Ventral axial organs regulate expression of myotomal Fgf-8 that influences rib development

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Received for publication 26 October 2001, revised 25 September 2002, accepted 8 November 2002

Abstract

Fgf-8 encodes a secreted signaling molecule mediating key roles in embryonic patterning. This study analyzes the expression pattern, regulation, and function of this growth factor in the paraxial mesoderm of the avian embryo. In the mature somite, expression of Fgf-8 is restricted to a subpopulation of myotome cells, comprising most, but not all, epaxial and hypaxial muscle precursors. Following ablation of the notochord and floor plate, Fgf-8 expression is not activated in the somites, in either the epaxial or the hypaxial domain, while ablation of the dorsal neural tube does not affect Fgf-8 expression in paraxial mesoderm. Contrary to the view that hypaxial muscle precursors are independent of regulatory influences from axial structures, these findings provide the first evidence for a regulatory influence of ventral, but not dorsal axial structures on the hypaxial muscle domain. Sonic hedgehog can substitute for the ventral neural tube and notochord in the initiation of Fgf-8 expression in the myotome. It is also shown that Fgf-8 protein leads to an increase in sclerotomal cell proliferation and enhances rib cartilage development in mature somites, whereas inhibition of Fgf signaling by SU 5402 causes deletions in developing ribs. These observations demonstrate: (1) a regulatory influence of the ventral axial organs on the hypaxial muscle compartment; (2) regulation of epaxial and hypaxial expression of Fgf-8 by Sonic hedgehog; and (3) independent regulation of Fgf-8 and MyoD in the hypaxial myotome by ventral axial organs. It is postulated that the notochord and ventral neural tube influence hypaxial expression of Fgf-8 in the myotome and that, in turn, Fgf-8 has a functional role in rib formation.

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Keywords: Fgf-8; Somite; Myotome; Notochord; Neural tube; Sonic hedgehog; MyoD; Chick embryo

Introduction

Secreted polypeptides of the fibroblast growth factor (Fgf) family are important mediators of intercellular signaling during animal development. Fgf-8 has proven to be an especially versatile signaling molecule, acting at many places and times throughout the course of development. It is active during gastrulation and is involved in setting up asymmetry and in the development of heart, brain, branchial arches, and limbs (Boettger et al., 1999; Crossley et al., 1996a,b; Furthauer et al., 1997; Joyner et al., 2000; Moon and Capecchi, 2000; Reifers et al., 2000; Shamim et al., 1999; Shanmugalingam et al., 2000; Sun et al., 1999; Trumpp et al., 1999; Vogel et al., 1996). Fgf-8 is prominently expressed in the presomitic mesoderm, where it controls somite formation (Dubrulle et al., 2001). Although Fgf-8 expression has been observed in the somite, its regulation and function in the somite are still unclear (Stolte et al., 2002).

Epithelial somites are repetitive segmental units that form from the unsegmented paraxial mesoderm in response to numerous signals from the surrounding embryonic structures (Christ et al., 1992; Dietrich et al., 1997; Marcelle et al., 1997; Pourquier et al., 1995 and 1996; for reviews, see
Borycki and Emerson, 2000; Brand-Saberi et al., 1996; Christ and Ordahl, 1995). The dorsal somite develops into the dermomyotome, while the ventral somite forms the sclerotome (Christ et al., 1978). The definitive myotome forms from the dermomyotome in response to signals from the notochord, neural tube, surface ectoderm, and lateral plate. Inductive influences from the notochord (Brand-Saberi et al., 1993; Pourquie et al., 1993; see Cossu et al., 1996b, for review) influence the ventral somite to become the sclerotome, that gives rise to vertebrae and ribs (Huang et al., 2000). Sonic hedgehog protein (Shh) (Johnson et al., 1994) is a principle mediator of these actions on the dorsal and ventral domains of the somite, having effects not only on myogenesis and chondrogenesis, but also on somitic cell survival and proliferation (Cann et al., 2000; Teillet et al., 1998; Teillet and Le Douarin, 1983). As a result of these influences from the ventral axial organs, the dorsal neural tube, surface ectoderm, and lateral plate, the definitive myotome establishes a dorsomedial (epaxial) and a ventrolateral (hypaxial) domain (Ordahl and Le Douarin, 1992; for review, see Ordahl et al., 2000). The epaxial myotome differentiates into the intrinsic back muscle, while the hypaxial myotome gives rise to the musculature of the ventrolateral body wall and the limbs.

The prevailing view is that the axial structures (notochord and neural tube) are essential for the formation of epaxial, but not hypaxial musculature (Rong et al., 1992; Teillet et al., 1998). Studies show that a separation of the notochord from early somites results in a down-regulation of myogenic regulatory factors in the epaxial region of the myotome (Borycki et al., 1998); and that in the Danforth Short-tail (sd) mouse mutant, where the notochord degenerates during somite formation, Myf5 fails to be activated in the epaxial myotome, whereas it is expressed normally in the hypaxial region (Asakura and Topscott, 1998). Likewise, in Shh null mutant mice, neither Myf5 nor MyoD is activated in the epaxial domain, whereas they are expressed in the hypaxial domain (Borycki et al., 1999). However, late effects on limb muscle formation in Shh null mouse mutant suggest that Shh may have a survival or proliferative function in the hypaxial limb musculature (Krüger et al., 2001). While it may be correct that those aspects of myogenesis regulated by Shh are predominately focused on the epaxial musculature, we have sought to determine whether it may also have an important role in the regulation of other signaling molecules that are crucial for hypaxial muscle and sclerotome-derived tissue formation.

Principal among the other signaling molecules that are important in somite cell fate are the Fgf family of receptors and their ligands (reviewed by Borycki and Emerson, 2000). Fgf family members have a role in regeneration (Floss et al., 1997), and both muscle and chondrogenic tissues contain receptors for Fgf family members. For example, myogenic cell lines expressed Fgfr-1 (Templeton and Hauschka, 1992), and this receptor is found in the myotome, limb bud, and other muscle groups (Grote et al., 1996). The Fgf receptors, Fgfr1 in particular, also have a role in the formation of the musculature of the limb, where their signaling is necessary to maintain myoblast number and organization (Flanagan-Steet et al., 2000). Mutations in receptors for Fgf are responsible for a number of cranial facial and limb skeletal syndromes in humans and animals, demonstrating the importance of these receptors and their ligands (see Burke et al., 1998). Fgf receptors are expressed in the forming ribs (Stark et al., 1991) and in a specific sequence during the chondrogenesis of the limb bones (Szébenyi et al., 1995). Thus, the Fgf family of receptors and their ligands play key roles in the formation of the skeletal elements, both those that derive from the somites (ribs) and those that derive from the limb bud (limb bones).
Fig. 2. Excision of the dorsal neural tube results in a shortened dermomyotome and myotome. (A) Diagram of operative scheme. (B, b) After reincubation for 1 day, the expression pattern of Fgf-8 is confined (small arrowheads) within the operated region (outlined by large arrowheads). (b) Transverse section through operated region of embryo shown in (B). On the operated side, dermomyotome and myotome are shortened (arrowheads) and the myotome is thickened. Nevertheless, expression of Fgf-8 is initiated in the absence of the dorsal neural tube. (C, c) After removal of the complete neural tube half on the right side, Fgf-8 is not expressed on that side in the operated region (arrowheads). (c) Transverse section through operated region. Organization of paraxial mesoderm is severely confined on the operated (right) side. (D, d) Pax-3 is not expressed in the epaxial domain after 1 day of reincubation, but is expressed in the hypaxial domain. The operated region is indicated by a red bracket. (d) Transverse section through operated region of embryo shown in (D). (E, e) MyoD expression is lacking in the medial part of the epaxial domain. The operated region is marked by a red bracket. (e) Transverse section through operated region of embryo shown in (E). Abbreviations: nc, notochord; nt, neural tube (the neural tube in Fig. 2d and e is outlined).
Fig. 3. Blocking signals from the lateral plate mesoderm by insertion of aluminum or gold foil alters the expression pattern of Fgf-8 (A–C) and MyoD (D–F) in the paraxial mesoderm. (A) Left (control) side of an embryo after 1 day of reincubation. Note the typical expression pattern of Fgf-8 and MyoD reflecting the longitudinal arrangement of muscle fibers in the myotome (arrowheads). (B) Right (experimental) side of embryo shown in (A). Large arrowheads outline regions where the barrier was placed. Expression pattern of Fgf-8 is dorsoventrally shortened (small arrowheads). Line indicates level of transverse section shown in (C). (C) Transverse section through manipulated region of embryo shown in (B). Note the dorsoventrally shortened expression domain of Fgf-8 on the side with the barrier (right) side. Faint MyoD expression detected in red color can be seen in the dorsal and ventral end of the myotome. (D–F) Expression pattern of MyoD is disorganized in the operated (right) side (E). (D) Control (left) side of embryo shown in (E). (F) Transverse section through operated region. Note the missing hypaxial expression of MyoD. Abbreviations: nc, notochord; nt, neural tube.

Fig. 4. Ablation of surface ectoderm results in altered expression of Fgf-8 in the myotome. (A) Control side of embryo after 1 day of reincubation. Arrowheads show normal expression of Fgf-8 in the myotomes. (B) Manipulated side of embryo shown in (A). Note condensed expression pattern in the somites (small arrowheads) where the ectoderm was removed (outlined by large arrowheads). Line indicates level of transverse section shown in (C). (C) Transverse section through operated region of embryo shown in (B). On the operated side, Fgf-8-expressing cells are clustered (arrowheads), instead of dispersing dorsoventrally within the myotome (see left side). Arrows indicate Fgf-8 expression in the nephron. (D) Control side and (E) operated side of embryo after 1 day of reincubation. Expression of MyoD in the myotome is shortened but stronger within the operated region (outlined by arrowheads). (F) Transverse section of the embryo in (E) showing no ventrolateral expansion of MyoD expression in the hypaxial domain. The myotome is much thicker than that in control side. Abbreviations: nc, notochord; nt, neural tube; lb, leg bud; wb, wing bud.
In this study, we have examined the regulation of Fgf-8 expression in the myotome. We performed gain-of-function and loss-of-function experiments to determine the role of Fgf-mediated signaling during late somite development, especially in rib formation. Using specific ablation experiments, we were able to discern between ventral, dorsal, and lateral signals in controlling Fgf-8 expression in the myotome. Our results show that Shh activates Fgf-8 expression not only in the epaxial but also in the hypaxial myotome, providing experimental evidence for a patterning influence of ventral axial structures on the hypaxial muscle compartment. High concentration of Fgf-8 introduced into mature somites leads to an altered expression of dermomyotomal and myotomal markers. However, Fgf-8 did not prevent their expression in the myotome, and the introduction of Fgf signaling inhibitors did not induce any change in muscle cell gene expression profile. However, either low or high concentrations of Fgf-8 resulted in significant increase in sclerotomal cell division and lead to increased rib cartilage formation. In contrast, FGF signaling inhibitor reduced the rate of cell division in this compartment and led to truncation of the ribs. We suggest that axial organs regulate the development of the ribs through their influence on the expression of FGF8 in the hypaxial myotome.

Materials and methods

Maintenance and staging of embryos

Fertilized eggs of White Leghorn chickens (Gallus gallus) were obtained from a local hatchery and incubated to the desired embryonic stage under controlled temperature (37.8°C) and humidity conditions (75%). Embryos were staged according to Hamburger and Hamilton (1951). Developmental stages are indicated by “HH-stage” followed by stage number.

Ablation of notochord–floor plate complex

After opening the vitelline membrane, neural tube and overlying ectoderm were cut open along the dorsal midline with a tungsten needle (see Fig. 1A). Two longitudinal cuts were made on both sides of the ventrolateral neural tube laterally to the floor plate, isolating the notochord–floor plate complex from the remaining neural tube tissue. A segment of the complex was then removed with a tungsten needle or fine forceps.

Ablation of dorsal neural tube

The vitelline membrane was opened along the dorsal midline. A tungsten needle was used to make a cleft between neural tube and adjacent paraxial mesoderm, either on one or both sides, for unilateral or bilateral excision of the dorsal neural tube, respectively (see Fig. 2A). The neural tube was then opened along the dorsal midline, and a longitudinal cut was made along the ventrolateral portion of the neural tube, thereby isolating the dorsal neural tube tissue, which was removed with a tungsten needle or fine forceps.

Ablation of surface ectoderm

The surface ectoderm was cut along the boundary between neural tube and paraxial mesoderm to isolate the ectoderm on the control side from the ectoderm on the experimental side. Surface ectoderm on the experimental side was then removed with a tungsten needle, and the defect was covered with gold foil to prevent regrowth of ectoderm.

<p>| Table 1 |
| Effects of dorsal neural tube excision on somite Fgf-8 expression |</p>
<table>
<thead>
<tr>
<th>Excision of dorsal neural tube</th>
<th>Normal expression</th>
<th>Expression lost in operated region</th>
<th>Altered expression pattern</th>
<th>Altered pattern + partial loss of expression</th>
<th>Not determinable</th>
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<tr>
<td>bilateral (n = 4)</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>unilateral (n = 6)</td>
<td>0</td>
<td>2a</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>total (n = 10)</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
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</table>

* Spina bifida.

<p>| Table 2 |
| Somitic expression of Fgf-8 after barrier insertion between segmental plate and lateral plate mesoderm |</p>
<table>
<thead>
<tr>
<th>Reincubation time</th>
<th>Normal expression</th>
<th>Expression domain dorsoventrally shortened</th>
<th>Expression downregulated</th>
<th>Not determinable</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day (n = 3)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2 days (n = 11)</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>total (n = 14)</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
Separation of the paraxial mesoderm from the lateral plate mesoderm

A longitudinal slit through all three germ layers was made between the paraxial and lateral plate mesoderm (Fig. 3A). Then, a gold or aluminum foil barrier was inserted into the slit.

Beads for Shh protein

Shh protein solution was prepared as described (Cann et al., 1999). Blue Affigel beads (BioRad Laboratories) were washed in Tris–NaCl buffer (pH 8.5) and incubated in 8 mg/ml Shh protein solution for at least 1 day at 4°C. As negative controls, Blue Affigel beads were incubated in 8 mg/ml of BSA in PBS for the same length of time. Shh beads were implanted immediately after notochord and floor plate ablation.

Beads for Fgf-8 protein

Purified recombinant mouse Fgf-8 protein (R&D Systems) was diluted in PBS to a concentration of 1–2.5 μg/μl. We used heparin-coated acrylic beads (diameter: 200 μm; Sigma) or, in one instance, Blue Affigel beads (BioRad Laboratories). The beads were incubated in the Fgf-8 solution for 1 h to 2 weeks at 4°C before implantation. Control beads were incubated in PBS for the same length of time. Beads were implanted into embryos at HH-stage 15–19 at the interlimb level.

Beads for SU 5402 Fgf signaling inhibitor

AG 1-X2 Resin carrier beads (diameter: 100 μm; BIO-RAD) were incubated for a minimum of 1 h up to 2 days in 10 mM SU5402 (Calbiochem; Mohammadi et al., 1997) dissolved in dimethyl sulfoxide. Control beads were incubated in dimethyl sulfoxide. The beads were then washed in Minimum Essential Medium Alpha (Sigma) several times to remove dimethyl sulfoxide. Beads were implanted into embryos at HH-stage 15–19 at the interlimb level.

Whole-mount in situ hybridization

Embryos were washed in DEPC–PBT and fixed in DEPC–PBT with 4% paraformaldehyde (PFA) overnight at 4°C. In situ hybridization was performed as described by Nieto et al. (1996). Briefly, fixed embryos were dehydrated with methanol, then rehydrated in PBT and treated with proteinase K. Proteinase K digestion was performed at room temperature for a duration of 1 min per developmental stage according to Hamburger and Hamilton (1951). From HH-stages 25 and up, digestion time was increased as follows: HH-stage 25: 30 min, HH-stage 26: 40 min, HH-stage 27: 1 h, HH-stage 28–30: 1.5 h. Proteinase K was used at a working concentration of 20 μg per ml DEPC–PBT. After proteinase K treatment, the embryos were washed in DEPC–PBT and refixed in DEPC–PBT containing 4% PFA and 0.25% glutaraldehyde for 20 min. After fixation, embryos were washed in DEPC–PBT and prehybridized in hybridization buffer (50% formamide, 5× SSC, 0.1% Chaps (Sigma), 0.1% Triton X-100, 5 mM EDTA, 50 μg/ml heparin, 1 mg/ml tRNA, 20 mg/ml Blocking Reagent (Roche) at 65°C. After 1–2 h, the hybridization solution was changed, and the embryos were incubated overnight at 65°C.

In situ hybridization was performed with one or two, occasionally three RNA probes differentially labeled with digoxigenin or fluorescein. Hybridization was performed at 65°C, for a minimum duration of 24 h. Probes were used at concentrations between 50 and 150 ng per ml of hybridization buffer. After hybridization, embryos were washed several times in 2× SSC and 0.2× SSC to remove unbound probe. To minimize nonspecific binding of the antibody fragments, embryos were incubated in KTBT (150 mM NaCl, 20 mM KCl, 1% Triton X-100, 25 mM Tris, pH 7.3) containing 10% horse serum for approximately 4 h at room temperature. After preblocking, embryos were incubated in fresh KTBT containing 10% horse serum and alkaline phosphatase-conjugated Fab fragments against digoxigenin or fluorescein (Roche) at a 1:2000 dilution (overnight at 4°C). Embryos were stained at room temperature with 4.5 μl NBT (Roche) and 3.5 μl BCIP (Roche) per ml alkaline phosphatase buffer (0.1 M Tris, pH 9.5, 50 mM MgCl₂, 0.1 M NaCl, 1% Triton X-100). For Fast Red staining, embryos were stained by using Fast Red tablets (Roche) dissolved in 0.1 M Tris, pH 8.0, containing 1% of Triton X-100. If more than one probe was to be detected, the Fast Red staining was performed first, and the alkaline phosphatase Fab fragment of the first detection round was inactivated with 30% glacial acetic acid in methanol. The embryos were then incubated

Table 3

<table>
<thead>
<tr>
<th>Operated region, reincubation time</th>
<th>Normal expression</th>
<th>Expression dorsoventrally shortened</th>
<th>Expression downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>segmental plate, 1 day (n = 6)</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>segmental plate, 2 days (n = 4)</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>trunk somites, 1 day (n = 2)</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>trunk somites, 2 days (n = 2)²</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total (n = 14)</td>
<td>0</td>
<td>11</td>
<td>3</td>
</tr>
</tbody>
</table>

² Fgf-8 only detectable in transverse sections.
FGF-8 bead implantation

Pax-1

Pax-3

Myf5

MyoD

Myogenin

SU5402 bead

Pax-1

Pax-3

Myf5

MyoD

Myogenin
in the second antibody, and staining was performed as described above. Stained embryos were stored at 4°C in PBS containing 4% paraformaldehyde. The following probes were used for in situ hybridization: cFgf-8 (800 bp; Crossley et al., 1996b), cMyf5, cMyoD (1518 bp; Lin et al., 1989), cMyogenin (1200 bp; Fujisawa-Sehara et al., 1990), cPax1 (Ebensperger et al., 1995), qPax3 (950 bp), and cShh (Riddle et al., 1993).

Preparation of cryostat sections

One of two methods was used to stain the cartilage/bone. (1) Fixed embryos were washed in 0.1 M potassium phosphate buffer at 4°C over the course of 1 h. Embryos were then immersed in 0.1 M potassium phosphate buffer containing 5% sucrose and then in 0.1 M potassium phosphate buffer containing 15% sucrose, each time for several hours. Embryos were embedded in Jung tissue freezing medium (Leica) and frozen with liquid nitrogen. Frozen embryos were sectioned to 35 μm with a Jung CM 3000 cryostat (Leica). (2) Embryos were fixed in ethanol for 1–3 days and then stained in 0.015% Alcian Blue made in 80% ethanol and 20% glacial acetic acid for 9 days. Subsequently, embryos were cleared in 100% methyl salicylate. After photography, embryos were prepared for paraffin section of 8-μm thickness.

BrdU method

Proliferative activity was determined by the BrdU method. After Fgf-8 (n = 6), SU5402 (n = 5), or control (n = 2 for each substance) bead implantation and 1-day reincubation, embryos were incubated in ovo with 0.2 ml of a 40 mM 5-bromo-2′-deoxyuridine (BrdU) solution in PBS for 30 min. After rinsing, the embryos were fixed in a mixture of 3% glacial acetic acid and 97% methanol and embedded in paraffin. The sections were stained with anti-BrdU antibody (Dako).

For quantitative evaluation, a systematic random sample of histological sections through the beads was obtained which included every third (Fgf-8) or second (SU 5402) section. In a ring around the beads of 5-cell-diameter thickness, BrdU-positive nuclei were counted in the region of the sclerotomes. Total counts over all sections within each specimen were compared by using the Mann–Whitney U test at P < 0.05 significance level.

Results

Regulation of Fgf-8 expression in the myotome

Notochord and floor plate are necessary to activate Fgf-8 expression in the epaxial and hypaxial myotome

To determine whether signals from the ventral axial organs (notochord and floor plate) are required for the myotomal expression of Fgf-8, the notochord–floor plate complex was excised in the segmental plate region of HH-stage 10–14 embryos, i.e., before somite formation and onset of somitic Fgf-8 expression (Fig. 1A). The operated embryos were fixed after 1 day (HH-stage 17–20) and examined for Fgf-8 expression by whole-mount in situ hybridization. To demonstrate that the notochord–floor plate complex was completely removed, Shh expression was also examined in operated embryos. Excision of the notochord–floor plate complex resulted in the complete absence of Fgf-8 expression in the paraxial mesoderm (Fig. 1B). Even after longer reincubation (2 days), expression of Fgf-8 could not be detected in the somites (Fig. 1C, c). In operated animals, the only expression of Shh was in the endodermal epithelium (Roberts et al., 1995, 1998). In nonmanipulated embryos of comparable stage, Fgf-8 was strongly expressed in all somites.

Fig. 5. Application of Fgf-8 to somites. Beads soaked in 2.5 μg/μl Fgf-8 were implanted at stages 15–19 at the interlimb level. (A) Expression of Pax-1 was not affected after Fgf-8 implantation for 1 day and was identical to (B) implantation of the control bead. (C) Transverse section of embryo following implantation of Fgf-8 bead showing normal expression of Pax-1 in the sclerotome. (D) Expression of Pax-3 is altered near the Fgf-8 bead (arrowhead) after 1 day. (E) Expression of Pax-3 after implantation of control bead after 1 day. (F) Transverse section of embryo with Fgf-8 bead on left and control bead on right side. Expression of Pax-3 is lower and ventral extension is reduced (arrowhead). (G) Expression of Myf5 is altered in both epaxial (white arrowhead) and hypaxial (arrow) myotome following Fgf-8 bead implantation after 1 day. (H) Expression of Myf5 is altered following control bead implantation after 1 day. (I) Transverse section showing expression of Myf5 after implantation of Fgf-8 bead on left and control bead on right. Myf5 expression is lower in both epaxial (arrowhead) and hypaxial (arrow) myotome. Note the diffuse hypaxial expression of Myf5 compared with compact domain in the control side (white arrowhead). (J) Expression of MyoD following implantation of Fgf-8 bead for 1 day. Expression is altered in both the epaxial (arrowhead) and the hypaxial (arrow) myotome. (K) Expression of MyoD following implantation of control bead for 1 day. (L1–L2) Transverse section showing expression of MyoD after implantation of Fgf-8 bead on left (L1) and control bead on right side (L2) after reincubation of 1 day. MyoD expression is reduced in the epaxial (arrowhead) and almost totally depleted in the hypaxial domain (arrow). (M) Expression of Myogenin following Fgf-8 bead implantation after 1 day. Expression was lower (arrowhead) compared with (N) implantation of control bead. (O) Transverse section showing expression of Myogenin 1 day after implantation of Fgf-8 bead (left) and control bead (right). Fgf-8 caused a reduction in the levels of Myogenin transcripts (arrow). (P) Expression of Myogenin following Fgf-8 bead implantation after 2 days. Expression was reduced in both the epaxial (arrow) and hypaxial (arrowhead) compared with levels in embryo following the implantation of control bead (Q). Transverse section showing expression of Myogenin 2 days after implantation of Fgf-8 bead (left, R1) and control bead (right, R2). Expression of Myogenin is down-regulated (arrow) by Fgf-8 (R1).

Fig. 6. Implantation of Fgf signaling inhibitor SU5402. Beads soaked in 10 mM SU5402 implanted at HH-stage 15–19 at interlimb level for 1 day (A, C, E, G). Control beads implanted for an identical period (B, D, F, H). No altered expression of Pax-1 (A, B), Pax-3 (C, D), Myf5 (E, F), or MyoD (G, H) was detected.
MyoD expression was obtained after longer reincubation (60 h). After 60 h reincubation, the effects on the expression of Fgf-8 could not be assessed, because in the trunk region, Fgf-8 is naturally downregulated at that stage (Stolte et al., 2002). This demonstrates that notochord and floor plate are essential for the activation of Fgf-8 expression in the epaxial as well as in the hypaxial compartment of the myotome.

Shh protein can replace notochord and floor plate in the activation of Fgf-8 expression

We hypothesized that the regulatory effect of the notochord and the floor plate on somitic Fgf-8 expression could be mediated by Shh, which is secreted by the ventral axial organs (Echelard et al., 1993). Moreover, Borycki et al. (1998) have shown that Shh protein is sufficient to restore expression of MyoD after ablation of floor plate and notochord. To determine whether the expression of Fgf-8 is regulated by Shh, we excised the notochord–floor plate complex in the segmental plate region of HH-stage 10 and HH-stage 14 embryos and implanted beads soaked in Shh protein into the paraxial mesoderm of the operated region (n = 3, Fig. 1F, f). The embryos were reincubated for 1 day, and expression of Fgf-8 and MyoD was analyzed by single or double whole-mount in situ hybridization. In all embryos, Fgf-8 was strongly expressed in the myotomes of one to two segments cranially and caudally to the bead (Fig. 1F, f). MyoD was up-regulated in the myotomes, several segments cranially and caudally to the Shh bead as well (Fig. 1G, g). Fgf-8 and MyoD were activated in some embryos at longer, and in others, at shorter distances from the bead, due to the differences in bead size and quantity of protein. To control for the nonspecific effects of bead implantation, beads soaked with bovine serum albumin (BSA) were implanted into the segmental plate of control embryos after removal of the notochord–floor plate complex (n = 3).

As expected, mesodermal Fgf-8 expression was absent from the operated region in embryos with BSA beads (n = 3) (not shown). These results show that hypaxial expression of Fgf-8 is regulated by Shh.

Fgf-8 protein is not able to initiate transcription of Fgf-8 in the absence of ventral axial organs

To determine whether or not Fgf-8 can activate its own expression in the absence of inducing signals from the ventral axial organs, we excised the notochord–floor plate complex and implanted a bead soaked in purified Fgf-8 protein into the paraxial mesoderm of the operated region. Excision of ventral axial organs and Fgf bead implantation were performed in the segmental plate of HH-stage 10 embryos, which were then reincubated for 1 day before assay for Fgf-8 expression. Fgf-8 expression was not detected in the operated region, suggesting that the growth factor is not able to induce its own expression in the absence of ventral axial organs (Fig. 1H).

Signals from the dorsal neural tube are necessary for dermomyotome morphogenesis but dispensable for myotomal Fgf-8 expression

Because the dorsal neural tube is a known source of signals patterning the dorsal somite (Dietrich et al., 1997; Marcelle et al., 1997; Münsterberg and Lassar, 1995; Stern et al., 1995, 1997), we determined whether the dorsal neural tube was required for somitic expression of Fgf-8. The dorsal neural tube was excised either on one (unilateral excision; Fig. 2A), or both sides of the embryo (bilateral excision) at HH-stages 11–13, reincubated for 1 day, and analyzed by whole-mount in situ hybridization with a Fgf-8 probe (Table 1). After dorsal neural tube removal, Fgf-8 was expressed in the somites in an altered pattern in 40% of the manipulated embryos (4 of 10). The amount of Fgf-8 expression appeared to relate to the amount of ventral neural tube that remained. In these embryos, expression appeared to be absent from the medial domain (Fig. 2B). Transverse sections through the operated regions of these embryos revealed a shortened dermomyotome and a compromised expression pattern of Fgf-8 (Fig. 2B, b). In the remaining embryos, Fgf-8 was either completely absent from the operated region (30%, 3 of 10), or short stretches with altered expression pattern alternated with stretches of complete absence (20%, 2 of 10). Two embryos that did not express Fgf-8 on one side showed a spina bifida in the operated region. Transverse sections prepared from these two embryos showed that, at some positions, one complete half, rather than just the dorsal portion of the neural tube, had been ablated (Fig. 2C, c). In those embryos lacking neural tubes, no proper dermomyotome and myotome structures were observed and Fgf-8 expression was mostly absent. At positions where ventral, but not dorsal neural tube was present, dermomyotome, myotome, and Fgf-8 expression were all present. We interpret these results to indicate that Fgf-8 expression is not dependent on the dorsal neural tube.
To more precisely determine the effects of dorsal neural tube excision, transverse sections were prepared from three embryos whose dorsal neural tube had been removed in the trunk region before the formation of somites. A total of 120 sections from the operated regions from three embryos were pooled and analyzed. A positive correlation was found between presence of neural tube tissue and morphological differentiation of the dermomyotome. The more neural tube tissue ablated along its dorsoventral dimension, the less frequently a morphologically distinct dermomyotome formed and the fewer Fgf-8-positive cells were found in the paraxial mesoderm. In the region of dorsal neural tube ablation, the dermomyotome was shortened in its mediolateral extension. Although the position of these shortened dermomyotomes was shifted ventrolaterally, a medial dermomyotome lip and a myotome were observed in most cases. Concomitantly with shortened dermomyotomes, the myotomes were thicker in some sections (Fig. 2b). When both dorsal and ventral neural tubes were excised on one side, most sections did not show a morphologically distinct dermomyotome nor expressed Fgf-8.

We also examined markers for the dermomyotome, Pax-3, and the myotome, MyoD, in somite following unilateral removal of the dorsal neural tube and a reincubation period of 1–2 days. The expression of the dermomyotome marker Pax-3 was also shortened in its mediolateral extension and located more ventrolaterally (Fig. 2D, d, e). Pax-3 expression in the operated embryos (n = 6) showed that the more the unilateral neural tube was removed, the less the Pax-3 expression extended in the epaxial domain. Similar phenomenon was observed in the operated embryos with MyoD expression (n = 5, Fig. 2E, e).

**Separation of the lateral plate mesoderm from the paraxial mesoderm alters the spatial distribution of Fgf-8-expressing myotomal cells**

The lateral plate mesoderm is a known source of signals participating in somite patterning (Dietrich et al., 1998; Pourquier et al., 1995, 1996). Therefore, to determine whether the lateral plate mesoderm has a role in regulating myotomal expression of Fgf-8, we separated the lateral plate from the paraxial mesoderm by a gold or aluminum foil barrier. The operation was performed in the segmental plate region of HH-stage 10–14 embryos, which were reincubated for 1 or 2 days and analyzed by whole-mount in situ hybridization for the expression of Fgf-8 (Fig. 3). While the level of Fgf-8 expression on the side with the barrier did not appear to be different from the control side, 57% of the manipulated embryos (8 of 14) exhibited an alteration in their somitic Fgf-8 expression pattern (Table 2) (Fig. 3B and C). Instead of showing its typical, somewhat fuzzy appearance reflecting the longitudinal alignment of myofi bers in the myotome (Fig. 3, arrowheads), expression on the manipulated side was condensed and shortened in its dorsoventral extent (Fig. 3B, small arrowheads). This observation was confirmed by transverse sections through the operated regions of the stained embryos (Fig. 3C). Analysis of MyoD expression after 2 days of reincubation showed that barrier insertion prevented a ventrolateral extension of MyoD expression in the hypaxial domain (Fig. 3). Thus, the lateral plate is required for normal morphological formation of the myotome, but not for the expression of Fgf-8.

**The surface ectoderm is necessary for the spatial distribution of Fgf-8-expressing cells and for the extension of the hypaxial myotome domain**

The surface ectoderm, which has been shown to be necessary for the development of the hypaxial myotome domain (Cossu et al., 1996a; Dietrich et al., 1998), was extirpated to determine whether this tissue influences Fgf-8 expression. The ectoderm overlying the paraxial mesoderm was removed and replaced with gold foil to prevent ectodermal regrowth. The manipulation was performed in the interlimb region either before (HH-stage 10–12) or after (HH-stage 16) the formation of thoracic somites. The manipulated embryos were analyzed for Fgf-8 (Fig. 4A, B, c) or MyoD (Fig. 4D–F) expression after 1 and 2 days of reincubation. Table 3 shows an overview of the results for the expression of Fgf-8 in the somites. Removal of the surface ectoderm resulted in a condensed and dorsoventrally shortened expression domain of somitic Fgf-8 in 79% of the operated embryos (n = 14) (Fig. 4B and C), similar to the pattern observed after separation of the lateral plate mesoderm. Fgf-8 expression in the somites appeared to be down-regulated in the remaining 21%. Regardless of whether surface ectoderm was removed before or after formation of somites, Fgf-8 was expressed in a dorsoventrally shortened pattern compared with the control side. Moreover, this expression domain was much thicker in its mediolateral extent than in its normal counterpart. The accumulation of Fgf-8-positive cells appeared to correspond to the region where epaxial and hypaxial myotome regions meet (Fig. 4A–C). Analysis revealed that this altered expression domain of Fgf-8 colocalized with myotomal MyoD expression (Fig. 4D–F). Whereas both genes were found to be expressed in the epaxial myotome and in the region close to the epaxial–hypaxial border, no Fgf-8- or MyoD-positive cells were observed in more ventral locations. This suggests that, after ectoderm removal overlying the segmental plate or epitelial somites, the myotome did not extend ventrolaterally. Instead, MyoD- and Fgf-8-positive cells accumulated at the epaxial–hypaxial border, resulting in the observed thickened expression pattern. Thus, the surface ectoderm is not required for Fgf-8 expression.

**Fgf-8 does not regulate its own expression in the myotome**

To determine whether Fgf-8 protein can regulate its own expression in the somite, heparin acrylic beads coated with purified Fgf-8 protein were implanted next to recently formed somites 20–22 of HH 14 embryos (n = 9), before the onset of myotomal Fgf-8 expression. After 1 day of
reincubation, no change in the expression of Fgf-8 could be detected, indicating that Fgf-8 expression in the somite neither positively nor negatively regulates its own expression.

**Role of myotomal Fgf-8 during somite differentiation**

**Application of Fgf-8 to chick somites only has effects at high concentrations**

To examine the functional relevance of myotomal Fgf-8, we implanted beads soaked in Fgf-8 into mature somites and determined the consequences of this manipulation in adjacent tissues with a panel of molecular markers.

Fgf-8 implantation for 1 day resulted in an alteration in the Pax-3 expression pattern in the ventrolateral lip of the dermomyotome (3 of 4, Fig. 5D and E). Transverse section revealed that the Pax-3-expressing cells appeared to be less compact compared with the sham-operated side (Fig. 5F). Expression of Myf5 was altered 1 day after the application of Fgf-8-soaked bead (6 of 8, Fig. 5G and H). The Fgf-8 bead had caused a bulge ventral to the bead (Fig. 5I). Myf5 expression was affected in both the epaxial and hypaxial regions. In the epaxial domain, expression was detected at slightly lower levels compared with those in sham-operated embryos. In addition, the dorsal extension was also reduced. Ventral to the bead, Fgf-8 prevented the development of a Myf5-expressing hypaxial myotome. However, we were able to detect dispersed expression of the gene in the expanded tissue. Unlike Pax-3 and Myf5, MyoD was unequivocally down-regulated in the epaxial domain 1 day after Fgf-8 bead implantation (7 of 7, Fig. 5J–L2). A similar profile was seen after 2 days (2 of 6; not shown). The extent rather than levels of expression of MyoD were reduced in the hypaxial region (Fig. 5L1 and L2). Expression levels of Myogenin were slightly lower on the operated side compared with the control side (Fig. 5M–R2; n = 6/8). These subtle effects were not detected following implantation of beads soaked in Fgf-8 at a concentration of 1 μg/μl (Pax-3, n = 14; Myf5, n = 10; MyoD, n = 29; Myogenin, n = 5).

We also used SU 5402-soaked beads (Mohammadi et al., 1997), an inhibitor of Fgf receptor, to determine whether blocking Fgf signaling would have any effect on these genes. Implantation of beads soaked in 10 μM SU 5402 failed to affect the expression of Pax-1, Pax-3, Myf-5, or MyoD (Fig. 6). However, implantation of beads at an identical concentration into the developing limb inhibited outgrowth.

These results show that overexpression of Fgf-8 results in a down-regulation of myogenic markers in the epaxial domain. In the hypaxial region, both the dermomyotome and myotome were less compact and did not extend as laterally as the sham-operated side. However, application of SU5402 had no effect on the expression of either dermomyotomal or myotomal marker genes. These results suggest that Fgf signaling does not play a significant role in the development of dermomyotome and myotome at embryonic stages investigated in this study.

**Influence of Fgf signaling on cell proliferation in the sclerotome**

Having failed to reveal dramatic effects on the dermomyotome and myotome following Fgf-8 implantation, we concentrated on sclerotome development. One day after the implantation of Fgf-8 beads, expression of the sclerotome marker Pax-1 was found to be unaltered compared with the levels of expression and distribution found in both unoperated regions as well as in regions implanted with beads soaked in PBS (n = 3, Fig. 5A and C). These results suggest that Fgf-mediated signaling does not play a major role in the early development of the sclerotome.

We examined the effect of Fgf-8 on cell proliferation in the sclerotome by implanting beads soaked in either 1 μg/μl (n = 3) or 2.5 μg/μl (n = 5) and assaying for the incorporation of BrdU (n = 5). Implantation of Fgf-8-soaked beads had two effects on somite development: cell proliferation was increased in the sclerotome directly adjacent to the Fgf-8 source; in addition, the dermis overlying the bead appeared to be less compact and dorsomedial and ventrolateral lips were less distinct (Fig. 7A–C). Implantation of beads soaked in 10 μM SU 5402 had a markedly different effect than Fgf-8 (n = 8; Fig. 7D). Application of SU 5402 resulted in a considerable decrease in the amount of BrdU label around the bead compared with the unoperated side and control operation, and the embryos also had epaxial and hypaxial lips that resembled the control side. Furthermore, application of SU 5402 led to lower amounts of dermal tissues than on the unoperated side. Implantation of a control bead did not have any effect on BrdU incorporation. Thus, Fgf-8 increases proliferation in sclerotomal cells. This observation was confirmed by quantitative evaluation (see Fig. 9): all six Fgf-8 values (three at 1 μg/μl, three at 2.5 μg/μl) were well above control counts, but only four of five SU 5402 values were well below, one slightly above control counts.

**Effect of Fgf signaling in rib development**

Implantation of Fgf8-coated beads for long durations (3–5 days) had no effect on myogenesis, but markedly affected rib development. To determine the long-term consequence of Fgf-8, beads coated with Fgf-8 protein (1 μg/μl) or SU5402 were implanted into the paraxial mesoderm of HH 12–15 embryos (approximately on the level of somite pair 20, shortly before or shortly after somite formation in this region). In each embryo, one Fgf-8 bead was implanted into the right paraxial mesoderm and one control bead (PBS) into the left paraxial mesoderm. The embryos were reincubated for 5 to 7 days, fixed around HH 35, and subjected to cartilage staining for morphological analysis of sclerotomal derivatives and for muscle development using...
Fig. 7. Cell proliferation and Fgf signaling. BrdU incorporation following implantation of beads soaked in 2.5 μg/μl Fgf-8 (A–C) or 10 mM SU5402 (D). Asterisk marks bead position. (A–C) Increased sclerotomal BrdU incorporation adjacent to the beads. The dorsal medial lip of the dermomyotome is less clearly evident (arrowhead) due to expanded subectodermal mesenchyme (arrows). (D) SU5402 results in lower BrdU incorporation adjacent to bead (arrow). The dermomyotome has very distinct morphology (arrowhead), and very little subectodermal mesenchyme (arrow) is found.

Fig. 8. Fgf-8 and the Fgf-inhibitor, SU 5402, cause alterations in rib morphology. (A, C) Implanted with control bead (left side of embryo). (B, D) Implanted with beads soaked in Fgf-8 1 μg/μl (B) or SU 5402 10 mM (D). (B) Cartilage formation in the rib anlagen is expanded and bifurcated (red arrow). (D) Conversely, deletions in the rib cartilage are seen in proximal region of the rib adjacent to the SU5402 bead (Red arrow). (E) Coronal section through thoracic region showing expanded rib anlage (arrowhead). Position of Fgf-8 bead shown by arrow. (F) Transverse section through thoracic region showing discontinuous rib anlage (arrowhead). Position of the SU5402 bead shown by arrow.
in situ hybridization for MyoD expression and desmin immunohistochemistry. There were no significant differences between Fgf-8 bead implanted embryos and controls (n = 16; data not shown) with regard to these markers of myogenesis. However, significant effects were detected in the morphology of the ribs in 9 of 9 embryos examined, which had been implanted with Fgf-8 at 1 μg/μl, and 5 of 5 implanted with beads of higher concentration (2.5 μg/μl, n = 5). Two rib phenotypes were detected at the lower concentration: 6 out of 9 showed thickening of the ribs (Fig. 8B and E), whereas the remaining three had bifurcation of ribs, often accompanied with shortening of the rib in the ventral domain (Fig. 8B and F). Abnormal distribution of myogenic cells was only detected in regions where rib development was perturbed (Fig. 8E and F). In contrast to increased cartilage formation caused by Fgf-8, SU 5402 caused the loss of cartilage in most cases (5 of 10), with the proximal part of the ribs failing to undergo the chondrogenesis. Interestingly, the distal aspect of the ribs developed normally (Fig. 8C and D). At the control side, we did not find any morphological alteration.

Discussion

Regulation of Fgf-8 expression in the myotome

Sonic hedgehog from the ventral axial organs regulates epaxial and hypaxial expression of Fgf-8

The formation of the hypaxial domain of the myotome appears to be dependent on signals from the notochord and ventral neural tube. In matured somites, expression of Fgf-8 defines two subpopulations of myotomal cells: those that express Fgf-8 and MyoD, and those that express MyoD but not Fgf-8. This pattern indicates that, at the molecular level, the cells comprising the myotome are distinct not only with regard to epaxial and hypaxial identity, but also within these two regions. In agreement with previous results showing that MyoD is expressed laterally in the myotome in the absence of notochord (Asakura and Tapscott, 1998) and in Shh null mutant embryos (Borycki et al., 1999), we also find that ablation of notochord and floor plate prevents epaxial, and spares hypaxial expression of MyoD. During later stages, chicken embryos deprived of neural tube and floor plate do not form any back but do form well-developed limb and body wall musculature (Rong et al., 1992). These findings argue for regulatory independence of the hypaxial muscle compartment from influence from axial organs. However, we show that, in contrast to MyoD, expression of Fgf-8 in both the epaxial and hypaxial myotome requires the notochord–floor plate complex. Thus, the axial organs do influence gene expression in the hypaxial myotome. This effect on the hypaxial myotome does not appear to be mediated by Fgf-8, because beads with Fgf-8 protein do not induce expression of Fgf-8 when implanted into the paraxial mesoderm of embryos deprived of notochord and floor plate. On the other hand, implantation of beads coated with Sonic hedgehog protein into the paraxial mesoderm of embryos deprived of notochord and floor plate fully restored expression of Fgf-8 in the paraxial mesoderm. These observations demonstrate: (1) a regulatory influence of the ventral axial organs on the hypaxial muscle compartment; (2) regulation of epaxial and hypaxial expression of Fgf-8 by Shh; and (3) independent regulation of Fgf-8 and MyoD (at least in the hypaxial myotome) by the ventral axial organs, despite the fact that both genes are expressed in overlapping domains in the myotome.

While it is unclear what the role is of Fgf-8 expression in the myotome, it may have a role in cell survival and proliferation. In the somite, Fgf-8 appears to also be regulated by retinoic acid (Maden et al., 2000). In the absence of retinoic acid, Fgf-8 expression is down-regulated in the somite, and the lateral somite halves undergo apoptosis. Trumpp et al. (1999) suggest that Fgf-8 can also function as a survival factor in the branchial arch region of the mouse embryo. Likewise, Sonic hedgehog has been shown to be a survival and proliferation factor for hypaxial progenitors (Krüger et al., 2001) and somites (Cann et al., 1999). During head development, local retinoic signaling coordinates forebrain and facial morphogenesis by maintaining the expression of Fgf-8 and Shh (Schneider et al., 2001). Sonic hedgehog signaling may coordinate Fgf-8 in this signaling process. Therefore, it is possible that retinoic acid signaling in the somite initiates a regulatory cascade that controls somite differentiation. In this signaling cascade, Sonic hedgehog might be a link between retinoic acid and Fgf-8 expression.
Expression of Fgf-8 in the myotome is not dependent on signals from dorsal neural tube, lateral plate mesoderm, and surface ectoderm

In agreement with others, we find that the dorsal neural tube is not required for the formation of the myotome, but the ventral neural tube is. The more dorsal neural tube tissue was excised in our experiments, the more frequently a dorsally shortened dermomyotome was observed. With incomplete unilateral excision of the neural tube, the shortened dermomyotome was usually located at a more ventrolaterally position than on the control side. In these cases, the distance between the dorsalmost neural tube edge and the medial edge of the dermomyotome was larger than on the control side. However, a dorsomedial dermomyotome lip was always present, arguing for the presence of the epaxial domain. Concomitantly with the shortened dermomyotomes, the myotomes were thickened, resulting in an altered domain of expression of Fgf-8. Dietrich et al. (1997; Fig. 1G) have observed almost normal dermomyotomes and MyoD-positive myotomes after ablation of the dorsal third of the neural tube, but morphologically distinct dermomyotomes did not form after unilateral ablation of the neural tube. In these embryos, expression of Fgf-8 was absent as well, as there was no myotome that formed. We conclude that the dorsal neural tube is involved in the extension of the dermomyotome, but does not directly regulate Fgf-8 expression in the myotome.

Likewise, the lateral plate is also not required for the expression of Fgf-8 expression in the myotome. Blocking signals from the lateral plate mesoderm by barrier insertion did not prevent expression of Fgf-8 in the somites. However, the expression domain was shortened dorsoventrally and thickened mediolaterally. Following 2 days of reincubation, hypaxial myotome did not extend mediolaterally, as expected from previous studies (Dietrich et al., 1998; Pourquier et al., 1995, 1996). An explanation for the more condensed expression of Fgf-8 in embryos lacking lateral plate signals is that myotomal cells fail to arrange properly due to the lack of a hypaxial dermomyotome lip, which is not induced in the absence of the lateral plate influences. It is assumed that the myotome grows by apposition of new cells from the dorsal and ventral dermomyotome lips (Denetclaw and Ordahl, 2000; Denetclaw et al., 1997; Ordahl et al., 2001). The failing ventral expansion of the hypaxial myotome observed after separation of the lateral plate from the segmental plate could, therefore, be due to a missing hypaxial dermomyotome lip. As a result, the dermomyotome could not elongate properly, causing the myotomal cells originating at the dorsomedial lip to accumulate rather than expand ventrally. These results demonstrate that the lateral plate mesoderm is not necessary to establish expression of Fgf-8 in the somites but that it is necessary for the spatial distribution of myotomal cells.

The ectoderm overlying the dermomyotome influences the distribution of Fgf-8 expression, but as with the dorsal neural tube and lateral plate, it does not determine whether or not Fgf-8 is expressed. Removal of the ectoderm results in down-regulation of Pax3 and concomitant up-regulation of MyoD, indicating a premature burst in myogenic differentiation. Following ablation of the surface ectoderm we consistently observed accumulation of Fgf-8- and MyoD-expressing cells at the boundary between epaxial and hypaxial myotome regions. In these embryos, Fgf-8 was expressed in a dorsoventrally shortened and mediolaterally broadened domain. In addition, embryos costained with MyoD revealed that the hypaxial myotome portion had failed to extend ventrally. These results are in agreement with previous studies demonstrating the necessity of the surface ectoderm for hypaxial muscle development (Cossu et al., 1996a; Dietrich et al., 1998). Moreover, the condensed expression of Fgf-8 following ectoderm removal most likely reflects compromised cell movements of Fgf-8-expressing cells. Instead of dispersing dorsoventrally, the cells accumulate in the region of the epaxial–hypaxial border. This could mean that signals from the surface ectoderm are necessary to provide cues for proper spatial allocation of Fgf-8-expressing cells. Alternatively, removal of surface ectoderm could interrupt the generation of muscle precursors from the dermomyotome. Amthor et al. (1999) have shown that, in the trunk region, the surface ectoderm is necessary to keep the underlying dermomyotome in a Pax3-expressing and thus proliferative state. According to this model, myogenic cells do not switch from proliferation to differentiation until they invade the underlying myotome, thereby escaping from the influence of the surface ectoderm. It appears that, in somites lacking overlying ectoderm, the myotome is established by early muscle precursors, but no new cells are added to the myotome after the surface ectoderm is removed. This leads to an accumulation of Fgf-8-positive cells at the center of the myotome, as no additional myotomal cells enter at the dorsal and ventral dermomyotome lips. These findings show that the surface ectoderm is not necessary to initiate or to maintain myotomal Fgf-8 expression. In summary, our results demonstrate that the dorsal neural tube, the lateral plate mesoderm, and the surface ectoderm do not directly regulate expression of Fgf-8 in the somitic mesoderm.

No autoregulative function of Fgf-8 in the myotome

Recently, it was shown that implantation of Fgf-8 beads causes expansion of the expression domain of Fgf-8 in the segmental plate, suggesting positive autoregulation (Dubrulle et al., 2001). We tested the ability of Fgf-8 beads to induce expression of Fgf-8 in the myotome in the absence of the notochord–floor plate complex as well as in presence of these structures. In the absence of the notochord–floor plate complex, Fgf-8 beads produced no expression in the operated region, whereas in the presence of these structures, Fgf-8 beads did not alter Fgf-8 expression within the myotome. Thus, there does not appear to be positive autoregulative feedback loop for Fgf-8 in the myotome.
A role of myotomal Fgf-8 during somite differentiation

To address the function of myotomally expressed Fgf-8 on somite differentiation, we analyzed the expression of somitic markers in response to enhanced Fgf-8 protein signaling or after the inhibition of Fgf signaling by SU 5402. One hypothesis was that Fgf-8 could influence the development of adjacent tissue, in particular, the dermomyotome. Application of beads soaked in 2.5 μg/μl Fgf-8 resulted in an altered expression of genes expressed in both the dermomyotome (Pax-3) and myotome (Myf5, MyoD, and Myogenin). In most cases, there was an alteration in the tissue morphology rather than in gene expression levels. These results clearly show that both the dermomyotomal and myotomal genes in this study by using SU 5402, a potent inhibitor of Fgf-mediated signaling, and by the observation that these beads are active when tested in the limb. While we have shown that the expression of Fgf-8 is under the control of axial tissues, these concentration-dependent effects suggest that there may not be a direct role of Fgf8 in the functioning of the dermomyotome or myotome. Taken together, these observations suggest that the expression of Pax-3, MyoD, Myf5, and Myogenin is not regulated by Fgf signaling at embryonic stages examined in this study, and therefore, this signaling molecule may play a role in other domains of the somite. However, a recent paper has shown that overexpression of Fgf-8 leads to a dramatic phenotype in the somites (Marcic et al., 2002). The authors have interpreted their results by suggesting that MyoD is induced following Fgf-8 overexpression. However, it is clear in the examples shown in the study, that the morphology of the somites has been greatly altered, with no expression in the epaxial domain and fused hypaxial domains. These and our results suggest that Fgf-8 may be responsible for the distribution of the myogenic cells in the somites as opposed to controlling the differentiation process itself.

A possible site for Fgf-8 action is on sclerotomal derivatives. The BrdU incorporation data suggest that Fgf-mediated signaling may regulate the growth of the sclerotome. The application of Fgf-8 (at either 1.0 or 2.5 μg/μl) caused a 50% increase in the number of proliferating cells around the bead compared with the unoperated side (P < 0.05). Furthermore, application of SU 5402 resulted in a 20% decrease in the number of BrdU-positive sclerotomal cells compared with the unoperated side. These results suggest that Fgf-mediated signaling may mediate sclerotome growth (Fig. 10). We did not detect any significant difference in the expression of the early sclerotomal marker Pax-1 following application of either Fgf-8 or SU 5402. One possible explanation for this result is that alteration in Pax-1 expression levels due to changes in cell number may be masked by the very high expression levels of this gene. An alternative explanation is that Fgf signaling only effects non-Pax-1-expressing sclerotomal cells. This population of sclerotomal progenitors is located in the ventrolateral regions of the somite at embryonic stages examined in this study. This hypothesis is supported by the observation that the Fgf-mediated effect on cell proliferation was found in ventrolateral regions and not in the Pax-1-expressing ventral portions of the somite (Christ et al., 2000).

Fgf-8 signaling influences rib formation

Enhanced chondrogenesis was induced by both high and low levels of Fgf-8 and inhibited by agents that block Fgf effects. This supports a hypothesis that an important function of Fgf-8 expression may be related to the formation of ribs. Although we have shown that there is an increase in cell division in the sclerotomal population in response to Fgf-8, alterations in rib formation could have resulted from the fusion of cells from adjacent ribs or recruitment of previously nonchondrogenic cells to the chondrogenic lineage. In the limbs, Fgf-5 has a proliferative effect on peri-
chondrial fibroblasts (Clase et al., 2000), and application of Fgf-2 causes duplications of cartilaginous structures (Montero et al., 2001; Riley et al., 1993). If similar functions can be ascribed to Fgf-8, the observed enhanced rib growth could represent duplications of single ribs. However, the formation of duplications does not distinguish between whether the additional cartilage material is formed from preexisting chondrogenic cells or from reprogrammed, previously nonchondrogenic cells. To further complicate this issue, different concentrations of Fgfs produced different responses in the limb. Experiments in vitro showed that low concentrations of Fgf-2 enhanced proliferation of anterior neural crest cells, whereas higher concentrations promoted cartilage differentiation (Sarkar et al., 2001).

In contrast to limb cartilage, which arises from the lateral plate mesoderm, ribs are of sclerotomal origin (Huang et al., 2000). Thoracic somites start to form ribs around embryonic stage 21. It is interesting to note that, as the somite matures, Fgf-8 is strongly expressed in the myotome. Furthermore, Fgf-8 expression is confined to more central myotome regions when compared with the distribution of MyoD (Fig. 1C), a region where a subpopulation of myotomal cells expresses Fgf-8, thus localizing its expression directly adjacent to the region where sclerotomal cells aggregate to form the anlagen for the vertebral arches, pedicles, and ribs (see Christ and Ordahl, 1995, their Fig. 17). In more advanced somites in the trunk, the expression of Fgf-8 follows the ventral extension of the myotome, where the rib-forming anlagen and the sclerotome are located. Taken together, the expression domain of Fgf-8 in the somite maintains a strong spatial association with the developing rib primordia, lending further support to a role of Fgf-8 in rib chondrogenesis. This is in line with the observation made by Kato and Aoyama (1998) that rib development depends on the dermomyotome.

In summary, we have shown that myotomal Fgf-8 expression is regulated by signals originating from axial structures. Application of Fgf or Fgf signaling inhibitors affected the distribution of Fgf-8 expression, but did not affect the expression of dermomyotomal or myotomal genes. Application of Fgf-8 increased the rate of cell division in the ventrolateral portion of the sclerotome, and Fgf signaling inhibitors decreased cell division, implying that endogenous Fgfs are responsible for the expansion of this cell population. These results combined with previous studies of sclerotome development suggest a two-stage model of rib sclerotome development. In the first instance, axial organs induce the development of the sclerotome through the action of Shh (Brand-Saber et al., 1993; Pourquié et al., 1993; see Cossu et al., 1996b, for review), while Shh also initiates expression of Fgf-8 in the myotome. Secondly, the ventrolateral domain of the sclerotome is expanded indirectly via the myotome through the action of Fgf family members. The results from this study provide a model to explain the observation of Teillet et al. (1998), who showed that rib formation was dependent on Shh originating from axial structures.

Acknowledgments

We thank M. Bronner-Fraser and C. Marcelle for the qPax3 probe, A. Neubüser for the cFgf-8 and cShh probe, B. Paterson for the cMyoD probe, and H.H. Arnold and A. Buchberger for the cMyogenin probe. We are indebted to W. Nikovits for helpful critical reading on the manuscript. We thank Mr. G. Frank for his contribution to the statistical analysis. We also thank Mrs. E. Gimbel, Mrs. L. Koschyna, Mrs. U. Pein, Mrs. M. Schüttoff, Mrs. S. Antoni, and Mrs. C. Micucci for their excellent technical assistance. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 592, A1 and Ch44/14-1) (to B.C.) and USDA Grant 2000-03280 (to F.E.S.). K.P. wishes to acknowledge generous funding from the Wellcome Trust (Grant 065213).

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