position to form the attachments associated with the axial musculoskeletal system. Motility of the vertebral column is ensured during differentiation of the somite derivatives through the process of somite resegmentation, in which the position of the future vertebrae relative to the somite boundaries shifts one half segment (Brand-Saberi and Christ, 2000). Chick-quail chimera and cell-labeling experiments have shown that a single

somite gives rise to the anterior and posterior halves of two adjacent vertebral bodies as well as the intervertebral tissues (Aoyama and Asamoto, 2000; Bagnall et al., 1988; Huang et al., 2000; Huang et al., 1996). By contrast, the myotome and syndetome do not undergo resegmentation, with the result that a single somite provides the information for one segmental epaxial muscle, including its tendon attachments (Aoyama and Asamoto, 2000; Bagnall et al., 1988; Brent et al., 2003; Huang et al., 2000; Huang et al., 1996). Because the connection of one epaxial segmental muscle to two adjacent vertebrae allows for free movement of the vertebral column, the somite has been described as generating the ‘vertebral motion segment’ a functional unit consisting of two adjacent vertebrae, the intervertebral tissues, and the tendons, ligaments and muscles that act on that segment (Huang et al., 1996). But in order for the segmented epaxial muscles derived from a single somite to properly attach to the resegmented vertebrae, it is crucial that the tendons develop at the anterior and posterior ends of the segmented muscle. Thus, the restriction of FGF-dependent induction of *Scx* to the anterior and posterior somite is essential to proper development of a functional and fully motile axial musculoskeletal system.

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