Expression of the *met* receptor tyrosine kinase in muscle progenitor cells in somites and limbs is absent in *Splotch* mice

Xiu-Ming Yang¹,³, Kyle Vogan², Philippe Gros² and Morag Park*¹,²,³,⁴

¹Molecular Oncology Group, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1
Departments of ²Biochemistry, ³Medicine and ⁴Oncology, McGill University, Montreal, Quebec, Canada

*Author for correspondence

**SUMMARY**

Hepatocyte growth factor/scatter factor (HGF/SF) stimulates proliferation, dissociation, migration and morphogenesis of cells in culture. To investigate a possible role for HGF/SF and its receptor, the Met tyrosine kinase, in embryonic development, we have analyzed their expression in mouse embryos from day 7.5 of gestation by whole-mount in situ hybridization. *Met* expression is first detected in the ventral portion of somites at day 9.25 of gestation (22 somite embryo) at the level of fore limb buds. As somites mature, *met* expression is detected in caudal somites, and is confined to the lateral and medial tips of the dermomyotome and dermomyotome/myotome respectively. In contrast, HGF/SF is expressed exclusively in the mesodermal core of the limb bud. As the dermomyotome elongates ventrolaterally, the *met*-expressing cells at the lateral tip appear to detach from the somite, invade the limb bud and localize at the dorsal and ventral limb sides in close proximity to HGF/SF-expressing cells. At later stages, both *met*- and HGF/SF-expressing cells appear to migrate distally and localize to the digit forming area of the developing hand plate. *Met* expression in the lateral dermomyotome and limb bud coincides with expression of *Pax-3*, a marker for migrating muscle precursor cells in the somite and limb. *Splotch-2H* and *Splotch-delayed* mice, which harbor mutations in *Pax-3*, show major disruptions in early limb muscle development. Significantly, no *met*-expressing cells were observed in the limbs of homozygous *Splotch-2H* and *Splotch-delayed* animals, whereas HGF/SF expression was not affected. The restricted expression of *met* to a sub-population of *Pax-3*-expressing cells in the lateral tip of the dermomyotome, demonstrates that *met* represents a unique molecular marker for this migratory cell population. From these observations, together with the biological activities of HGF/SF, we propose that in homozygous *Splotch* embryos the failure of muscle precursors to migrate into and populate the limb bud results from a loss of *met* expression in the cells at the ventrolateral edge of the somitic dermomyotome.

Key words: Met receptor tyrosine kinase, hepatocyte growth factor/scatter factor, somites, *Splotch*, *Pax-3*, cell migration, dermomyotome, myoblasts, mouse

**INTRODUCTION**

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a multifunctional cytokine secreted by embryonal fibroblasts with activities on epithelial and endothelial cells. HGF/SF promotes proliferation, dissociation and motility of epithelial cells and endothelial cells in culture (Stoker et al., 1987; Grant et al., 1993), and stimulates the invasion of carcinoma cells (Weidner et al., 1990) and the branching morphogenesis of tubular epithelial cells (Montesano et al., 1991). HGF/SF is essential for embryonic development (Schmidt et al., 1995; Uehara et al., 1995), is a potent angiogenic factor (Grant et al., 1993) and is involved in organ regeneration (Matsumoto and Nakamura, 1992). The high affinity receptor for HGF/SF has been identified as the product of the *met* proto-oncogene, which encodes a receptor tyrosine kinase (RTK) (Bottaro et al., 1991; Naldini et al., 1991). Activation of the Met RTK is sufficient to mediate the pleiotropic biological activities of HGF/SF in culture (Weidner et al., 1993; Komada and Kitamura, 1993; Zhu et al., 1994), suggesting that the Met RTK mediates the physiological responses to HGF/SF in vivo.

The *met* and HGF/SF genes are expressed in the majority of adult and embryonic tissues (Chan et al., 1988; Iyer et al., 1990; Tashiro et al., 1990; Sonnenberg et al., 1993), with *met* showing highest levels of expression in epithelia that line tubules and ducts (Di Renzo et al., 1991; Yang and Park, 1995). During embryogenesis, HGF/SF is expressed in mesenchymal cells in close proximity to *met*-expressing epithelia (Sonnenberg et al., 1993), supporting a role for Met and HGF/SF in mesenchymal/epithelial cell interactions important for organogenesis during embryonic development. However, while mice lacking a functional HGF/SF protein die in utero with developmental defects in liver and placenta, they fail to show defects in other epithelial organs (Schmidt et al., 1995; Uehara et al., 1995). Moreover, the observation that the ectopic expression of HGF/SF in chick embryos at gastrulation induces the formation of neuronal tissues and markers (Stern et al., 1990; Streit et al., 1995), plus the ability of HGF/SF to stimulate dis-
sociation, motility and invasion of epithelia, have also suggested a role for HGF/SF and its receptor, Met, during early murine development, at stages prior to organogenesis.

To investigate the possible function of the Met RTK and HGF/SF during early development, we have examined the temporal and spatial expression pattern of met and HGF/SF in mouse embryos from day 7.5 of gestation by whole-mount in situ hybridization. We demonstrate that HGF/SF is expressed in the mesodermal core of the developing limb buds, whereas the met RTK is expressed in somites in two distinct domains; one localized to the lateral tip of the dermomyotome marking a population of cells that will migrate into the limb bud, and a second localized to the medial tip of the dermomyotome/myotome. Moreover, we show that the cell population that expresses met in the lateral tip of the dermomyotome and in the limb is missing in the Pax-3 mutants Splotch-2H (Sp 2H) and Splotch-delayed (Sp 2) which lack limb bud musculature, whereas HGF/SF expression is apparently normal in these mutant animals. These results identify met as a unique marker for limb muscle progenitor cells, and suggests a possible interaction between Pax-3 and met during the development of this specific cell population at the ventrolateral edge of the somitic dermomyotome.

MATERIALS AND METHODS

Mouse embryos and staging
Embryos for in situ hybridizations were dissected from the F1 female mice of a B6 and C3H cross. Sp 2H/+ mice on a mixed C3H/HeH and 101/H background (MRC Radiobiology Unit, Harwell, England) and Sp 2/+ on a C57BL/6J background (The Jackson Laboratory, Bar Harbor, ME) were maintained in our animal facility by brother-sister mating. Embryos were generated using timed matings, with the Harbor, ME) were maintained in our animal facility by brother-sister mating. Embryos were generated using timed matings, with the Harbor, ME) were maintained in our animal facility by brother-sister mating.

Genotyping of Splotch embryos
As probes for hybridization studies, an 0.5 kb EcoRI fragment (pmet SC1) (Yang and Park, 1993) derived from the 5’-portion of the murine met cDNA (pD) (Iyer et al., 1990), or a 1.5 kb EcoRI fragment (pmet SC2) located at the 3’-portion of pmet SC1 were subcloned into the pBluescript KSII+ vector (Stratagene). The mouse HGF/SF cDNA was kindly provided by Dr. Walter Birchmeier. Non-radioactive antisense and sense riboprobes were synthesized by in vitro transcription using digoxigenin-UTP following the manufacturers instructions (Boehringer Mannheim). The met antisense probes used in these experiments were as follows: met1, corresponding to bp 479-720 in the murine cDNA; met2, bp 268-720; met3, bp 1876-2298 (Chan et al., 1988). HGF/SF antisense probes were as described by Sonnenberg et al. (1993). The myogenin, MyoD antisense probes (Sassoon et al., 1989), and Pax-3 probes (Goulding et al., 1991), have been described previously.

Whole-mount in situ hybridization
Whole-mount in situ hybridization was performed according to a revised protocol kindly provided by Dr. Janet Rossant (Consol and Rossant, 1992). Embryos were dissected free in DEPC (diethyl pyrocarbonate) treated phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 2 hours at 4°C with rocking. After washing three times, embryos were incubated for 1 hour with cold PBS containing 0.1% Tween 20 (PBT) at 4°C, then stored in 100% methanol at −20°C for less than a month. For hybridization, embryos were incubated at 65°C overnight in hybridization buffer with 0.4 μg/ml digoxigenin-labeled RNA probes.

To determine the hybridization at the cellular level, stained embryos were re-fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature, dehydrated with a TBST (137 mM NaCl, 25 mM Tris-HCl pH 7.6, 3 mm KCl, 0.1% Tween 20)/methanol series (30%, 50%, 75%, 95% and 100% twice, 5 minutes each), and incubated with 100% methanol twice for 1 hour each, xylene twice for 1 hour each, 1:1 xylene/paraffin twice for 30 minutes each at 60°C and paraffin three times for 1 hour each at 60°C. Embryos were then embedded in wax and sectioned at 7 μm. Sections were dewaxed for 2x 15 minutes in xylene and rehydrated in an ethanol series (100% ×2; 95%, 70%, 50% and water, 5 minutes each), followed by counterstaining with 0.002% eosin solution, and then mounted with Permount.

RNA isolation and RT-PCR
RNA was isolated from frozen sections prepared from mouse embryos of 11 d.p.c, and first strand cDNA was synthesized from 2 μg of total RNA as described previously (Vogan et al., 1993). Pax-3, met, HGF/SF, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) specific cDNA fragments were amplified by PCR from 50 ng of template using the following sets of primers under the reaction conditions outlined below: Pax-3 (sense: 5′-538GTTCGGCTCTCTAA-GATCTTGC558′; antisense: 5′-719GCGCTTCCTGGA-CAATTGC699′; 32 cycles, annealing: 62°C), met (sense: 5′-272AGATGAACGTGAAACAGTAAG281′; antisense: 5′-566CTAAT-GAGTGTACATCATAG546′; 32 cycles, annealing: 52°C), HGF/SF (sense: 5′-434CATGATTTGTCATG25′; antisense: 5′-760ACTGGAAGATGTCAG34′; 25 cycles, annealing: 55°C), and GAPDH (sense: 5′-375CCATGGAAG-GGCTGG393′; antisense: 5′-570CAAAGTGGTCATGGAT-GAC551′; 20 cycles, annealing: 62°C). Amplification products were radiolabeled by including 2.5 μCi of [α-32P]dATP in each reaction, and products were resolved on 12% polyacrylamide gels and exposed to X-ray film for autoradiography.

RESULTS

Localization of the met RTK transcripts to developing somites
To investigate the involvement of the Met RTK and its ligand
HGF/SF in early embryonic development, we have studied the expression of these genes in mouse embryos from day 7.5 of gestation by whole-mount in situ hybridization. A comparison of embryos hybridized with digoxigenin-labelled antisense (Fig. 1A-C) and sense met cRNA probes (Fig. 1D) revealed that met transcripts are not detected at 7 or 8 days post coitum (d.p.c.) and are first identified in the ventral portion of somites at 9.25-9.5 d.p.c. (20-30 somite embryos; Fig. 1A). Somites first appear at day 8 of gestation and rapidly partition into the sclerotome, which will subsequently give rise to myotome and dermatome (reviewed by Emerson, 1993). Somites mature in a rostrocaudal direction where newly formed undifferentiated somites and fully differentiated somites can be found in the same embryo in caudal and cranial regions respectively.

Met expression was localized to two discrete domains within somites. By 9.25-9.5 d.p.c., met was first expressed in the ventral portion of rostral somites at the level where the fore limb buds are formed (Fig. 1A). Met expression progressed to more caudal somites and by 9.75 d.p.c. met was expressed in the ventral region of thoracic somites and in somites at the level of the hind limb buds (Fig. 1B) and in the ventral region of the most caudal somites by 10 d.p.c. (Fig. 1C). A second domain of met expression was observed in the dorsal portion of the somites. A low level of expression was first identified in the dorsal aspect of somites rostral to the fore limb bud at 9.5 d.p.c. (Fig. 1A). Met expression increased and progressed to more caudal somites such that met was expressed in both the ventral and dorsal tip of the same somite (Fig. 1C). From a comparison of 10 embryos at 9.75 d.p.c. (Fig. 1B), met expression within a given somite begins in the dorsal portion several hours later than in the ventral portion of the same somite. In addition, at 9.75 d.p.c., met is expressed in the dorsal tip of somites rostral to the fore limb bud; somites that do not appear to express met in their ventral domain (Fig. 1B,C).

**Location of met-expressing cells in the developing limb buds**

At 9.75 d.p.c. (Fig. 1B) and 10 d.p.c. (Fig. 1C,E,I), met transcripts were no longer detected in the ventral portion of somites at the level of the fore (Fig. 1I) and hind limb buds (Fig. 1C,E). Instead, at 9.75 d.p.c., met-expressing cells were detected as a diffuse signal localized to the mesodermal core at the base of the developing fore limb bud (Fig. 1B,I) and hind limb bud at 10 d.p.c. (Fig. 1C,E). Expression increased and progressed distally by 11.0 d.p.c. where it was concentrated in the distal half of the limb bud over the dorsal (data not shown) and ventral limb sides (Fig. 1J). Expression decreased by 11.5 d.p.c. and localized to the digit forming area in the developing hand plate (Fig. 1K,L). A similar localization of met-expressing cells was observed in the hind limbs lagging 0.5 days behind the fore limbs (Fig. 1E).

**Expression of HGF/SF in the developing limb buds**

To establish whether Met/HGF/SF interactions occur in the developing limb bud, we localized HGF/SF expression by whole-mount in situ hybridization. A diffuse signal for HGF/SF was detected at 9.75 d.p.c. (Fig. 1F). However, by early 10 d.p.c. (Fig. 1G,M), when compared with embryos hybridized with a sense riboprobe (Fig. 1H), HGF/SF-specific transcripts were concentrated to the mesodermal core of the limb bud (Fig. 1G) and were localized over the dorsal (Fig. 1M) and ventral (data not shown) limb sides. By 11-11.75 d.p.c., HGF/SF-expressing cells progressed distally and become concentrated ventrally to a subapical domain in the distal half of the fore limb (Fig. 1N) then subsequently to the digit forming area of the developing hand plate (Fig. 1P). The progression of HGF/SF-expressing cells to the distal half of the limb buds correlated spatially and temporally with met-expressing cells in the limb bud at this stage (Fig 1J-L). The amplification of HGF/SF-specific cDNA fragments from head, trunk, tail and limb sections of embryos by polymerase chain reaction further demonstrated that only basal levels of HGF/SF were present in head, trunk and tail sections, whereas high levels of HGF/SF were observed in the limbs (Fig. 5, left panel).

**Met expression overlaps with Pax-3, a marker for migratory muscle precursor cells**

Somites give rise to several myogenic cell populations, one derived from migratory muscle precursor cells at the lateral tip of somitic dermomyotome at the level of the limb buds, a second originates from the lateral tip of the somitic dermomyotome of the trunk from which abdominal muscles derive, and a third derives from the medial half of the dermomyotome and gives rise to axial, back and intercostal muscle (Christ et al., 1983; Ordahl and Le Douarin, 1992). Expression of met in the lateral tip of somites adjacent to the limb buds (Fig. 1A-C) and expression of HGF/SF (Fig. 1J-L) and met (Fig. 1N-P) in adjacent regions of the limb buds, suggest a potential role for met and HGF/SF in the migration of muscle precursor cells from the somite. In order to establish if met expression correlates with the location of muscle precursor cells in the developing embryo, met expression was compared with that of Pax3 and of myogenic regulatory factors myogenin and MyoD.

Pax-3, a member of the Pax family of transcriptional regulators, is expressed in the somitic dermomyotome and marks a population of cells that migrate from the lateral dermomyotome into the limbs (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). To identify the cell types in somites that express met transcripts following whole-mount in situ hybridization, embryos at various stages (9.25-10.5 d.p.c.) were postfixed in paraformaldehyde, sectioned and compared with sections from embryos hybridized with Pax3. At 9.75 d.p.c., Pax-3 is expressed in cells throughout the dermomyotome (Fig. 2A; Bober et al., 1994; Goulding et al., 1994) whereas met expression was concentrated in cells at the lateral tip of the dermomyotome (Fig. 2C), that overlap with a population of Pax3-expressing cells (Fig. 2A).

At 9.75 d.p.c., met- and Pax3-expressing cells localized to the lateral tip of the dermomyotome, appeared to detach from the somite domain, accumulate in clusters at the body-limb junction (Fig. 2D and B respectively), and then further invade the limb (Fig. 2E). The expression of met in the limb buds parallels that of Pax3-expressing cells (Fig. 2B) and by 10.5 d.p.c., both the met- (Fig. 3B) and Pax3- (Fig. 3C) expressing cell populations have segregated into dorsal and ventral limb regions. At this stage, met-expressing cells (Fig. 3B) overlap with the dorsal and ventral expression domains of MyoD (Fig. 3D and data not shown) and myogenin (Fig. 3E and data not shown), which later give rise to limb muscles (Sassoon et al., 1989; Ott et al., 1991). However, met-expressing cells (Fig. 3D and data not shown).
Fig. 1. Expression of *met* and *HGF/SF* during somite and limb development. Lateral views of whole-mount in situ hybridization with digoxigenin labelled antisense *met* (A-C,E-I-L), *HGF/SF* (F,G,M-P), sense *met* (D) or *HGF/SF* (H) cRNA probes. Specific hybrids are visualised as a blue precipitate. In embryos hybridized with sense probes (D,H), background staining is detected, mostly in the head, whereas (A) at 9.5 d.p.c. (30 somites), specific *met* transcripts are detected in the ventral portion (arrow) of somites at the level of the fore limb buds (f), in the dorsal tip of a few rostral somites (arrowhead), as well as in the tail bud. (B) At 9.75 d.p.c. (36 somites), *met* is no longer expressed in the ventral tip of somites at the fore limb, but is expressed in the fore limb bud (small arrowhead), the ventral region of thoracic (arrow), hind limb (h) and tail somites, and in the dorsal tip of somites (large arrowhead). (C) At 10 d.p.c. (45 somites), *met* transcripts are no longer observed in the ventral tip of somites at the level of the hind limb, but are concentrated in the hind limb bud (arrowhead). E is an enlargement of C showing *met* expression in the dorsal (large arrowhead) and ventral (arrow) tips of somites and *met* expression in the hind limb bud (small arrowhead). When compared with control sense *HGF/SF* cRNA probe (H), at 9.75 d.p.c., a diffuse signal for *HGF/SF* is observed throughout the whole embryo (F) which becomes concentrated in the fore limbs at 10 d.p.c. (45 somites) (G) (arrow). At 10 d.p.c., arrows indicate *met* (I) and *HGF/SF* (M) expression domains in the fore limb bud. At 11 d.p.c. (53 somites), expression of *met* (J) (arrow) and *HGF/SF* (N) (arrow) progresses distally and colocalizes on the ventral limb side. By 11.5 d.p.c. (58 somites), *met* is expressed in the dorsal (K, arrow) and ventral (L, arrow) sides of the fore hand plate at the digit forming area, whereas *HGF/SF* expression is not detected on the dorsal side (O) and appears to colocalize with the *met* expression domain on the ventral side (P, arrow) of the developing hand plate.
3B) were predominantly concentrated distal to that of MyoD (Fig. 3D) or myogenin (Fig. 3E) and localized adjacent to HGF/SF-expressing mesenchymal cells (Fig. 3A). Furthermore, from whole-mount in situ hybridizations at 10. 5 d.p.c. (Fig. 1E) and from transverse sections at the level of the fore limb bud at 9.75-10.75 d.p.c., we have also localized met expression to the dorsal/medial tip of the dermomyotome/early myotome (Fig. 2E).

Met-expressing cells are absent in the developing limbs of Splotch-2H embryos

The coexpression of met and Pax-3 in the lateral dermomyotome and the apparent progression of met-expressing cells into the limb buds, suggests a potential role for met in the development of limb musculature. In order to investigate whether met is expressed in the myoblast precursor cells of the limb buds, met expression was analyzed in Sp2H mutant mice. In Sp2H mice, which carry a mutant allele of the Pax-3 gene (Epstein et al., 1991), myoblast precursor cells expressing Pax-3 fail to migrate into the limb (Bober et al., 1994; Goulding et al., 1994). Thus, expression of myogenic genes is not detected in the limbs (Bober et al., 1994) and as a consequence, Sp2H mice are devoid of limb musculature (Franz, 1993; Franz et al., 1993).

To examine met and HGF/SF expression in Sp2H embryos, a series of whole-mount in situ hybridizations were performed between 9 and 11.5 d.p.c. In homozygous Sp2H embryos, distinct differences in the distribution of met transcripts were observed (Fig. 4G-I) when compared to the normal (Fig. 4A-C) and heterozygote (Fig. 4D-F) embryos, while those of HGF/SF remained normal (Fig. 4J). No positive cells hybridizing with met were observed in the ventral portion of somites at 9.75-11.5 d.p.c (Fig. 4G,H) (the development of Sp2H embryos is delayed approximately half a day compared to C3H/B6 F1 embryos). Significantly, no met-expressing cells that leave the somites or populate the limbs between 9.5-11.5 d.p.c. were observed in homozygous Sp2H embryos (Fig. 4H,I). Moreover, in homozygous Sp2H animals, met expression in the dorsal portion of the dermomyotome is severely decreased and/or dispersed in thoracic somites (Fig. 4G,H), but is maintained in somites rostral and caudal to the fore and hind limbs respectively. A second Splotch allele, Splotch-delayed, which contains a point mutation in the paired domain of Pax-3 (Vogan et al., 1993) with a similar defect in limb muscle (Franz, 1993), shows a pattern of met and HGF/SF expression similar to Sp2H (data not shown). RT-PCR analyses using RNA prepared from wild-type and mutant limbs confirm the observations that HGF/SF expression is not affected in mutant embryos (Fig. 5 right panel), whereas met expression is severely decreased.

DISCUSSION

The met RTK is expressed in migratory cells of the lateral dermomyotome; prospective myogenic precursors of the limbs

In this paper, we present data showing that the migratory precursor cells of limb bud muscle express the met RTK, whereas HGF/SF is expressed in the mesodermal core of the developing limb buds. Whole-mount in situ hybridizations on mouse embryos indicated that met is first expressed in the ventrolateral tip of the dermomyotome in somites at the level of the fore limb buds at 9.25 d.p.c. and at the level of the hind limb bud by 9.75 d.p.c (Fig. 1A,B). As somites mature, the

Fig. 2. Expression of met and Pax-3 in the dermomyotome (transverse sections obtained from whole-mount in situ embryos). Sections at fore limb level at 9.75 d.p.c. (36 somites) hybridized with Pax-3 (A,B) or met (C,D) antisense probes. Pax-3 is expressed throughout the dermomyotome, but is concentrated at the lateral tip (A, arrow). Met is expressed in cells located at the ventrolateral tip of the dermomyotome (C, arrow), colocalizing with a subpopulation of Pax-3-expressing cells. All met-expressing cells (D, arrow) and a subpopulation of Pax-3-expressing cells (B, arrow) at the ventrolateral tip of the dermomyotome, appear to migrate towards the base of the fore limb bud. At 10 d.p.c. (40 somites), met-expressing cells are found in the fore limb buds (arrow) and met expression is also detected in the dorsal-medial tip (arrowhead) of the dermomyotome/early myotome (E).
met-expressing cells in the lateral dermomyotome of 5-6 somites at the level of the fore and hind limb buds begin to invade adjacent limbs (Figs 1B,C,E, 2C,D). At later stages, met-expressing cells become organized into ventral and dorsal sides of the limb bud (Figs 1J-L, 3B) and appear to localize with muscle precursor cells that migrate into the mesodermal core of the fore and hind limb buds as identified from chick/quail grafting experiments (Christ et al., 1977, 1983; Ordahl and Le Douarin, 1992). As myogenic factors (MyoD and myogenin) begin to be expressed in the limb (Fig. 3D,E) and muscle differentiation occurs, the met signal disappears gradually from this region and is localized to a subapical domain on the dorsal and ventral limb side (Figs 1J-L, 3B) in close proximity to HGF/SF-expressing mesenchyme (Figs 1N,P, 3A).

This evidence suggests that the met-expressing cells from the lateral dermomyotome invade the limbs and correspond to muscle precursor cells. This is further supported by the observation that cells expressing met, in both the lateral tip of the dermomyotome (Fig. 2A,C) and in the limb bud (Fig. 3B), overlap with cells expressing Pax-3; a molecular marker for migratory precursor cells of limb muscle (Figs 2B,D, 3C) (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). Moreover, evidence for met expression in myogenic precursor cells, rather than the induction of met expression in the limb mesenchyme, is provided by the observation that no met-expressing cells are localized in the limbs of Splotch-2H (Fig. 4G-I) or Splotch-delayed mutant mice (data not shown), which have mutations in Pax-3 (Epstein et al., 1991; Vogan et al., 1993) and are devoid of limb musculature (Franz, 1993; Franz et al., 1993). Interestingly, met is also expressed in muscle derivatives which differentiate in cultures of pluripotent P19 teratocarcinoma cells (Yang and Park, 1993). However, met is not expressed in terminally differentiated muscles in the limb bud (Fig. 3B) or in skeletal muscles in the adult animal (Yang and Park, 1995). Thus, the expression of the met RTK appears restricted to proliferating and/or migrating muscle precursor cells.

**Met-expressing cells align adjacent to HGF/SF-expressing cells in the limb bud**

HGF/SF affects cultured epithelia by stimulating cell proliferation, breakdown of cell junctions, increased cell motility and cell scatter, which are coupled with the ability of HGF/SF to induce an epithelial-mesenchymal transition of cells in culture (Stoker et al., 1987; Weidner et al., 1990). The spatiotemporal distribution of HGF/SF-expressing cells in the developing limb (Fig. 1G,M-P), their organization into dorsal and ventral cell masses (Fig. 3A), and their subsequent localization to a subapical domain in the digit forming area to which met-expressing cells concentrate (Fig. 1N-P), is in agreement with recent reports demonstrating expression of HGF/SF in the murine (Bladt et al., 1995; Takebayashi et al., 1995) and chicken (Myokai et al., 1995) limb bud and supports a role for HGF/SF in the localization of migratory met-expressing cells to the limb bud. Although it is formally possible that met expression is induced in cells in the limb bud in the vicinity of the HGF/SF-expressing cells, the observation that the Sp2H and Spd mutants express HGF/SF in the limb buds at apparently wild-type levels (Figs 4J, 5), whereas no met-expressing cells are observed in the limb buds by in situ hybridization (Fig. 4G-I), makes this unlikely. However, we cannot exclude that the low level of met expression detected by RT-PCR in the limb buds of Splotch-2H mutant mice (Fig. 5) corresponds to de novo met expression in the limb bud. While this manuscript was in preparation, Bladt et al. (1995) reported an absence of limb musculature in Met−/− and HGF/SF−/− mice, demonstrating a direct role for the Met RTK and its ligand HGF/SF in the formation of limb bud musculature. These data are in agreement with our data and support a role for HGF/SF in stimulating the transition of epithelial, met-expressing lateral dermomyotome cells, into migratory, proliferating mesenchymal cells that invade the limbs and give rise to muscle.

In addition to the migration of met-expressing cells from the somites, both met- and HGF/SF-expressing cells within the limb bud appear to migrate to the base of the digit forming area of the hand plate (Fig. 1J-L,N-P). The signal for the migration
of the HGF/SF-expressing cells is unknown. However, Takebayashi et al. (1995) have recently demonstrated expression of HGF/SF in the digit forming area of the hand plate and although they failed to detect met expression, they demonstrated that Met/HGF/SF interactions are involved in cell motility, proliferation and proteoglycan synthesis of chondrocytes in culture. Thus the met-expressing cells localized in the digit forming area of the developing hand plate (Fig. 1K,L) may mark precursor cells for chondrocytes.

**Met expression defines two distinct dermomyotomal domains**

During embryogenesis, cells located in the lateral portion of

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Fig. 4. Met expression is absent in the limb buds of the Splotch-2H mutant. In control (+/+), embryos at 11 d.p.c. (48 somites), met expression is detected in the mesodermal core of the developing fore and hind limb buds (A-C), in the ventral tip of thoracic and caudal somites (A,B), and in the dorsal tip of nearly all somites (A). In a heterozygous embryo (+/−) with spina bifida (D-F), the met expression pattern is similar to that of +/+ embryos. In mutant embryos (Sp2H/+; Sp2H) with exencephaly and spina bifida (G-I), met expression is no longer detected in the fore (G,H) and hind limb buds (G,I) or in the ventral tip of thoracic somites, and is severely decreased in the dorsal tip of thoracic somites (G,H). Expression of HGF/SF in the dorsal and ventral sides of the developing fore and hind limb buds is normal in (Sp2H/+; Sp2H) mutant embryos (J, arrowhead). f, fore limb.

Fig. 5. Correlation of met and HGF/SF expression in normal and Splotch-2H embryos. First strand cDNA was synthesized from total RNA isolated from the head, trunk, tail and limb bud segments of 11 d.p.c. normal embryos (left panel), or from 11 d.p.c. limb buds of normal (+/+), heterozygous (Sp2H/+) and mutant (Sp2H/Sp2H) embryos (left panel). Specific segments of Pax-3, met and HGF/SF cDNA were amplified by polymerase chain reaction using specific oligonucleotide primers (see Materials and Methods). A PCR amplification using oligonucleotide primers specific for GAPDH was used to control for the amount of cDNA in each reaction.
the somitic dermomyotome migrate and give rise to abdominal muscle and limb muscle, whereas cells in the dorsal medial quadrant of the somite give rise to myotome, which forms the axial, back and intercostal muscles and dermomyotome, which gives rise to cells of the dermis (Christ et al., 1983; Ordahl and LeDouarin, 1992; Williams and Ordahl, 1994). In addition to its expression in cells at the ventrolateral tip of the dermomyotome, met is also expressed in cells at the dorsal-medial tip of the dermomyotome/early myotome (Fig. 2E). These cells are the first cells in the medial portion of the somite to activate expression of muscle differentiation markers and express MyoD (Williams and Ordahl, 1994). Although the exact function of these cells is not known, the expression of MyoD is localized in the myotome at later stages (Sassoon et al., 1989; Williams and Ordahl, 1994). This has suggested that there are different cell populations within the myotome; one is first recognizable as a cell population at the anteriomedial edge of the somite (Christ et al., 1978; Kaelin et al., 1988) that expresses myf-5 (Ott et al., 1991), a second may derive from a Pax-3-expressing cell population at the cranial edge of the medial half of the dermomyotome (Williams and Ordahl, 1994), and a third may derive from the dorsal-medial tip of the dermomyotome/myotome which expresses met and MyoD (Pownall and Emerson, 1991; Williams and Ordahl, 1994). These results, together with the observation that met is expressed in muscle of the intercostal region of embryos at 11-15 d.p.c. (Sonnenberg et al., 1993), suggest that met-expressing cells of the dorsal-medial tip of the dermomyotome/early myotome may be involved in the formation of intercostal and axial musculature. Although axial muscle does not appear to be affected in the met RTK−/− mice (Bladt et al., 1995), there may be functional redundancy in this cell population. For example in MyoD D−/− or Myf 5−/− mice there is no defect in axial musculature and this is only observed in Myf 5/Myo D−/− mice (Braun et al., 1992; Rudnicki et al., 1992, 1993).

Met is aberrantly expressed in Splotch-2H and Splotch-delayed embryos

From studies of Sp2H and SpΔ mutant embryos, the absence of a functional Pax-3 protein has profound effects on met expression. met-expressing cells are absent from limb buds and no met-expressing cells are detected in the ventral tip of somites, thus supporting a role for Pax-3 in directly or indirectly regulating met expression in these domains, or in specifying the met-expressing cells in the lateral dermomyotome. In contrast, met expression is apparently retained at wild-type levels in the dorsal-medial tip of somites, rostral to the developing forelimb bud and caudal to the hind limb bud, but is severely decreased in the dorsal tip of somites within the trunk region (Fig. 4G,H). The dermomyotome of somites at the trunk level is disorganized in Sp2H embryos (Franz, 1993; Franz et al., 1993; Bober et al., 1994), which may directly or indirectly affect met expression, or disperse met-expressing cells such that they are no longer detected by whole-mount in situ hybridization. Interestingly, although facial and body wall muscles develop almost normally in the Sp2H mutant, a reduction in axial muscles in a rostral-caudal gradient has been observed (Franz, 1993; Franz et al., 1993) which is therefore concurrent with the decrease in met-expressing cells in the dorsal tip of the dermomyotome in the Sp2H mutant (Fig. 4G,H).

Several lines of evidence suggest that Pax-3 may be involved in maintaining muscle precursor cells in an undifferentiated proliferative state (Galili et al., 1993; Epstein et al., 1995). This plus the observation, that in the met−/− mice (Bladt et al., 1995), Pax-3 expression appears normal, yet no limb bud musculature is formed, supports a role for the Met RTK and HGF/SF in the dissociation and migration of limb muscle precursor cells from the somite. Therefore in Splotch embryos, the failure of muscle precursors to leave the somites and populate the limbs may result directly from the failure of mutant forms of Pax-3 to induce Met expression in the presumptive cells at the ventrolateral edge of the dermomyotome. The possibility that Pax-3 acts directly to activate Met expression in this cell population remains an interesting avenue for further study.

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REFERENCES

Pownall, M. E. and Emerson, C. P. Jr.

Franz, T.

Ordahl, C. P. and Le Douarin, N. M.

Komada, M. and Kitamura, N.

Kaehn, K., Jacob, H. J., Christ, B., Hinrichsen, K. and Poelman, R. E.

Epstein, D. J., Vekemans, M. and Gros, P.

Franz, T., Kothary, R., Surani, M. A. H. and Grim, M.


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