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The role of SF/HGF and c-Met in the development of skeletal muscle

Susanne Dietrich[1,*], Faikah Abou-Rebyeh[2,*], Henning Brohmann[2,*], Friedhelm Bladt[2,*,§], Eva Sonnenberg-Riethmacher[2,*], Tomoichiro Yamaai[2,¶], Andrew Lumsden[1], Beate Brand-Saberi[3] and Carmen Birchmeier[2,‡]

[1]King's College, London Bridge, London SE1 9RT, UK
[2]Max-Delbrueck-Center for Molecular Medicine, Robert-Roessle-Strasse 10, 13092 Berlin, Germany
[3]Institut für Anatomie II, Albert-Ludwigs Universität Freiburg, Germany
[§]Present address: Samuel Lunefeld Research Institute, Mount Sinai Hospital, Toronto, Canada
[¶]On leave of absence from the Okayama University, Japan
*These authors have contributed equally
[‡]Author for correspondence (e-mail: cbirch@mdc-berlin.de)

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SUMMARY

Hypaxial skeletal muscles develop from migratory and non-migratory precursor cells that are generated by the lateral lip of the dermomyotome. Previous work shows that the formation of migratory precursors requires the c-*Met* and *SF/HGF* genes. We show here that in mice lacking c-Met or SF/HGF, the initial development of the dermomyotome proceeds appropriately and growth and survival of cells in the dermomyotome are not affected. Migratory precursors are also correctly specified, as monitored by the expression of *Lbx1*. However, these cells remain aggregated and fail to take up long range migration. We conclude that parallel but independent cues converge on the migratory hypaxial precursors in the dermomyotomal lip after they are laid down: a signal given by SF/HGF that controls the emigration of the precursors, and an as yet unidentified signal that controls *Lbx1*. SF/HGF and c-Met act in a paracrine manner to control emigration, and migratory cells only dissociate from somites located close to SF/HGF-expressing cells. During long range migration, prolonged receptor-ligand-interaction appears to be required, as *SF/HGF* is expressed both along the routes and at the target sites of migratory myogenic progenitors. Mice that lack c-Met die during the second part of gestation due to a placental defect. Rescue of the placental defect by aggregation of tetraploid (wild type) and diploid (c-*Met*$^{-/-}$) morulae allows development of c-*Met* mutant animals to term. They lack muscle groups that derive from migratory precursor cells, but display otherwise normal skeletal musculature.

Key words: c-Met, Scatter factor, Hepatocyte growth factor, Pax3, Lbx1, Hypaxial muscle, Cell migration, Mouse

INTRODUCTION

During amniote development, the paraxial mesoderm segments to form somites, ball-like structures that consist of morphologically uniform epithelial cells (reviewed by Christ and Ordahl, 1995). The fate of these cells is determined by the environment of the somite. Extrinsic cues regulate differentiation into the ventral sclerotome and the dorsal dermomyotome. Cells in the dermomyotome give rise to all skeletal muscles of the body and some head muscles. Skeletal muscle precursors are, however, restricted to specific territories of the dermomyotome. The medial lip of the dermomyotome generates the medial myotome and gives rise to the epaxial musculature (deep muscles of the back; cf. Ordahl and Le Douarin, 1992; Denetclaw et al., 1997). The lateral lip of the dermomyotome will generate the hypaxial musculature (superficially, laterally and ventrally located muscles; cf. Ordahl and Le Douarin, 1992; Christ and Ordahl, 1995).

Hypaxial musculature is not an homogenous entity, as it develops from a migratory and a non-migratory precursor population (reviewed by Christ and Ordahl, 1995). Migratory cells are formed from the dermomyotome of somites that are located occipitally, cervically and at the level of the limbs. In such somites, the epithelial cells of the lateral dermomyotomal lips delaminate, and scattered cells are released that take up long range migration. They give rise to the tongue musculature, the muscular diaphragm and the limb and associated shoulder musculature, respectively (Nishi, 1967; Grim, 1970; Chevallier et al., 1977; Christ et al., 1977; Jacob et al., 1978, 1979; Schemainda, 1979; Noden, 1983; Bladt et al., 1995; this study). The migratory cells, although destined to form muscle, do not express myogenic determination factors or contractile proteins until they reach their target sites (Bober et al., 1991; Pownall and Emerson, 1992; Smith et al., 1994; Williams and Ordahl, 1994). In contrast, in somites on flank levels the ventral dermomyotome generates non-migratory precursors of hypaxial musculature and gives rise to the intercostal muscles and the various muscles of the body wall (Christ et al., 1983; Denetclaw et al., 1997).

c-*Met* (encoding a tyrosine kinase receptor), *Sim1* (encoding a basic helix-loop-helix transcription factor), and, at elevated levels, *Pax3* (encoding a paired and homeodomain containing

transcription factor) are expressed in the lateral dermomyotome of all somites. Thus, expression of these genes does not discriminate between migratory and non-migratory muscle precursors (Goulding et al., 1994; Williams and Ordahl, 1994; Bladt et al., 1995; Fan et al., 1996; Pourquié et al., 1996; Yang et al., 1996). Conversely, expression of the *Lbx1* gene (encoding a homeobox transcription factor) is confined to migratory precursors. *Lbx1* is induced in the dermomyotomal lips of only those somites that are destined to generate migratory cells. This expression continues during precursor cell migration and declines when the cells settle down at their target sites to differentiate (Jagla et al., 1995; Dietrich et al., 1998; Mennerich et al., 1989).

Mice deficient for the c-Met tyrosine kinase receptor or its ligand, SF/HGF, lack muscle groups that derive from migratory hypaxial precursor cells (Bladt et al., 1995; Maina et al., 1996). In these mutants, myogenic precursors fail to populate the limb bud. Furthermore, SF/HGF is expressed in the limb bud, suggesting that SF/HGF driven activation of c-Met is responsible for the release of migratory cells (Bladt et al, 1995). This is supported by the finding that application of SF/HGF to flank somites of the chick causes de-epithelialisation of their lateral dermomyotomal lips (Brand-Saberi et al., 1996; Heymann et al., 1996). It is not clear, however, whether the interaction of receptor and ligand is required for the earlier specification of migratory muscle precursors. Additional functions of c-Met during myogenesis have been suggested, for instance in the expansion of the myogenic precursor pool (Maina et al., 1996); indeed, c-*Met* transcripts are associated with all muscle groups at late stages of myogenesis, and are also found in satellite cells of the adult (Sonnenberg et al., 1993; Cornelison et al., 1997; Anastasi et al., 1997; Gal et al., 1998; Tatsumi et al., 1998).

Similar to mice that lack c-Met or SF/HGF, in *Pax3* mutant mice, Splotch, migratory muscle precursors in the limbs are absent and limb muscles, shoulder muscles and diaphragm fail to develop (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996; Tajbakhsh et al., 1997; Tremblay et al., 1998). Interestingly, expression of c-*Met* is barely detectable in Splotch lateral dermomyotomes (Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996). In addition, the c-*Met* promoter contains a Pax3 binding site, and Pax3 can drive reporter gene expression from the c-*Met* promoter in vitro (Epstein et al., 1996). Thus, Pax3 may control the release of migratory muscle precursors in vivo by activating c-*Met*. However, Pax3 serves additional functions during the development of the lateral dermomyotomal and of non-migratory muscle precursors (Daston et al., 1996; Tajbakhsh et al., 1997; Tremblay et al., 1998).

Here we examine the role of the c-Met-SF/HGF dependent signalling cascade in the formation of skeletal muscle. Dermomyotome development in *SF/HGF* or c-*Met* mutant embryos was analysed histologically and by the use of various markers. We demonstrate that in the absence of c-Met or SF/HGF, the dermomyotome develops appropriately. Migratory myogenic precursors are correctly specified, as monitored by the expression of *Lbx1*. However, instead of taking up migration, the cells remain aggregated and stay in close proximity to the somite. We furthermore show that *SF/HGF* is expressed close to all somites which generate migratory precursor cells, along the migratory paths and at the target sites. The expression pattern is thus in accordance with a role of SF/HGF as an inducer of delamination of dermomyotomal cells, and indicates additional functions during the migratory process. Mice that lack c-Met die during the second part of gestation due to a independent placental phenotype, which precludes the analysis of skeletal muscle at late developmental stages. To overcome this limitation, we rescued the placental defect of c-*Met* mutant mice by the use of tetraploid wild-type cells. Such rescued animals develop to term; they lack hypaxial muscle groups generated from migratory myogenic precursors, but display otherwise normal skeletal muscle.

MATERIALS AND METHODS

Strains of mutant mice

The generation of targeted mutations in the mouse *SF/HGF* and c-*Met* genes and the establishment of strains that carry the mutations have been described (Bladt et al., 1995; Schmidt et al., 1995). The analysis was performed in animals with a mixed 129Ola/C57BL6 genetic background. Homozygous mutant animals were obtained by heterozygous matings; the genotype of the embryos and animals was determined by PCR.

Preparation of probes and in situ hybridisation

RNA probes were prepared as previously described: mouse c-Met and SF/HGF cDNA (Sonnenberg et al., 1993); Pax3 cDNA (500 bp) encoding the C-terminal part of the protein that includes the homeobox sequence (Goulding et al., 1991); Sim1 cDNA fragment (1.2 kb) (Fan et al., 1996); a mouse Lbx1 fragment (520 bp) that corresponds to the 3′ coding region including the homeobox (Jagla et al., 1995). Labelled transcripts were synthesised either with T3 or T7 RNA polymerase. All probes revealed reproducible hybridisation patterns when used in antisense orientation. Transcripts in sense orientation revealed no specific hybridisation patterns.

In situ hybridisation on frozen sections (10 μm) of mouse embryos embedded in OCT compound (Tissue-Tek, Miles Inc., Elkhart, Illinios, USA) was performed using ^{35}S-labelled UTP and CTP labelled riboprobes (Sonnenberg et al., 1993). Whole-mount in situ hybridisation (Wilkinson, 1992) was performed with digoxigenin (DIG)-labelled mRNA probes (DIG-RNA labelling kit, Boehringer Mannheim).

Histological analysis

Embryos were fixed overnight in 4% PFA in PBS, dehydrated and embedded in hydroxyethylmethacrylate (Technovit 7100 resin, Kulzer GmbH, Wehrheim, Germany). The same embedding procedure was used for embryos stained by whole-mount in situ hybridisation. Serial sections (5-6 μm) were cut and counterstained with Delafield's hematoxylin and eosin. To determine the proportion of mitotic and apoptotic cells, sections that spanned the entire forelimb of E9.5 embryos were stained with DAPI (Sigma); intact, mitotic and pyknotic nuclei in the dermomytome were counted.

For vibratome sectioning, stained embryos were embedded in 20% gelatine in PBS and refixed in 4% PFA in PBS overnight. Sections were cut to 40 μm.

Generation of tetraploid embryos and morula aggregation

Embryos at the 2-cell stage were isolated from wild-type C57BL6 mice. Tetraploidy was achieved by electrofusion (Wang et al., 1997). The blastomeres were fused in M2 medium using an electric pulse for 100 mseconds in an effective field (92 V; 6 V AC field) generated by a CF-100 pulse generator (Biochemical Laboratory Service,

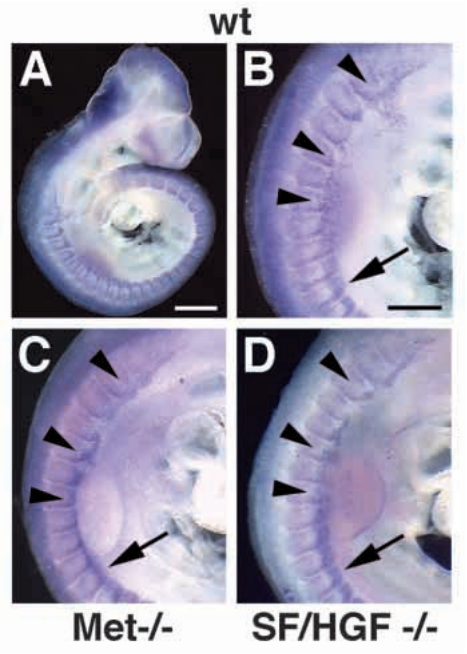

Fig. 1. *Pax3* expression in occipital somites of c-*Met* and *SF/HGF* mutants. Expression of *Pax3* (A-C) in wild-type (A,B), c-*Met*$^{-/-}$ (C) and *SF/HGF*$^{-/-}$ embryos (D) as analysed by whole mount in situ hybridisation. Arrows in B-D point towards the up-regulated *Pax3* expression domain in the lateral dermomyotome of control and mutant embryos; the arrowheads indicate occipital and cervical somites where migratory hypaxial precursors delaminate from the lateral dermomyotome in control, but not in mutant embryos. In mutant embryos (C,D), the *Pax3*-expressing cells at the lateral edge join, but migrating cells are not observed. Embryos were analysed at E10.0. Bars (A) 500 μm, (B) 300 μm.

Budapest). Fused embryos were selected and incubated overnight until they reached the 4-cell stage.

8-cell stage morulae were isolated from 2.5 days p.c. c-*Met*$^{+/-}$ females intercrossed with c-*Met*$^{+/-}$ males. 25% of these embryos are expected to have a c-*Met*$^{-/-}$genotype. The zona pellucida was removed by Tyrode's solution, and the diploid morulae from c-*Met*$^{+/-}$ intercrosses were aggregated with 4-cell stage tetraploid wild-type morulae, and further incubated under paraffin oil in a droplet of M16 medium. Under these conditions, the majority of the embryos spontaneously formed blastocysts composed of diploid and tetraploid cells. The blastocysts were transferred into the uterus of pseudopregnant mice, which were subjected to Caesarean section on day 16.5 to day 18.5 of gestation to isolate the embryos together with yolk sacs and placentas. Embryos with a c-*Met*$^{-/-}$ genotype were recognisable by the thinness of their extremities; the genotype of embryo and yolk sac was determined by PCR.

BrdU labelling

BrdU (75 μg/g body weight) was injected twice into pregnant foster mothers on E15 (8 am and 8 pm); mice were killed on E17.5 and the embryos dissected. BrdU-positive nuclei were identified on frozen sections using anti-BrdU antibodies (Sigma, monoclonal antibody clone BU-33). The sections were counterstained with anti-laminin antibodies to identify the myotubes surrounded by extracellular matrix. Nuclei inside myotubes were determined in intercostal muscle and deep muscle of the back. The number of labelled nuclei and of myotubes within a field were counted using a Leica confocal microscope.

RESULTS

Development of the dermomyotome in c-*Met* and *SF/HGF* mutant mice

Previous studies revealed that mice mutant for c-*Met* or *SF/HGF* fail to form muscles of the limbs, diaphragm and tongue; moreover, they lack myogenic precursors that invade the limb (Bladt et al., 1995). To clarify whether formation of all migratory myogenic precursors is arrested and to determine the stage at which the development of these precursors is interrupted, we studied dermomyotome development in c-*Met* and *SF/HGF* mutants using *Pax3* as

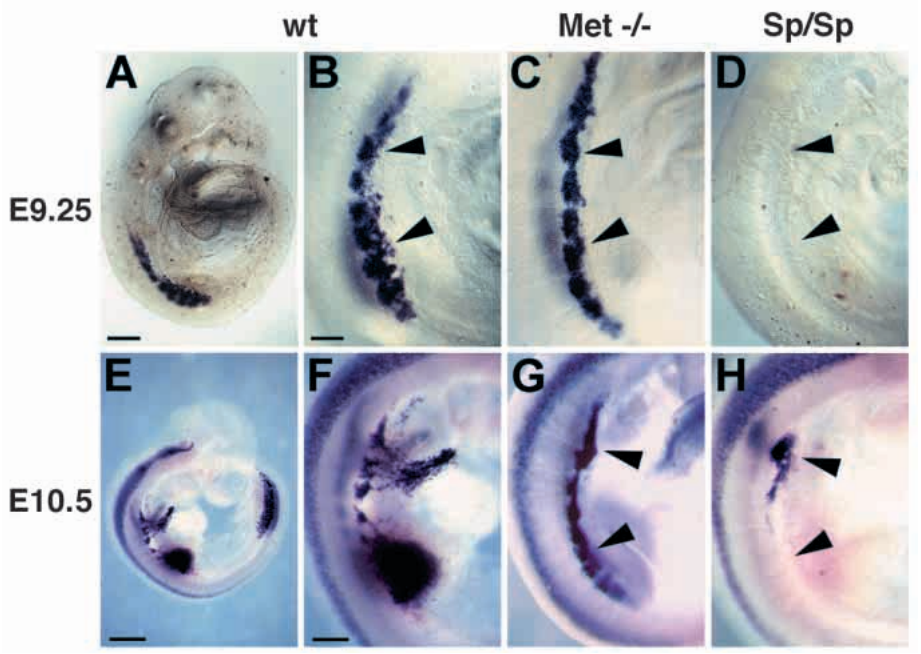

Fig. 2. Lbx1 expression in wild-type, c-Met$^{-/-}$ and Splotch embryos. Whole-mount in situ hybridisation of wild-type (A,B,E,F), c-*Met*$^{-/-}$ (C,G) and Splotch (*Sp/Sp*; D,H) embryos using a Lbx1-specific probe at E9.25 (A-D) and E10.5 (E-H). Arrowheads point to occipital and cervical somites adjacent to the forelimbs. Note that *Lbx1* is expressed in the lateral dermomyotomal lips in wild-type and c-*Met*$^{-/-}$ embryos, but migrating cells are absent in the mutant. In Splotch, *Lbx1* expression is absent in the trunk and delayed in the occipital region. Bars (A) 400 μm, (E) 900 μm, (B) 175 μm, (F) 375 μm.

marker (Goulding et al., 1994; Williams and Ordahl, 1994). In the dermomyotome of c-*Met* and *SF/HGF* mutant mice, *Pax3* expression commenced normally and lateral upregulation of *Pax3* was observed at E10.0 (Fig. 1A-D, arrows). However, in wild-type embryos the lateral dermomyotomal lips of occipital, cervical and forelimb somites dispersed and released migrating cells (Fig. 1A,B arrowheads); this was not observed at any axial level in c-*Met* or *SF/HGF* mutant embryos. In the mutants, the strongly *Pax3*-expressing cells remained closely associated with the dermomyotomal lip; in occipital somites, the *Pax3*-positive cells adhered to cells from adjacent somites, connecting individual somites (Fig. 1C,D arrowheads). *Pax7* is initially co-expressed with *Pax3* in the dermomyotome, but subsequently becomes down-regulated laterally (Jostes et al., 1990). We found identical expression pattern of *Pax7* in controls, and mice mutant for c-*Met* or *SF/HGF* by in situ hybridisation on sections (data not shown). *Sim1* serves as marker for the lateral somite half (Fan et al., 1996; Pourquié et al., 1996) and is expressed in identical patterns in control embryos and in embryos mutant for c-*Met* or *SF/HGF* at E10.0 and at later stages of development (data not shown).

Expression of *Lbx1* is confined to hypaxial muscle precursor cells destined to take up long range migration (Jagla et al., 1995; Dietrich et al., 1998; Mennerich et al., 1998). In wild-type embryos at E9.25, *Lbx1* was expressed in the lateral dermomyotomal lips of occipital, cervical and some forelimb somites as well as in cells that had dispersed from those somites (Fig. 2A,B, arrowheads). At this stage, embryos mutant for c-*Met* or *SF/HGF* showed similar expression in the dermomyotome. However, instead of migrating out, *Lbx1*-expressing cells remained attached to their source in the c-*Met* (Fig. 2C) or *SF/HGF* (not shown) mutants. In wild-type embryos at E10.5, *Lbx1*-expressing streams of cells lead from occipital somites to the branchial arches, and from limb somites into the limb bud (Fig. 2E,F). In addition, the dispersing lateral dermomyotomal lips of hindlimb levels also expressed *Lbx1* (Fig. 2E). Removal of the forelimb revealed an additional stream that began in cervical somites and bypassed the forelimb (see also below). In mice mutant for c-*Met* (Fig. 2G) or *SF/HGF* (not shown), somitic *Lbx1* expression was present at the appropriate axial levels at E10.5. Cells that expressed *Lbx1* adhered to the dermomyotomal lips and formed bridges to neighbouring somites. Migratory cells were not released at any axial level, although extensions that protruded into the direction in which cells would normally migrate, were frequently observed. We conclude that in the absence of c-Met or its ligand, migratory hypaxial muscle precursors are specified, as assessed by *Lbx1* expression, but fail to migrate from the lateral dermomyotomal lip.

In contrast, expression of *Lbx1* in Splotch was dramatically altered. At E9.25, Splotch homozygotes did not express *Lbx1* at all (Fig. 2D). At E10.5, some *Lbx1* expression was found in occipital somites (Fig. 2H) but emigration of tongue muscle precursors seemed delayed. In the trunk, no *Lbx1* expression was found. We conclude that in the trunk of *Pax3*-mutant mice, not only is delamination of migratory hypaxial muscle precursors compromised, but their specification in the dermomytome is already impaired (cf. also Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996).

Morphology of *Lbx1*-expressing cells at limb levels in c-*Met* mutant mice

We analysed the morphology of the lateral dermomyotomal lips at forelimb levels in the c-*Met* and *SF/HGF* mutants. Cross sections of control embryos revealed dispersed lateral lips at E9.5 and E10.5 (Fig. 3A,B, arrowhead). In the mice mutant for c-*Met*, these lips retained their epithelial arrangement at E9.5 (Fig. 3C, arrowhead).

Lbx1 expression was associated with cells located in the de-epithelialising dermomyotomal lips in wild-type embryos on E9.5 (Fig. 3D, E). In addition, dispersed cells that had lost their epithelial appearance expressed the gene. Many of these cells had moved away from the dermomyotome. In contrast, in mice mutant for c-*Met*, the *Lbx1*-expressing lateral dermomyotomal lips displayed an epithelial organisation (Fig. 3F). Cells outside the lateral dermomyotomal lips were found to express *Lbx1*. Nevertheless, they resided in close proximity to the lateral dermomyotomal lip and remained tightly clustered. In wild-type embryos at E10.5, the lateral dermomyotomal lip of somites in the centre of the forelimb was devoid of *Lbx1*-expressing cells, indicating that these cells had completely emigrated (Fig. 3G,H). In contrast, the dermomyotomal lip of c-*Met* mutant embryos contained *Lbx1*-positive cells, and the dermomyotomal lips appeared to extend further ventrally than in control embryos (Fig. 3I).

SF/HGF can not only scatter epithelial cells, but is also a potent growth and survival factor for many cell types in culture. To assess the influence on growth or survival of dermomyotomal cells, we compared the ratio of mitotic or apoptotic cells in the dermomytome of wild-type, *SF/HGF* and c-*Met* mutant embryos. The mitotic index in the dermomytome of cervical somites at forelimb levels was identical in wild-type (2.49%; s.d. ±0.33%), *SF/HGF* or c-*Met* mutant embryos on E9.5 (2.38%; s.d. ±0.21%). Apoptosis was assessed by TUNEL staining, and quantified by determining the proportion of pyknotic nuclei. The frequency of cell death in the dermomyotomes of individual embryos was highly variable; this was observed in wild-type, *SF/HGF* and c-*Met* mutant embryos. However, when average ratios from several individuals were determined, a similar proportion of pyknotic cells was observed in wild-type (2.7% ±s.d. 1.2%), *SF/HGF* and c-*Met* mutants (2.9% s.d. ±2.5%). We conclude that c-Met receptor and its ligand, SF/HGF affects the delamination, but not growth or survival of dermomyotomal cells.

Relation of SF/HGF expression and migration of hypaxial muscle precursors in the branchial arches and the trunk

We analyzed the spatial pattern of SF/HGF expression during delamination and migration of hypaxial precursors. *Lbx1* was used to visualise migrating precursors; it should be noted that these cells also express c-*Met*. Vibratome sections through the floor of the branchial arches of E10.5 control embryos revealed the path of *Lbx1*-expressing precursors that extend from occipital somites towards the branchial arches where the anlage of the tongue is located (Fig. 4A, compare to Fig. 4E,F). Most of this route was located laterally below the surface ectoderm. Finally, the stream turned anteriorly and towards the ventral midline, and the cells assembled in the centre of the 1st branchial arch (arrow in Fig. 4A). This stream of migrating muscle precursors was absent in embryos mutant for c-*Met*

(Fig. 4B). In situ hybridisation of corresponding sections revealed that *SF/HGF* expression coincided with the route of migratory tongue muscle precursors in wild-type embryos (Fig. 4C). Signals along this migratory path were detected as early as E9.25, when migratory cells began to de-laminate (data not shown). Mice mutant for c-*Met* showed identical expression of *SF/HGF* (Fig. 4D).

In the trunk, migrating streams of cells that generate the muscles of the limbs and the diaphragm are observed. Cross sections at the anterior border of the forelimbs showed that in wild-type embryos at E10.5, hypaxial muscle precursors have already reached their targets in the limb and attained a dorsal and ventral position (Fig. 5A, compare to Fig 2E,F). Cells that express *Lbx1* and *SF/HGF* co-localised (Fig. 5C,E). Expression of *SF/HGF* in the limb bud mesenchyme was detected already at the onset of cell migration at E9.25. The *SF/HGF*-expressing domain is located first in the proximal limb bud and subsequently in dorsal and ventral territories (not shown). In mice mutant for c-*Met*, the limb muscle precursors are absent (Fig. 5B). However, *SF/HGF* displayed its normal expression pattern (Fig. 5F).

The migratory precursors of the diaphragm muscle at E10.5 in wild-type mice were apparent as a stream of *Lbx1*-expressing cells that emerged from somites at the anterior border of the forelimb. These cells bypassed the limb buds ventrally and entered the septum transversum (Fig. 5A,C, arrowheads). Along their route and within the septum transversum, *SF/HGF* was expressed (Fig. 5E). In mice lacking a functional c-*Met* gene, cells migrating into the septum transversum were absent (Fig. 5B,D), while *SF/HGF* was expressed in the correct pattern (Fig. 5F). Thus, *SF/HGF* expression is independent from the presence of migratory muscle precursors along all routes of migration. The spatial correlation of *SF/HGF* expression and migratory pathways of hypaxial precursor cells suggests a prolonged requirement of the ligand during migration of hypaxial muscle progenitors.

Rescue of the embryonal lethality of c-*Met* mutant mice and analysis of skeletal muscle in the perinatal animal

Mice that lack c-Met or SF/HGF activity die during the second part of gestation. The embryonal lethality is probably caused by a placental defect (Uehara et al., 1995). This embryonal death has hitherto precluded an analysis of skeletal muscle at late developmental stages. To overcome this limitation, we rescued the animals by generating aggregation chimeras between tetraploid wild-type and diploid c-*Met* mutant embryos. Previous studies indicate that tetraploid cells can effectively contribute to all extraembryonal tissues, but not to the embryo itself (James et al., 1995; Wang et al., 1997). The tetraploid (wild type) cells should therefore complement the placental defect and allow the analysis of skeletal muscle of c-*Met* mutant embryos at late developmental stages. c-*Met*$^{-/-}$ tetraploid aggregation chimeras reached E17.5 or E18.5, indicating that extraembryonal and not embryonal defects are responsible for the embryonal lethality. PCR analysis demonstrated the existence of embryos containing wild-type and mutant c-*Met* alleles in the yolk sac, and solely the mutant c-*Met* allele in embryonal tissues (Fig. 6A). When compared to their littermates, the rescued c-*Met* mutants were normal in overall size, but displayed thin extremities. Histological analysis demonstrated a normal morphology of the placenta and a reduced size of the liver (not shown). The rescued c-*Met* mutants lacked muscles of the diaphragm (Fig. 6C, compare to the control embryo in Fig. 6B), limbs, shoulders and the hypoglossal cord (not shown). Other hypaxial and epaxial muscle groups of the trunk, like intercostal muscle (Fig 6B,C) or deep muscles of the back, appeared normal in size. BrdU-labelling was used to assess the expansion of the myogenic precursor pool late in intra-uterine development (cf. Material and Methods). The number of BrdU-labelled nuclei present in myotubes of intercostal or of deep muscles of the back were compared (Fig. 6D,E). The number of BrdU-positive nuclei in 100 myotubes was 20.9 (s.d. ±1.5) for control embryos (2 individuals), and 21.2 (s.d. ±0.9) in rescued c-met$^{-/-}$ embryos (2 individuals). Counting BrdU-positive cells within a field, we observed 7.6 (s.d. ±2.3) in control and 8.1 (s.d. ±3.2) in rescued c-*Met*$^{-/-}$ embryos. Thus, our analysis demonstrates an essential role of the c-Met receptor in the development of muscles that derive from migratory hypaxial precursors, but does not indicate a function in the development of other trunk muscles, i.e. epaxial muscle groups or of hypaxial muscle that derive from non-migratory precursors.

DISCUSSION

Hypaxial skeletal muscle derives from the lateral dermomyotome and is generated by a migratory and a non-migratory cell population. The c-*Met*, *SF/HGF* and *Pax3* genes control the generation of migratory hypaxial precursor cells as the mutants lacking these genes lack the muscle groups derived from them. Here we have directly compared c-*Met* mutant mice with the *Pax3* mutant Splotch and demonstrated that formation of migratory precursor cells is distinctly affected. *Lbx1* expression marks exclusively the migrating precursor cells and their progenitors in the lateral dermomyotome. In Splotch mice, *Lbx1* is not induced in somites of the trunk and appears delayed in occipital somites. In contrast, induction of *Lbx1* in the dermomyotome proceeds appropriately in c-*Met* or *SF/HGF* mutant embryos. Therefore, unlike mutations in *Pax3*, mutations in the SF/HGF/c-Met signalling system interfere specifically with the release of the migratory cells, but not with their prior development.

The role of c-Met and SF/HGF in development of hypaxial muscle precursors

Previous studies suggested that the release of migratory muscle precursors from occipital, cervical and limb somites depends on c-Met and its ligand, SF/HGF: Muscles of the limbs, shoulder, tongue and diaphragm that derive from migratory hypaxial precursors are absent, but other muscle groups form (Bladt et al., 1995). Our detailed analysis presented here demonstrates that c-Met and its ligand do not act during patterning of the dermomyotome. Using *Sim1*, *Pax3*, *Pax7*, c-*Met* and *Lbx1* as markers, we found that in both c-*Met* and *SF/HGF* mutants the patterning and differentiation of the dermomytome proceeds appropriately, suggesting that the general precursor pool for hypaxial muscles is established correctly.

Although the function of *Lbx1* has not been established, the expression of this gene serves as a marker for migratory

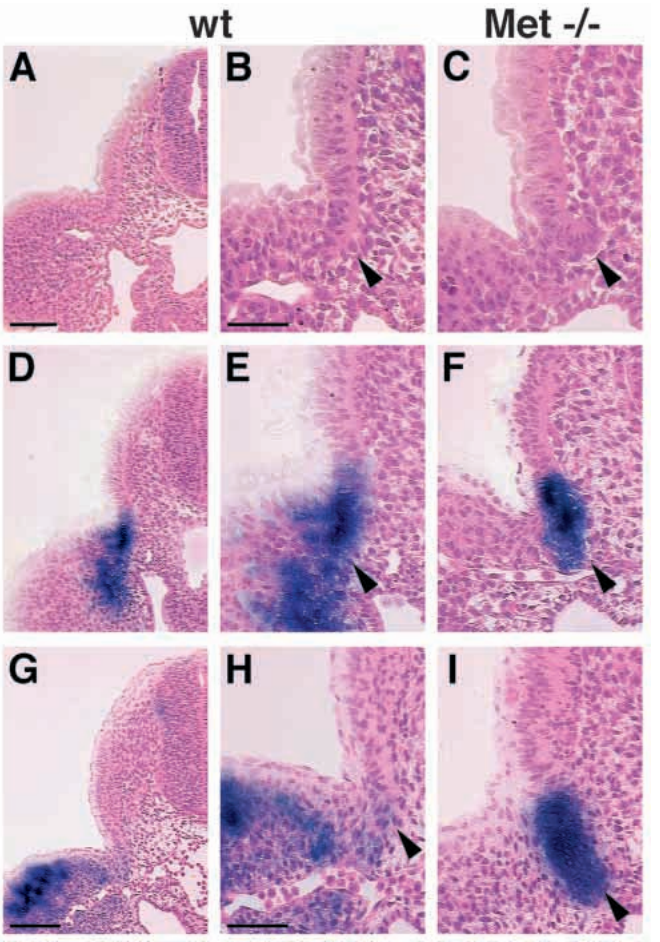

Fig. 3. Histological appearance of the dermomyotome and Lbx1-expressing cells in wild-type and c-Met$^{-/-}$ embryos. Cross sections of control (A,B), c-*Met*$^{-/-}$ (C) at the forelimb level were stained with eosin-hematoxylin. Note the epithelial organisation of the dermomyotome in control and mutant embryos. Arrowheads point towards the lateral lip of the dermomyotome. This lip disperses in the control embryo but maintains its epithelial organisation in the mutants. Cross sections of a control (D,E,G,H) and a c-*Met* mutant (F,I) embryos embedded in plastic after whole-mount in situ hybridisation with a Lbx1-specific probe. Sections are from forelimb levels of E9.25 (D-F) and E10.5 (G-I) embryos. Bars (A,G) 100 μm and (B,H) 50 μm.

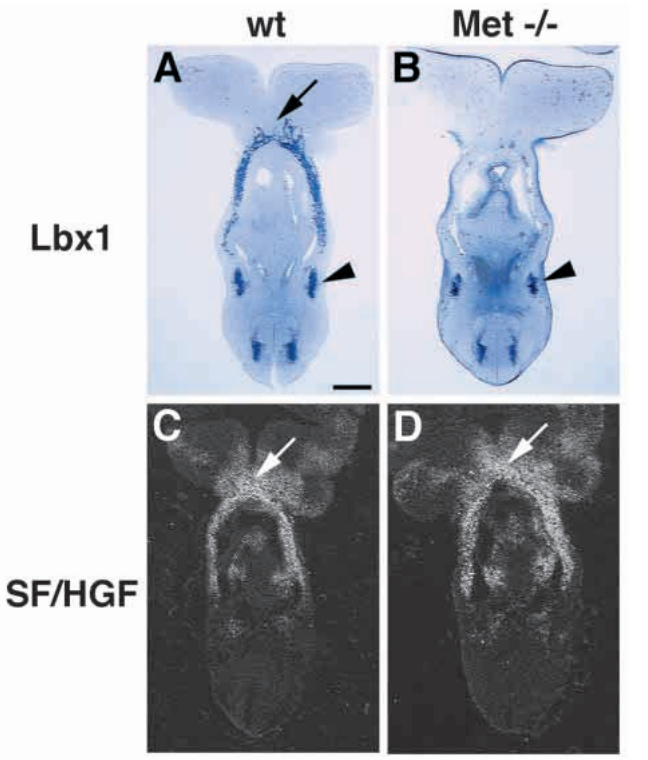

Fig. 4. Route and target of migrating hypaxial muscle precursors in the branchial arches and SF/HGF expression. Coronal vibratome sections of a wild-type (A) and a c-*Met*$^{-/-}$ (B) embryo after whole-mount in situ hybridisation with a Lbx1-specific probe. In the control but not the mutant embryo, precursor cells migrate from occipital somites (arrowhead) towards the centre of the first branchial arch (arrow), to form the anlage of the tongue muscles. SF/HGF expression in wild-type (C) and c-*Met*$^{-/-}$ (D) embryo, as determined by in situ hybridisation with a ^{35}S-labelled SF/HGF probe on frozen sections that are shown in dark-field. Note that the patterns are identical in the control and mutant embryos, although no migrating precursors are observed in the mutant. Arrows point to the centre of the first branchial arch. Embryos were used at E10.5 (33-35 somites). Bar, 250 μm.

precursors of hypaxial muscle, and allows the analysis of the specification of these cells prior to their emigration from the dermomyotome (Jagla et al., 1995; Dietrich et al., 1998; Mennerich et al., 1998). In c-*Met* and *SF/HGF* mutants, *Lbx1* expression is initiated correctly. All somites expressing the gene in wild-type embryos are also positive in the mutants. Thus, c-*Met* and *SF/HGF* are not required for the specification of hypaxial muscle precursors capable of long range migration. *Lbx1* and c-*Met* or *SF/HGF* thus act in spatially but not genetically connected pathways.

However, in the absence of functional c-Met or SF/HGF, de-epithelialisation of the lateral dermomyotomal lips and dispersal of muscle precursors is defective. In the mutants, the lateral dermomyotomal lips remain compact. Cells maintain their epithelial organisation and continue to express *Lbx1*, indicating that migratory precursors remain in the somite. Small groups of *Lbx1*-, c-*Met*- or *Pax3*-expressing cells are found outside the lateral dermomyotomal lip. However, these cells form densely packed aggregates that stay in close proximity of the somite. This suggests that c-Met and its ligand are essential to liberate migratory precursors from the lateral dermomyotomal lip and to scatter these cells. This is supported by the finding that SF/HGF applied to flank somites of the chick causes de-epithelialisation of the lateral dermomyotome and ectopic emigration of muscle precursors (Brand-Saberi et al., 1996; Heymann et al., 1996).

In contrast to *Lbx1*, c-*Met* is expressed in all the lateral dermomyotomal lips along the anteroposterior axis. However, mutations of both the c-*Met* and the *SF/HGF* genes specifically prevent the emigration of migratory muscle precursors while the development of non-migratory muscle precursors proceeds normally. We show here that *SF/HGF* expression is observed in occipital, cervical and limb somatopleura only, but not in the somatopleure of the flank. Signal transduction through the c-

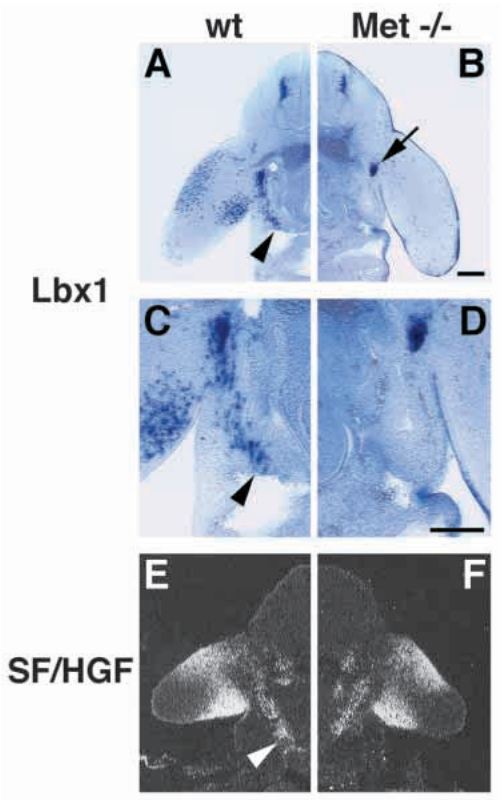

Fig. 5. Routes and targets of migrating hypaxial muscle precursors and SF/HGF expression in the trunk. Vibratome cross section at the anterior border of a wild-type (A,C) and c-*Met*$^{-/-}$ (B,D) forelimb after whole-mount in situ hybridisation with a Lbx1-specific probe. In the control embryo, hypaxial precursor cells migrate from cervical somites towards the septum transversum (arrowheads in A,C) where the anlage of the diaphragm is located. The muscle precursors have also assembled in the dorsal and ventral mesenchyme of the forelimb. In c-*Met*$^{-/-}$ embryos, the precursors remain mobile (arrow in B). *SF/HGF* expression in wild-type (E) and c-*Met*$^{-/-}$ (F) embryo, as determined by in situ hybridisation with a ^{35}S-labelled SF/HGF probe on frozen sections that are shown in dark-field. Note expression of *SF/HGF* along the route used by the migratory precursors. In the mutant, migrating precursors are absent, but *SF/HGF* is expressed normally. The arrowhead (E) points to the septum transversum. Embryos were used at E10.5 (33-35 somite stages). Bars (B,D) 100 μm.

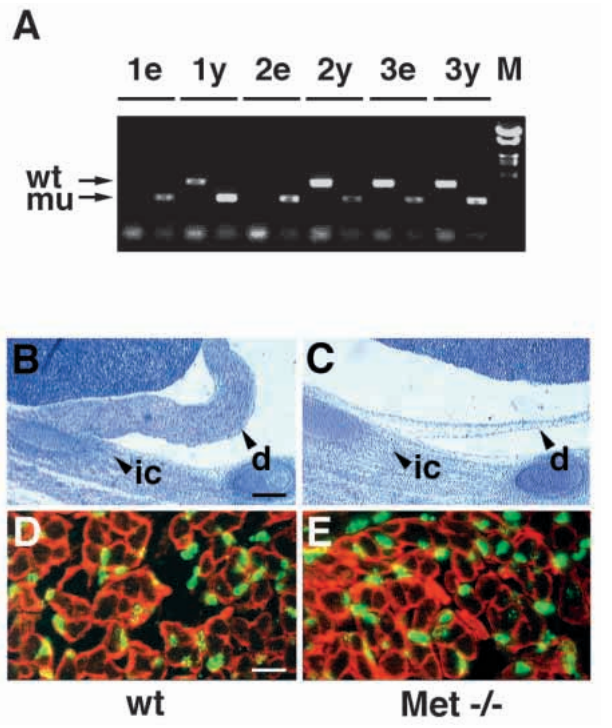

Fig. 6. Rescue of the placental defect of *c-Met*$^{-/-}$ mice and analysis of skeletal muscle in the perinatal animal. (A) Genotyping by PCR of embryos isolated on E18.5, which were generated by the aggregation of tetraploid wild-type morulae and diploid morulae obtained from matings of c-*Met* heterozygous animals. Note that embryo 1 and 2 contain wild-type and c-*Met* mutant alleles in the yolk sac, and only the c-*Met* mutant allele in the embryo. Embryo 3 contains wild-type and c-*Met* mutant alleles both in the embryo (e) and the yolk sac (y). (B,C) Histological appearance of intercostal (ic) and diaphragm (d) muscle of a control (B) and c-*Met*$^{-/-}$ (C) aggregation chimera on E17.5. (D,E) BrdU-positive nuclei (green) of intercostal muscle from a control (D) and c-*Met*$^{-/-}$ (E) aggregation chimera on E17.5. The extracellular matrix surrounding myotubes was visualized by anti-laminin antibodies (red). Bar, (B) 300 μm, (D) 200 μm.

Met receptor is thus confined by the spatially limited expression of the ligand.

Prolonged requirement of c-Met and SF/HGF during long range migration of muscle precursors

Interestingly, expression of *SF/HGF* is also found along all routes of migratory cells and at their target sites. During their course of migration, hypaxial muscle precursors continue to express c-*Met* (Bladt et al., 1995; Yang et al., 1996). Thus, they constantly receive the SF/HGF signal. Localisation of this signal suggests that SF/HGF directs muscle precursor migration towards their target sites. However, when SF/HGF was applied at high concentrations to the flank of chick embryos, the de-epithelialising cells did not approach the SF/HGF source (Brand-Saberi et al., 1996; Heymann et al., 1996). Considering that c-Met and SF/HGF act in the initial dissociation of migratory muscle precursors, their prolonged interaction may be required to prevent cell re-aggregation, thereby maintaining cell motility. They might also control additional properties of migratory muscle precursors such as suppressing the expression of myogenic determination factors.

Pax3 controls both specification and delamination of migratory muscle precursors

In the *Pax3* mutant Splotch, all muscles derived from migratory precursors are lacking in the trunk, while the formation of tongue muscles appears delayed (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996; Tajbakhsh et al., 1997; Tremblay et al., 1998). Since expression of c-*Met* is barely detectable in the lateral dermomyotome of Splotch, *Pax3* has been suggested to control the generation of migratory muscle precursors by transactivation of c-*Met* (Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996). This is supported by the finding that Pax3 can bind to the c-*Met* promoter to transactivate reporter genes in vitro (Epstein et al., 1996).

However, additional changes are present in mice mutant for *Pax3* (Bober et al., 1991; Daston et al., 1996; Tremblay et al., 1998). Splotch mice fail to upregulate *Pax3* in the lateral dermomyotome and display severely disorganised dermomyotomal lips. We detect no *Lbx1* expression in the trunk of Splotch mice, suggesting that migratory hypaxial muscle precursors are not specified. This is in agreement with a recent report by others (Mennerich et al., 1998). Moreover, the *Pax3* mutation affects not only migratory hypaxial precursors. In Splotch, all muscle masses are reduced, and the rectus abdominis that ventrally closes the body wall is missing (Tremblay et al., 1998). This reflects an early function of *Pax3* during expansion of the dermomyotomal progenitor pool and the mediolateral elongation of the dermomyotome. All of these additional changes are not present in mice that lack c-Met or SF/HGF, indicating that c-*Met* independent properties of muscle precursors are compromised.

c-Met and the expansion of the myogenic precursor pool

During development of skeletal muscle, c-*Met* is initially expressed in the lateral dermomyotome, and thus in precursors of hypaxial musculature (Bladt et al., 1995; Yang et al., 1996). At subsequent stages of development, expression of c-*Met* in all developing muscle can be observed, and c-*Met* expression is retained in satellite cells, the precursor cells of the adult muscle (Sonnenberg et al., 1993; Cornelison et al., 1997; Anastasi et al., 1997; Gal et al., 1998; Tatsumi et al., 1998). SF/HGF is a potent growth factor for satellite cells in culture; moreover, changes in the expansion of the myogenic precursor pool were reported in perinatal mice that carry a mutation which alters the bi-dentate docking site of c-*Met* (Maina et al., 1996; Cornelison et al., 1997; Anastasi et al., 1997; Gal et al., 1998; Tatsumi et al., 1998). Our detailed analysis presented here indicates that during the time migratory hypaxial precursors are generated, growth rates of the dermomyotome are not affected by a lack of functional SF/HGF or c-Met. Moreover, a tetraploid rescue of the placental defect phenotype does not reveal an effect of the c-*Met* mutation on the size of trunk muscle masses in the perinatal animal, or on the expansion of myogenic precursors during late intra-uterine development. Instead, the defects observed in the rescued perinatal c-*Met* mutants are restricted to muscle groups that develop from migratory hypaxial precursors.

In contrast to the results presented here indicating that c-Met is not necessary for growth of myogenic precursors prior to birth, it was reported in an independent study that a hypomorph mutation in c-*Met* reduces the myoblast precursor pool that participates in secondary myogenesis in the trunk (Maina et al., 1996). The biological basis for these differences in phenotypes are not yet clear, but a number of hypotheses can be proposed. First, the hypomorph allele previously produced changes the bi-dentate docking site of c-Met, which is phosphorylated in response to SF/HGF and responsible for binding various substrates that transmit the c-Met signal. The mutation allows signalling, but changes signalling specificity, and might therefore cause phenotypes not found in this study. The mutant allele used here deletes sequences encoding the ATP-binding site of c-Met and thus results in a non-functional receptor that does not possess tyrosine kinase activity and is not phosphorylated in response to SF/HGF (Bladt et al., 1995). Second, the hypomorph allele might also affect placental development, but in a manner more subtle than a null mutation. This might reduce myogenic precursor expansion due to insufficient supply of nutrients or oxygen to the embryos. Third, differences in genetic background could also account for the differences (mixed 129Sv/C57BL6 background vs. a mixed 129Ola/C57BL6 background).

The genetic hierarchy regulating the formation of migratory muscle precursors

As a summary of our analysis and of previous work by others, we propose the following genetic hierarchy for the development of migratory hypaxial muscle precursors. Hypaxial muscle progenitors are induced by a complex set of signals that up-regulate *Pax3* in the lateral dermomyotomal lip (Cossu et al., 1996; Pourquié et al., 1996; Dietrich et al., 1998). This upregulation is *Pax3* dependent and not observed in Splotch (Bober et al., 1994; Daston et al., 1996; Tremblay et al, 1998). *Pax3* is also required for the activation of *Lbx1* (Mennerich et al., 1998 and this study). However, to restrict *Lbx1* expression to occipital, cervical and limb somites, additional cues are required. Such signals might be provided by members of the FGF family, some of which are expressed in the correct spatial pattern (Ohuchi, et al. 1997; Webb et al, 1997). Alternatively, *Lbx1* may be negatively regulated in the flank. Besides *Lbx1*, *Pax3* activates expression of the gene that encodes the c-Met receptor. Now, cells in the lateral dermomyotomal lips can receive the SF/HGF signal provided in a spatially restricted manner by the mesoderm. As a consequence of receptor-ligand interaction, hypaxial muscle progenitors dissociate to take up long range migration.

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