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

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Accepted 17 May 2004

Development 131, 3885-3896
Published by The Company of Biologists 2004
doi:10.1242/dev.01275

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 (Finnbogadóttir 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; Maillieux 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 dermatomyotome – 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 paraaffin wax, and 10 μm sections were collected. Probes included chick Scx (Schweitzer et al., 2001), chick Fgf8 (Brent et al., 2003), quail Prc (Brent et al., 2003), chick Fgfr1, chick Pea3 (RT-PCR product using primers 5’ ACGTC-TAGAGTGCATAAATACATAGG 3’ and 5’ AGCGAAATTCTC-ATGAGGTTAAGCTTGGC 3’), chick Erm (RT-PCR product using primers 5’ ACGTCTAGCCCGCCACGCC-TGGCCG 3’ and 5’ AGCGAAATCAGATGGAAGCC-CCCTTCG 3’), chick Mkp3 (ChEST246m1 obtained from MRC genescience), chick Sef (ChEST52Sk13 obtained from MRC genescience), 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 phosho-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’ ACGGCTCTCCCCTATGCGCCAGGAGTGAAG 3’ and 5’ ACGG-ATCTCCTAGATAGATACCCCTG 3’), and the mouse Pea3 Ets DNA binding domain (RT-PCR product using primers 5’ ACGG-ATTCTCCCATGCAGCCCGGTTGCC 3’ and 5’ ACGG-ATTCCGGTCTCGACACACACACATTTGATAC 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).

Dermatomyotome 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
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/Fgfr4 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 myotomally expressed FGF signaling directly to the sclerotome to activate Scx.

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

If FGFs 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
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 injection, 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-
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 (Firmberg 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 Fgfl 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 Fgfl-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 Fgfl-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 Fgfl 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 Fgfl and Pea3. It has been shown that Fgfl 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).
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. 7I, red arrows). Interestingly, the sclerotome domains of Scx and Pea3 in somites XII and XIII (Fig. 7J) 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...
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 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.

![Fig. 8. Model for FGF-dependent activation of Pea3 and Erm, and subsequent induction of Scx in the somite.](image-url)

(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.
**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 sufficient 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 FGFs. 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 Fgfr8: while Fgfr8 is expressed throughout somite development, Pea3, Erm and Scx only become restricted to their respective sclerotomal and dermomyotomal domains after Fgfr8 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 determinacy 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 Fgfr8 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 Fgfr8 mRNA is localized to the center of the myotome, it remains possible that Fgfr8 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 Scl to the anterior and posterior somite is essential to proper development of a functional and fully motile axial musculoskeletal system.

The authors thank the McMahon laboratory for sharing unpublished data, and Andrew McMahon, Romen Schweitzer and Jennifer Mansfield for their critical readings of the manuscript and for many helpful discussions. Tendon research in C.J.T.’s laboratory is supported by PO1 DK56246 from the NIH. A.E.B. is supported by an NSF Predoctoral Fellowship.

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