Fgf8 drives myogenic progression of a novel lateral fast muscle fibre population in zebrafish

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Summary

Fibroblast growth factors (Fgfs) have long been implicated in regulating vertebrate skeletal muscle differentiation, but their precise role(s) in vivo remain unclear. Here, we show that Fgf8 signalling in the somite is required for their precise role(s) in vivo. Some cultured myoblasts require Fgf for differentiation, whereas in others differentiation is repressed by Fgf (Seed and Hauschka, 1988). Fgfs signal through tyrosine kinase receptors, the activated receptor phosphorylates several downstream proteins, including the MAP kinases (Seed and Hauschka, 1988). However, Fgf8-independent medial fast muscle precursors are lacking in floatinghead mutants, suggesting that they require another ventral midline-derived signal. We conclude that Fgf8 drives terminal differentiation of a specific population of lateral muscle precursor cells within the early somite.

Key words: Fibroblast growth factor 8, Muscle, Zebrafish, Fast, Myod, Somite

Introduction

During zebrafish muscle development, two distinct cell populations give rise to slow and fast muscle fibres (Stickney et al., 2000). The location and origin of these populations has been well characterised (Devoto et al., 1996; Hirsinger et al., 2004). The slow muscle derives from the medially located adaxial cells and depends on Hedgehog (Hh) signals from the midline (Barresi et al., 2000; Blagden et al., 1997; Du et al., 1997; Wolff et al., 2003). Slow fibres fall into two sub-populations: the superficial slow fibres (SSF), which subsequently migrate to the lateral somite surface; and the muscle pioneers (MPs) that remain medial and express engrailed genes (Devoto et al., 1996; Hatta et al., 1991; Ekker et al., 1992). More lateral somite cells do not migrate and later differentiate into fast muscle fibres (Blagden et al., 1997; Devoto et al., 1996; Wolff et al., 2003). A medially located fast fibre sub-population expresses low levels of Engrailed (Hatta et al., 1991; Ekker et al., 1992; Devoto et al., 1996). Like MPs, these multinucleate fibres, dubbed medial fast fibres (MFF), express Engrailed in response to Hh signalling (van Eeden et al., 1996; Schauerte et al., 1998; Roy et al., 2001; Wolff et al., 2003). How the cells of the lateral somite are specified to make fast muscle is unknown.

Expression of members of the Myod family of myogenic regulatory transcription factos (MRFs) is known to commit cells to myogenesis and control their terminal differentiation (Buckingham, 2001). The MRF myf5 is expressed in posterior presomitic mesoderm in presumptive fast muscle precursors. myf5 expression declines in anterior presomitic mesoderm, but is transiently re-expressed in the posterior border of each somite as it forms (Chen et al., 2001; Coutelle et al., 2001). Expression of the MRF myod coincides with that of myf5 in the posterior lateral somite from the six somite stage (6s) (Coutelle et al., 2001; Weinberg et al., 1996). myod expression is maintained in the posterior region of the first six and all subsequently formed somites until the cells begin to differentiate into fast muscle around the 15-somite stage, when it is downregulated (Weinberg et al., 1996). A third MRF, myogenin, which is often associated with terminal differentiation, is expressed in posterior somite border cells after myod but prior to their terminal differentiation (Weinberg et al., 1996). The signals regulating MRF expression in fast muscle precursors are unknown.

During a search for signals that might regulate fast myogenesis, we noticed that zebrafish fibroblast growth factor 8 (fgf8) gene is expressed in a stripe in the anterior of zebrafish somites with a similar timecourse to myod expression in the posterior (Furthauer et al., 1997; Reifers et al., 1998). The expression of fgf8 and MRFs are also temporally alike in amniote somites (Crossley and Martin, 1995; Maruoka et al., 1998; Stolte et al., 2002). Thus, Fgf8 is expressed in space and time such that it could regulate myogenesis.

The seminal work of Hauschka showed that distinct myogenic cell populations are differentially sensitive to Fgfs, but the relevance of these findings to myogenesis in vivo have been unclear. Some cultured myoblasts require Fgf for differentiation, whereas in others differentiation is repressed by Fgf (Seed and Hauschka, 1988). Fgfs signal through tyrosine kinase receptors to activate the MAP kinases, which are known to be involved in myogenesis. However, the signals that might regulate fast myogenesis are not yet known.
kinase Fgf receptors, several of which are expressed in zebrafish somites, although their role there is unknown (Klint and Claesson-Welsh, 1999; Thisse et al., 1995; Tonou-Fujimori et al., 2002). In fish, a mutation in fgf8 has been reported to diminish myod expression (Reifers et al., 1998). In Drosophila, the heartless Fgf8 receptor is required for formation of a subset of somatic muscles (Michelson et al., 1998). Manipulation of Fgf levels or signalling pathways in amniotes alters early muscle patterning. In the chick limb, both Fgfr1 and Fgfr4 signalling appear to promote differentiation (Flanagan-Steet et al., 2000; Marics et al., 2002). By contrast, blockade of somitic Fgfr1 results in premature muscle differentiation and prevents muscle precursors from migrating (Itoh et al., 1996). In the chick somite, Fgfr1 is widely expressed, whereas Fgfr4 is particularly abundant in precursors of body wall, limb and oculomotor muscles (Marcelle et al., 1994). Thus, the effect of Fgf signalling may depend on the receptor and the recipient cell type. Overexpression of fgf4 or fgf8 in the limb decreases the expression of myod, fgfr4 and the number of muscle cells and induces tendon-specific markers (Edom-Vovard et al., 2001; Edom-Vovard et al., 2002). In the somite, however, the formation of muscle and tendon can be promoted by the addition of Fgf (Brent and Tabin, 2004; Itoh et al., 1996; Marics et al., 2002). In conclusion, several Fgfs, including Fgf8, are implicated in regulating myogenesis in vivo.

Fgf8 is expressed in the tail bud of vertebrate embryos, as well as in the somites. This tail bud expression is implicated in the formation of somite boundaries (Dale and Pourquie, 2000; Dubrulle et al., 2001; Sawada et al., 2001). However, ablation of fgf8 in the mouse leads to embryonic lethality by day E9.5, owing to malformations of the heart (Frank et al., 2002; Moon and Capechci, 2000; Sun et al., 1999). Compound heterozygotes and conditional mutants have implicated fgf8 in various aspects of development but have yet to test its role in myogenesis (Abu-Issa et al., 2002; Frank et al., 2002; Meyers et al., 1998; Meyers and Martin, 1999; Moon and Capechci, 2000; Trumpp et al., 1999). Overall, the pleiotropic patterning defects observed in Fgf manipulations and the difficulty of distinguishing myogenic cell subpopulations in amniotes have severely hampered analysis of the role of Fgfs in amniote myogenesis in vivo.

In the zebrafish, Fgf signalling promotes posterior mesoderm development and can influence somite border positioning (Cao et al., 2004; Draper et al., 2003; Griffin et al., 1995; Sawada et al., 2001). The loss-of-function fgf8 mutant acerebellar (ace) exhibits only mild somite defects (Draper et al., 2003; Reifers et al., 1998). Despite morphological changes, somites form and some embryonic myogenesis is present. Overall, however, the function of somitic fgf8 expression and its role in myogenesis is unclear.

Here, we identify a new cell population in the lateral somite, the lateral fast myoblasts (LFM) that is dependent on Fgf signalling. Fgf8 is required for the initiation and maintenance of myod and myogenin, but not myf5, expression in LFM. Lack of Fgf signalling leads to failure of dermomyotomal marker downregulation and lateral fast fibre (LFF) differentiation. So Fgf8 drives progression of the myogenic program but not the initiation of myogenesis in these cells. Strikingly, residual medial fast fibres are unaffected by the loss of Fgf signalling, even in the absence of Hh signalling, indicating that MFFs and LFFs may constitute distinct cell populations. Our findings reveal a specific pro-myogenic role for Fgf8 in the lateral somite.

Materials and methods
Zebrafish lines and maintenance
Mutant lines ace<sup>282a</sup> (Reifers et al., 1998), yot<sup>119</sup> (van Eeden et al., 1996) and flhn<sup>2</sup> (Coulette et al., 2001) were maintained on King’s wild-type (Kwt) background. Staging and husbandry were as described previously (Westerfield, 1995).

In situ mRNA hybridisation and immunohistochemistry
In situ mRNA hybridisation was as described previously (Coulette et al., 2001). Embryos were fixed in 4% paraformaldehyde (PFA) for at least 30 minutes at 28°C and fluorescein- or digoxigenin-tagged probes made with Roche labelling mix to full-length myf5-coding sequence (pFG1-Myf5), myl2 (Xu et al., 1999), pax3 (Seo et al., 1998), myod or myogenin (Weinberg et al., 1996). Embryos were fixed for antibody staining with 4% PFA for 30 minutes at 28°C or Carnoy’s (Barresi et al., 2000), mounted in 1.5% agarose blocks in 5% sucrose, which were subsequently soaked overnight in 30% sucrose. Cryosections were cut at 10-15 μm, dried, washed with PBTw (PBS 0.1% Tween20), blocked with 5% goat/horse serum in PBTw (according to host of secondary antibody) for 1 hour at room temperature, incubated with primary antibodies A4.1025 (Blagden et al., 1997), SS8 (Devoto et al., 1996) or EB165 (Blagden et al., 1997) diluted in block overnight at 4°C, and detected as described (Blagden et al., 1997). Wholemounts were treated similarly, but using HRP-conjugated class-specific antibodies (Vector) and DAB detection or triple stained for 4D9 (DSHB), A4.1025 and DAPI using Alexa-conjugated sub-class-specific secondary antibodies (Molecular Probes) and Citifluor mount.

Embryo manipulations
Embryos were injected at the one- to two-cell stage with fgf8 morpholino (gagctcatgtttatgcctcagta, 7-10 ng from 2.5 mg/ml stock) and rhodamine as described (Westerfield, 1995). Cyclopamine (200 μM, or ethanol control) was added from 50% epiboly. Embryos were dechorionated at the six-somite stage (Westerfield, 1995), placed in 1% agarose-coated dishes with SU5402 (Calbiochem, 60 μM) or DMSO control. Fgf8 bead implantation above nascent somites was performed at the 10-somite stage and analysed at the 15-somite stage as described (Reifers et al., 2000b).

Results
Correlation of Fgf8 signalling with fast muscle precursor formation
We examined the expression of fgf8 and fgfr receptors in relation to somite myogenesis (Fig. 1). Fgf8 is expressed in nascent presumptive mesoderm during gastrulation and later in the tailbud (http://www.zfin.org/). Expression declines in the anterior presumptive mesoderm, but is then upregulated again during somite formation (Fig. 1A). At this stage, expression of an early response gene for Fgf signalling, the ets domain transcription factor erm, closely matches that of fgf8, as reported previously (Roehl and Nüsslein-Volhard, 2001). No myod mRNA is detected in the lateral anterior presumptive mesoderm before the two-somite stage (2 s), expression being restricted to the medially located slow muscle precursors (data not shown). By 5 s, fgf8 expression is highest in the anterior region of somites and is declining in the posterior region of the oldest somites, where myod mRNA is now abundantly detected in lateral ‘wings’ of cells which are thought to be fast muscle
precursors. *erm* expression encompasses the region of *myod* expression, suggesting that Fgf8 may signal within the posterior somite (Fig. 1A,B,E). Between 10 and 21 s, *fgf8* expression persists in the tailbud, is lower in anterior presomitic mesoderm and rises in each nascent somite as it forms. Expression persists as somites mature, but becomes restricted to the anterior and medial region in more mature somites, reciprocal with *erm* (Fig. 1A,B,D,E). *myod* is expressed in the posterior somite border (Fig. 1B). At later stages, when slow muscle precursors migrate laterally and fast precursors undergo terminal differentiation, *fgf8* mRNA declines anteriorly (Fig. 1D). Genes encoding Fgf receptors *fgfr1* and *fgfr4*, but not *fgfr3*, are expressed in presomitic and somitic mesoderm in locations consistent with a role in mediating *erm* activation (Fig. 1F-H). Thus, *fgf8* is expressed in the somite during the formation of early muscle fibre populations and correlates in space and time with the expression of *myod* in lateral fast muscle precursors.

**Fgf8 signalling is required for normal fast muscle differentiation**

To examine the role of Fgf8 in muscle development, we ablated signalling using Fgf8 antisense morpholino (MO) oligonucleotides and analysed fast myosin light chain expression, a marker of differentiated fast muscle cells (Xu et al., 1999). In unmanipulated embryos, fast myosin expression is first apparent at low levels in anterior somites at 15 s and subsequently increases in these cells (data not shown). By 22 s, fast myosin is expressed across the mediolateral extent of somites 1–17 (Fig. 2A). Embryos that lack Fgf8 show a reduction in fast muscle formation: fast myosin expression is lacking in the lateral somite, although a significant quantity is detected medially (Fig. 2A; 72/72 injected embryos). A similar defect is observed in *ace* mutant fish, which have a loss of Fgf8 function owing to a point mutation at a splice junction (Reifers et al., 1998). Next, embryos were exposed to SU5402, a drug that blocks the phosphorylation of Fgf receptors and so prevents downstream signalling, as indicated by the ability of SU5402 to block all somitic *erm* expression (Fig. 1I) (Roehl and Nüsslein-Volhard, 2001). Early treatment with SU5402 causes severe gastrulation and tailbud outgrowth defects (data not shown). However, later treatment with SU5402 from 6 s yields embryos with a reduced overall length but a normal number of somites. Such embryos lose lateral fast myosin expression, but retain medial expression (Fig. 2A; 78/78 treated embryos). Thus, Fgf8 signalling is required for normal fast myogenesis in nascent zebrafish somites.

The early defect in fast muscle terminal differentiation persists. We examined accumulation of fast MyHC, which normally marks all fast fibres, at 33 hpf. embryos with blocked Fgf signalling have a reduced population of fibres with lower levels of fast MyHC in the median somite, and significantly less fast MyHC in the lateral somite, although a significant quantity is detected medially (Fig. 2A; 72/72 injected embryos). By 22 s, fast myosin expression is lacking in the lateral somite, although a significant quantity is detected medially (Fig. 2A; 72/72 injected embryos). A similar defect is observed in *ace* mutant fish, which have a loss of Fgf8 function owing to a point mutation at a splice junction (Reifers et al., 1998). Next, embryos were exposed to SU5402, a drug that blocks the phosphorylation of Fgf receptors and so prevents downstream signalling, as indicated by the ability of SU5402 to block all somitic *erm* expression (Fig. 1I) (Roehl and Nüsslein-Volhard, 2001). Early treatment with SU5402 causes severe gastrulation and tailbud outgrowth defects (data not shown). However, later treatment with SU5402 from 6 s yields embryos with a reduced overall length but a normal number of somites. Such embryos lose lateral fast myosin expression, but retain medial expression (Fig. 2A; 78/78 treated embryos). Thus, Fgf8 signalling is required for normal fast myogenesis in nascent zebrafish somites.

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Fgf8 does not appear to be required for slow myogenesis. At 15 s, slow MyHC is detected in two medial stripes adjacent to the notochord of SU5402- or Fgf8 MO-treated or *ace* mutant embryos, just as in controls (Fig. 2C and data not shown). By 33 hpf in unmanipulated embryos, most slow fibres have migrated to lie close to the superficial somite surface (Fig. 2D). After blockade of Fgf8, slow muscle fibres

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**Fig. 1.** Fgf8 signalling correlates with myogenic marker expression in the lateral somite. In situ mRNA hybridisation for *myod* (A–C), *fgf8* (A,B,D) and *erm* (A,B,E) at 4–6 s, 15 s and 19–20 s, and *fgfr1* (F), *fgfr3* (G) and *fgfr4* (H) at 15 s. (A,B) Discreet expression of *fgf8* overlaps or is immediately adjacent to locations of *erm* expression in somites. *Myod* expression parallels *fgf8* and *erm* in the somites, but not elsewhere. Flatmount dorsal view, anterior towards the top. (Insets) Lateral view, anterior towards the top, dorsal towards the left. By 15 s, *erm* expression is becoming excluded from regions of high *fgf8* expression in anterior somites (white arrows). *myod* and *erm* expression overlap within the posterior somite. Somite boundaries in the newest five somites are indicated. (C–E) *myod*, *fgf8* and *erm* expression persist as somites mature. (F) *fgfr1* is expressed in presomitic mesoderm and nascent somites. (G) *fgfr3* shows little expression in nascent somites. (H) *fgfr4* is expressed segmentally in maturing somites and in neural tube. (F–H) Dorsal flatmounts of whole embryo, anterior towards the top. (I) Treatment with SU5402 blocks *erm* expression throughout the embryo. (C–E,I and insets in F–H) Lateral view, anterior towards the top.
Fgf signalling is required for myod initiation

To determine at what step in fast myogenesis Fgf8 is required, we examined expression of MRF genes. Wild-type embryos express myod in the adaxial cells, which differentiate into slow muscle, and in presumed fast muscle precursors in a stripe at the posterior of each somite (Fig. 3A). Analysis of Fgf8 MO-injected embryos reveals a lack of myod in the lateral cells of the somite (100/100 rhodamine-labelled embryos), a phenotype that is not observed in control non-injected and rhodamine-dextran-injected embryos. Myod expression in the adaxial cells is unaffected. Similarly, SU5402-treated embryos and those mutant at ace display a lack of lateral myod, despite the presence of adaxial somitic myod mRNA (147/151 SU5402-treated and 14/88 embryos from an heterozygous ace cross). None of the three Fgf manipulations prevents formation of the normal number of somites (Fig. 3A and data not shown). Thus, Fgf8 function is required for normal initiation of myod expression in lateral somite cells.

The medial myod expression remaining after blockade of Fgf8 signalling is not confined to slow muscle cells. A group of cells in the medioposterior region of each somite retains myod expression (Fig. 3A, black arrows). The extent of lateral myod expression across the posterior somite was measured by counting cells in somites 4-6 and 13-15 of flatmounted 15 s embryos. Although somite width is not significantly altered in either Fgf8 MO- or SU5402-treated embryos, at around 11 cells in each case, ~70% the cells of the posterior somite border lose myod mRNA in response to either treatment (Table 1). In the case of ace, somite shape is altered such that the posterior somite border contains fewer cells. Nevertheless, the number of residual myod-expressing cells is similar to that in morphant and SU5402-treated embryos (Table 1). Therefore, Fgf8 morphant, SU5402-treated and ace mutant embryos are similar with respect to loss of myod expression, indicating that Fgf8 is required for myod initiation in the zebrafish lateral somite.

The MRF gene myogenin is a known direct target of Myod in myoblasts (Bergstrom et al., 2002). Zebrafish myogenin is expressed in lateral posterior somite cells after myod, but prior to myosin accumulation (Weinberg et al., 1996). Ablation of Fgf8 signalling decreases myogenin expression in the lateral somite, although significant expression remains in both adaxial cells and medial regions (Fig. 3A; 42/45, 49/49 and 22/30; Fgf8 MO, SU5402 and ace embryos, respectively). The reduction of myogenin expression is less marked than that of myod (Fig. 3A, black arrows). As myogenin promotes myoblast terminal differentiation (Hasty et al., 1993; Nabeshima et al., 1993), these data suggest that the failure of myogenin accumulation in the lateral somite may be the immediate cause of the lack of fast muscle differentiation in this location.

Although myod has been implicated as a key regulator of

Table 1. Residual medial myod expression at posterior somite border after Fgf blockade

<table>
<thead>
<tr>
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<th>Cell number</th>
<th>myod-positive cells</th>
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<tr>
<td></td>
<td>(mean±s.e.m. (n))</td>
<td>(mean±s.e.m. (n))</td>
<td>myod positive (%)</td>
</tr>
<tr>
<td>Control</td>
<td>11.1±0.3 (11)</td>
<td>11.1±0.3 (11)</td>
<td>100</td>
</tr>
<tr>
<td>SU5402</td>
<td>11.1±0.2 (12)</td>
<td>2.9±0.4 (12)</td>
<td>26.3</td>
</tr>
<tr>
<td>Fgf8 MO</td>
<td>10.9±0.2 (12)</td>
<td>3.0±0.2 (12)</td>
<td>27.5</td>
</tr>
<tr>
<td>ace/ace</td>
<td>7.0±0.3 (10)</td>
<td>3.1±0.2 (10)</td>
<td>44.3</td>
</tr>
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Data derived from somites 13-15 in 15 s embryos.
myogenesis in the lateral amniote somite, *myf5* is also expressed in lateral myogenic cells of both amniotes and fish (Coutelle et al., 2001; Hadchouel et al., 2003). In fish, *myf5* is the first MRF gene expressed in fast muscle precursors, being expressed in the tailbud, declining in anterior presomitic mesoderm and then re-accumulating transiently coincident with *myod* expression in posterior somite border cells as each somite forms (Coutelle et al., 2001). Ablation of Fgf8 function or signalling does not prevent *myf5* expression in the lateral somite; indeed, expression often appears to persist at higher than control levels in anterior somites (Fig. 3A; 215/215, 56/56 and 9/35; Fgf8 MO, SU5402 and ace heterozygous cross embryos, respectively). Thus, the Fgf system is required to permit myogenesis to proceed from the initiation of *myf5* to the upregulation of *myod* and *myogenin*.

Treatment with SU5402 does not diminish *myf5* expression in nascent somites or adaxial cells, but severely downregulates *myf5* in the presomitic tailbud region, as does Fgf8 MO in some treated embryos (Fig. 3A; 215/215, 56/56 and 9/35; Fgf8 MO, SU5402 and ace heterozygous cross embryos, respectively). Thus, the Fgf system is required to permit myogenesis to proceed from the initiation of *myf5* to the upregulation of *myod* and *myogenin*.

Fgf8 induces *myod* within the somite

The fact that addition of SU5402 after somite segmentation has commenced leads to downregulation of *myod* expression in all somites strongly suggests that Fgf signalling within the somite drives *myod* (Fig. 3A). To prove that Fgf8 can induce *myod* expression locally within the somite, we applied a bead with Fgf8 protein above the somites on one side of the animal. Fgf8-soaked beads induce a dramatic unilateral or bilateral upregulation of *myod*, whereas control beads either do not
Fig. 4. Pax3 marks the somitic external cell layer and is expanded when Fgf8 signalling is blocked. (A) Pax3 in situ mRNA hybridisation (blue/purple) with whole mounts showing expression in somites throughout the axis at 15 s, but more highly expressed in nascent somites by 20 s. Cryosections at the levels indicated reveal that somitic expression is in a discontinuous layer in the lateral region at 20 s (arrows), weakening further by 24 hpf and most prominent at the dorsal and ventral somitic extremes and the horizontal myoseptum level. There is persistent expression in dorsal neural tube. (B) Dorsal flat mount, anterior towards the top showing pax3 expression in all somites at 15 s. (CD) Electron micrographs in transverse (C) and longitudinal (D) section of 24 hpf lateral somite at midbody level showing external cells (black arrows, ext) superficial to mononucleate slow muscle cells with pronounced medially located myofilbrils (white arrows), which themselves overlie multinucleate fast fibres. The epidermis (epi) is separated from the external cells by amorphous material. Scale bar: 1.43 μm in C; 10 μm in D. (E) Oblique section through a heavily developed 24 hpf embryo showing pax3 expression in the region of the external cells (arrows) clearly superficial to slow MyHC (brown). (F) Compared with control 23 s siblings (con), SU5402-treated embryos show upregulation and maintenance of somitic pax3 both in whole mount and in cryosections. (G) Fgf8 MO reduces the downregulation of meox expression as somites mature (22/23 injected embryos). (H) wnt11r marks the dorsomedial somite edge (arrows) and neural tube. (Left) Dorsal flat mount at 15 s. (Right) Cryosections. (I) SU5402 upregulates wnt11r expression in both spinal cord and dorsomedial somite (56/56 treated embryos at 15 s).

Pax3 downregulation requires Fgf8 signalling

To address the fate of the somite cells that fail to form fast muscle in the absence of Fgf8, we characterised potential markers of pre-myogenic tissue within the somite. In amniotes, pax3 is expressed in early epithelial somites, becomes restricted to dermomyotome and is required for generation of some lateral somite-derived muscle (Bober et al., 1994). In X. laevis, after an initial widespread expression in nascent somites, pax3 marks a dermomyotome-like cell layer (Grimaldi et al., 2004). Pax3 is first expressed in zebrafish around 80% epiboly in the germ ring and rapidly becomes localised to the anterior presumptive mesoderm and nascent somites as somitogenesis begins (Fig. 4A). Expression is initially widespread in nascent somite and subsequently becomes restricted to the lateral somite edge as fast muscle differentiates in an anterior to posterior wave around the 20 s stage (Fig. 4A,B). By 24 hpf, pax3 mRNA is further downregulated such that it is primarily detected in groups of cells at the dorsal and ventral extremes of the somite and in small cells at the outer somite surface (Fig. 4A,E). At this stage, electron micrographs reveal flattened external cells, often only 500 nm thick, on the somite surface (Waterman, 1969). The cells do not contain sarcomeric material and therefore lie outside the superficial slow muscle fibre layer (Fig. 4C,D). Cells in this position express pax3 (Fig. 4E). Thus, in zebrafish somites, pax3 mRNA is a marker of lateral somite cells and declines as these cells generate muscle, becoming restricted to non-muscle external cells.

When lateral fast myogenesis is inhibited with SU5402 or by Fgf8 MO injection, lateral somite cells retain pax3 expression (Fig. 4F; data not shown). Transverse sections reveal that the normal restriction of most readily detectable pax3 expression to the dorsal and ventral extremes of the lateral somite fails to occur in embryos lacking Fgf signalling, leaving pax3 mRNA in a broad lateral layer (Fig. 4F). SU5402 also upregulates pax3 in the dorsal neural tube. A second marker of nascent somitic cells is meox mRNA (Neyt et al., 2000). After somite formation, meox is downregulated and becomes restricted to lateral somite cells in a manner similar to pax3 (Fig. 4G). Upon Fgf8 MO injection, meox is upregulated in the lateral somite region where fast muscle fails to form (Fig. 4G). A third gene, wnt11r, is expressed transiently in a superficial region of the dorsal somitic extreme and neural tube of control embryos, similar to pax3 (Fig. 4H). wnt11r is also more highly expressed in both dorsal somitic tissue and neural tube of SU5402-treated embryos (Fig. 4I). Thus, Fgf8 signalling is required for downregulation of pax3, meox and probably wnt11r, and upregulation of myod and myogenin in the somite.
Fgf signalling is required for myod maintenance

The persistent expression of fgf8 and erm in maturing somites suggested that Fgf signalling continues until the period of fast muscle differentiation (Fig. 1). To determine whether Fgf signalling continues to be required for maintenance of somitic myod expression, SU5402 was applied to older embryos (schematised in Fig. 5A). From 6-10 s, myod is expressed strongly in a posterior stripe in all somites (Fig. 1A, Fig. 5B). When SU5402 treatment is initiated between 6 s and 10 s, lateral expression of myod begins to decline within 30 minutes and is lost throughout the axis 1 hour later, both in new somites formed during the treatment period and in old somites that were expressing myod laterally prior to treatment (Fig. 5C-G). However, medial myod expression in adaxially derived cells is retained. SU5402 treatment from 10 s to 15 s prevents myod expression in nascent somites and also in older somites, although the reduction is less marked in the most anterior somites (Fig. 5H,I). Conversely, washout of SU5402 at 15 s is followed by a rapid recovery of lateral myod expression (Fig. 5J). The Fgf signalling pathway is required, therefore, for maintenance of myod expression in the lateral somite.

Fgf signalling is required at late stages for fast muscle differentiation

Treatment of embryos from 18 s with SU5402 has less effect than at earlier stages on myod expression in mature anterior somites (data not shown, also Fig. 5H-J, anterior). Nevertheless, even at this late stage, SU5402 exposure diminishes the accumulation of mylz2 transcripts, suggesting that persistent Fgf signalling is required for fast fibre terminal differentiation (Fig. 5K-M).

Residual medial fast muscle is not Hh dependant

Although reduction of Fgf signalling ablates lateral fast muscle differentiation, medial fast myogenesis still occurs. Hh proteins derived from ventral midline tissues are required for adaxial slow myogenesis (Barresi et al., 2000; Blagden et al., 1997; Du et al., 1997) and for engrailed expression in the specialized medial fast fibres (MFF) (Wolff et al., 2003). We investigated the relationship between the Fgf8-independent fast fibres and MFFs (Fig. 6). Treatment of wild-type embryos with Hh signalling inhibitor cyclopamine leads to loss of myod expression in adaxial slow muscle cells but has no noticeable effect on myod expression in the fast muscle precursors (Fig. 6A,C; 86/92 treated embryos). Exposure to both cyclopamine and SU5402 leads to embryos with a residual medial posterior group of myod-expressing cells in each somite but lacking both adaxial and lateral expression (Fig. 6D, 90/98 treated embryos). Thus, the effects of the two drugs appear additive. Subsequently, a small quantity of residual fast muscle forms, but not slow muscle (data not shown). Similar results are obtained after SU5402 treatment of you-too (yot) mutant...
embryos, which lack slow myogenesis owing to a mutation in the Gli2 component of the Hh signalling pathway (Fig. 6E,F). Taken together, these data show that Hh signalling is not responsible for the resistance of residual fast fibres to reduction in Fgf signalling.

To investigate the relationship of residual medial fast muscle and MFFs the effect of SU5402 on engrailed expression was examined. Treatment does not prevent expression of eng1 mRNA or Engrailed protein in either MPs or MFFs (Fig. 6I-L). In addition, in ace mutants, engrailed expression appears normal (Fig. 6M). Thus, the residual fast muscle has the capacity to differentiate into MFFs under the influence of Hh signalling.

The Hh- and Fgf8-independence of medial fast muscle raised the possibility that a distinct population of MFF precursors form in the medial somite dependent on another midline signal. We tested this hypothesis by examining the role of Fgf signalling in floatinghead (flh) mutants, which lack ventral midline tissues and have bilateral somites fused at the midline beneath the neural tube (Halpern et al., 1995). flh mutants lack adaxial myod expression at 15 s but retain a single stripe of myod expression in the posterior of each forming somite (Coutelle et al., 2001). Treatment with SU5402 essentially ablates the posterior somitic myod stripes (28/108 treated embryos from heterozygous flh/+ crosses; Fig. 6H). However, SU5402 does not suppress anterior myod expression underlying and flanking the neural tube, which may be within the delayed differentiating slow muscle observed in flh mutants (Blagden et al., 1997). In conclusion, the flh gene is required for formation of most, if not all, medial Fgf8- and Hh-independent myod-expressing cells but not for lateral Fgf8-dependent myod-expressing cells.

**Discussion**

Fast muscle is the major force-producing system in zebrafish. Despite extensive genetic screens, no mutations that specifically ablate fast myogenesis have been described, although numerous genes are essential for early slow myogenesis. Here, we suggest a reason: two populations of fast muscle fibres form independently during development. One fast fibre population, arising in the lateral somite, is Fgf8 dependent and ventral midline independent. The other, arising medially within the somite, is Fgf8 independent but can be ablated by mutations that abolish midline patterning. We characterise a new pathway controlling somite muscle development (Fig. 7).

**Fgf8 drives lateral fast myogenesis**

Defects in Fgf8 morphants and ace mutants demonstrate that...
lateral cells in the posterior zebrafish somite require Fgf8 signalling to initiate the expression of myoD and, subsequently, to undergo terminal differentiation into fast muscle fibres. Several lines of evidence indicate that Fgf8 signalling is required during and after somite border formation, rather than earlier in development. First, fgf8 is expressed in anterior regions of both immature and mature somites. Second, expression of the immediate Fgf target gene, ern, and its inhibition by exposure to the Fgf receptor antagonist SU5402 after somite border formation, indicate that Fgf-like signalling persists in the posterior region of maturing somites. Third, ectopic Fgf8 expression after somite border formation upregulates myoD expression. Fourth, SU5402 rapidly blocks lateral myoD expression in somites. Fifth, the effect of SU5402 is reversible, leading to myoD re-expression in the posterior somite. Sixth, loss of myoD induction is accompanied by persistence of markers of the immature posterior somite border, such as myf5, pax3 and meox, which themselves are essential markers of subsets of myogenic precursors in mouse (Kassar-Duchossoy et al., 2004; Mankoo et al., 1999; Tajbakhsh et al., 1997). Thus, Fgf8 functions as a muscle differentiation factor in this in vivo context in zebrafish.

Fgf8 expression within the somite itself is necessary and sufficient for myoD expression in the lateral somite. The evidence quoted in the preceding paragraph supports this view. Additional evidence shows that tailbud-derived Fgf8 is not essential. First, the reduction in myoD expression caused by Fgf8 MO can be rapidly reversed by application of Fgf8 directly to the somites. Second, ern expression is low in anterior presomitic mesoderm, suggesting that tailbud Fgf8 does not diffuse anteriorly into the somites at significant levels. Third, myoD expression in anterior somites of no tail mutant embryos, which lack tailbud fgf8 expression, is normal (Coutelle et al., 2001; Draper et al., 2003). Taken together, these data indicate that neither prior Fgf8 exposure during gastrulation or within the tailbud, nor diffusion of tailbud-derived Fgf8 into the somites, account for somitic fgf8 dependence of lateral myoD expression. Instead, we favour the parsimonious model that local diffusion of Fgf8 from the anterior somite, perhaps acting through either Fgfr1 or Fgfr4, promotes myoD and myogenin expression, and presumably fast muscle differentiation, in nearby cells (Fig. 7).

Myogenin is the MRF directly responsible for most murine muscle differentiation (Venuti et al., 1995). In zebrafish lateral somite, myogenin expression follows that of myoD after a delay (Weinberg et al., 1996). As Fgf8 blockade prevents lateral myogenin expression, an explanation could be that in zebrafish, as in mice, Myod drives myogenin expression in the lateral somite (Bergstrom et al., 2002; Rudnicki et al., 1993). Moreover, lack of myogenin may contribute to the failure of terminal differentiation we observe.

Decline in myoD expression in ace mutants has previously been suggested to result from effects on earlier mesodermal patterning during gastrulation or tail bud outgrowth (Draper et al., 2003; Reifers et al., 1998). However, myf5, pax3 and meox expression are initiated in a near normal pattern at presumptive somite boundaries in ace. Fgf8 MO- and SU5402-treated embryos, suggesting that anterior presomitic mesoderm is capable of undergoing normal myogenic patterning. Other signals in addition to Fgf8 must pattern myf5 expression in this region.

Our data confirm that Fgf-like signalling has a significant role in tail bud outgrowth. Early blockade of Fgf signalling with dominant-negative constructs or SU5402 truncates embryos and disrupts gastrulation (Draper et al., 2003; Griffin et al., 1995). We also demonstrate that expression of the earliest known myogenic marker, myf5, is disrupted in the tailbud region and posterior presomitic mesoderm by SU5402 inhibition of Fgf-like signalling. However, in zebrafish, other Fgf genes contribute to tailbud Fgf signalling so that ablation of fgf8 function does not prevent reasonable somite development (Draper et al., 2003; Reifers et al., 1998).

Although fgf8 is expressed in amniote somites (Edmondson and Duprez, 2004; Maruoka et al., 1998; Stolte et al., 2002; Vogel et al., 1996), the role of Fgf8 in somite myogenesis is unknown owing to the pleiotropic roles of Fgf8 (Brent et al., 2003; Frank et al., 2002; Moon and Capecchi, 2000; Sun et al., 1999). However, numerous experiments show that Fgfs can promote formation of differentiated muscle both in vivo and in vitro (Flanagan-Steet et al., 2000; Marics et al., 2002; Seed and Hauschka, 1988). The ‘community effect’ that regulates myogenesis in Xenopus is mediated by early-acting Fgf signalling (Fish et al., 2002; Standley et al., 2001). Although eFGF (an fgf4/6 homologue) is a candidate mediator of the community effect in vivo, fgf8 is also expressed in nascent Xenopus somites just when fast muscle is differentiating and in a pattern remarkably similar to that in zebrafish (Grimaldi et al., 2004; Moreno and Kimble, 2004). Moreover, interruption of Fgf signalling can inhibit somitic myogenesis (Marics et al., 2002). Our findings do not rule out involvement of other Fgfs, particularly the close homologues Fgf17 and Fgf17b (Cao et al., 2004; Reifers et al., 2000a), in somitic myogenesis in zebrafish. Interestingly, in Drosophila, Fgf8-
like factors have recently been identified as signals required for generation of a subset of somatic muscles (Gryzik and Muller, 2004; Michelson et al., 1998). We speculate that triggering myogenic progression within the mesoderm may be an evolutionarily conserved function of Fgf8 signalling.

**Fgf signalling maintains myod**

Fast fibre differentiation in rostral somites is significantly delayed (about 3 hours at 28°C) relative to initiation of myod expression (Blagden et al., 1997). Fgf signalling maintains myod expression during this delay. SU5402 treatment decreases lateral myod expression in somites that express myod prior to treatment. This maintenance function may persist until fast fibre terminal differentiation. Maintenance may not be required in caudal somites as fast fibres differentiate soon after myod initiation.

The normal decline of myod expression in rostral somites of wild-type embryos with age parallels terminal differentiation of the lateral fast cells. However, residual myod expression remains in older anterior somites and this is relatively insensitive to SU5402 treatment. We suggest this Fgf-independent myod expression is in other cell populations, such as the migrating SSFs (see discussion of flh below). A requirement for Fgf8 only up until terminal differentiation is consistent with the loss of most somitic expression of fgf8 at later stages (Roehl and Nüsslein-Volhard, 2001).

It is notable that loss of Fgf8 function does not reduce fast muscle as much as might be predicted from the severe early reduction of myod expression in the lateral somite. Consistently, myogenin reduction is less marked than that of myod. Some recovery of fast differentiation could be driven by later-acting Fgf8s. Alternatively, differentiation of somitic cells that do not express myod early may partially rescue fast myogenesis. The extent to which residual medial fast fibres are structurally and functionally normal remains to be determined.

**A Fgf8- and Hh-independent population of medial fast fibres**

Our results show that medial somite cells, including both slow and medial fast muscle precursors, are not dependent on Fgf signalling and can express myod and undergo terminal differentiation in the presence of SU5402, Fgf8 MO and in acel mutant. This may explain why previous studies have concluded that there is only a mild posterior mesoderm or anteroposterior patterning defect in ace and Fgf8 MO-injected embryos (Draper et al., 2003; Reifers et al., 1998).

A unique population of medial fibres distinct from MPs express low levels of Engrailed (Devoto et al., 1996; Hatta et al., 1991). These multinucleate fast fibres express Engrailed in response to late Hh signalling and have been named MFF (Roy et al., 2001; Wolff et al., 2003). The Fgf8-independent residual fast fibres include cells capable of forming MFFs, based on their location, fast character and continued Engrailed expression in flh mutants or after SU5402 treatment. Strikingly, however, our cells do not require Hh signalling for myod expression or terminal differentiation. Engrailed expression is not extensive enough to account for all residual fast fibres. Thus, late Hh may act upon these cells to promote Engrailed expression (Wolff et al., 2003). Fast fibre terminal differentiation may be required prior to Hh signalling.

The medial location of our residual fast fibres indicates that midline patterning may be important for their formation. Although Hh signalling is required for medial slow muscle formation (Barresi et al., 2000; Blagden et al., 1997; Du et al., 1997; Schauerte et al., 1998), our results demonstrate that neither Hh signalling nor slow muscle is required for residual fast fibre initiation, as medial myod expression is observed in yot mutants and in embryos treated with the Hh signalling antagonist cyclopamine. Moreover, blockade of Hh signalling after initiation of myod expression demonstrates that Hh signalling is not required for the maintenance of myod expression in these cells. Nevertheless, the flh mutation leads to ablation of essentially all myod expression in the posterior somite. The homeobox transcription factor Flh is required for notochord formation but is expressed more broadly in midline tissue at early stages (Talbot et al., 1995). So either a non-Hh notochord-derived signal or a cell-autonomous action of Flh in somite precursors may control medial fast fibre initiation. Residual snail1 expression is present in medial cells of ace mutant embryos, highlighting a possible role for Snail1 in the initiation of myod expression (Reifers et al., 1998). The cellular and molecular origin of our residual fast fibres requires further study.

**Slow fibres form and migrate without Fgf8**

Slow MP and SSF fibres form in the absence of Fgf8. Whether other Fgf signalling might be required for slow fibre formation is uncertain because early SU5402 treatment disrupts gastrulation. SSF normally migrate laterally to lie superficial to fast muscle, but under a dermomyotome-like external cell layer (see below). Although SSFs come to lie lateral to the residual fast fibres in embryos with defective Fgf8 signalling, they fail to cross the enlarged domain of undifferentiated lateral somitic tissue. This correlation raises the possibility that the lateral displacement of slow muscle is dependent on differentiation of fast muscle.

**Expansion of dermomyotome-like tissue in the absence of Fgf8 signalling**

We examined expression of several zebrafish homologues of molecules that mark amniote dermomyotome. pax3 and meox genes mark many/most cells in nascent somites, but subsequently become more restricted. Pax3 is observed in thin cells on the somite surface, probably the ‘external cells’ (Waterman, 1969). We have also observed Pax7 protein in this superficial cell layer in a variety of fish species (Devoto et al., 2005). meox is expressed in a subset of cells at the somite surface. A zebrafish gene denominated wnt11r has greater sequence homology to murine Wnt11 than the gene originally named wnt11 in zebrafish (J. Minchin, unpublished, see www.ensembl.org). Like mouse Wnt11 (Christiansen et al., 1995), zebrafish wnt11r is expressed in the dorsomedial corner of the nascent zebrafish somite. Thus, cells near the superficial surface of the zebrafish somite share a number of molecular characteristics with amniote dermomyotome.

In the absence of Fgf8 signalling, the dermomyotome-like markers are upregulated in the lateral somite, suggesting that cells failing to undergo myogenesis maintain a character similar to that of immature somites. Thus, lack of Fgf signalling does not lead to death of lateral somite cells, but causes them to remain in a less differentiated state, perhaps awaiting further signals. High, possibly unphysiological, levels
of Fgf8 from implanted beads appear to drive ectopic myod expression in anterior somite cells. Nevertheless, the block on differentiation caused by Fgf signalling blockade can be relieved by removal of the blockade or application of exogenous Fgf8 with the recovery of a near-normal myod expression pattern. It seems the myogenic program can be arrested and resumed in lateral cells with no obvious delay or defect from control embryos. Thus, the blocked cells appear to retain a ‘memory’ for their fate that allows Fgf signalling to promote rapid myod mRNA accumulation. These findings suggest that raising Fgf8 within the somite controls the timing of myod expression in the lateral cells, but may not be responsible for the spatial restriction of expression to the posterior somite border.

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