The role of Lbx1 in migration of muscle precursor cells

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SUMMARY

The homeobox gene Lbx1 is expressed in migrating hypaxial muscle precursor cells during development. These precursors delaminate from the lateral edge of the dermomyotome and form distinct streams that migrate over large distances, using characteristic paths. The targets of migration are limbs, septum transversum and the floor of the first branchial arch where the cells form skeletal muscle of limbs and shoulders, diaphragm and hypoglossal cord, respectively. We used gene targeting to analyse the function of Lbx1 in the mouse. Myogenic precursor cells delaminate from the dermomyotome in Lbx1 mutants, but migrate in an aberrant manner. Most critically affected are migrating cells that move to the limbs. Precursor cells that reach the dorsal limb field are absent. In the ventral limb, precursors are present but distributed in an abnormal manner. As a consequence, at birth some muscles in the forelimbs are completely lacking (extensor muscles) or reduced in size (flexor muscles). Hindlimb muscles are affected strongly, and distal limb muscles are more affected than proximal ones. Other migrating precursor cells heading towards the floor of the first branchial arch move along the appropriate path in Lbx1 mutants. However, these cells migrate less efficiently and reduced numbers of precursors reach their distal target. At birth, the internal lingual muscle is therefore reduced in size. We suggest that Lbx1 controls the expression of genes that are essential for the recognition or interpretation of cues that guide migrating muscle precursors and maintain their migratory potential.

Key words: Pax3, c-Met, Hypaxial muscle, Homeobox gene, Lbx1, Directed migration

INTRODUCTION

Homebox genes encode DNA-binding transcription factors that can regulate axial patterning, segment or cell identity, and proliferation. The classical homebox genes are organised in clusters and determine the basic body plan of animals (Maconochie et al., 1996). In addition, homeobox genes exist that are clustered or dispersed in the genome. The two ladybird genes of Drosophila belong to a cluster of homeobox genes involved in mesoderm development and are located on the same chromosome, but some distance away, from the classical homeobox gene clusters (Jagla et al., 1994). They function in cell fate decisions of somatic muscle and cardiac cells, and participate in development of epidermal cells (Jagla et al., 1997a, b, 1998). Ladybird-like genes were recently also identified in mammals. The first member characterised, Lbx1, is expressed in developing skeletal muscle and the nervous system (Jagla et al., 1995). During muscle development, Lbx1 expression is restricted to migrating hypaxial muscle precursor cells and provides thus an excellent marker for the identification of this lineage (Jagla et al., 1995; Dietrich et al., 1998, 1999). The restricted expression of Lbx1 indicates that it regulates properties that are specific for the migratory lineage of muscle precursors.

Ordered and directed cell migration is an essential developmental mechanism, by which precursor cells reach targets distal from their site of origin. Muscle precursors, neural crest cells, primordial germ cells and other cell types move over large distances, using characteristic paths. Attractive and repulsive cues are thought to direct the migrating cells. The elucidation of the molecular nature of such cues has been an area of active research. Cell-cell and cell-matrix adhesion molecules play important roles in migration in vitro and in vivo, and the corresponding ligands are thought to provide the appropriate substratum for migration (Montell, 1999). Ligands recognised by tyrosine kinase receptors can provide positive or negative cues for cell migration in mice and C. elegans; mutations that affect signalling components of these receptors can result in mislocation and misrouting of cells (Donovan, 1994; Robinson et al., 1997; Wehrle-Haller and Weston, 1997; Britsch et al., 1998; Chen and Stern, 1998; Montell, 1999). Netrins that function in the guidance of developing axons can also provide cues that direct the migration of muscle precursor cells in C. elegans (Culotti and Merz, 1998).

In vertebrates, migrating myogenic precursor cells are generated from the lateral dermomyotome, a derivative of the somite (Chevallier et al., 1977; Christ et al., 1977). The lateral dermomyotome in the trunk gives rise to hypaxial muscles (superficially, laterally and ventrally located muscles), which are formed by a migratory and a non-migratory cell population (reviewed by Christ and Ordahl, 1995). The migratory
precursors are generated from distinct somites only. These somites are located occipitally, cervically and on the levels of the limbs, where cells delaminate from the epithelial dermomyotome to take up long-range migration (Nishi, 1967; Grim, 1970; Chevallier et al., 1977; Christ et al., 1977; Jacob et al., 1979; Schemainda, 1979; Noden, 1983; Bladt et al., 1995; Dietrich et al., 1999). In mice, the migration targets are the limbs, septum transversum and floor of the first branchial arch, where these cells form skeletal muscle of the extremities (muscles of the limbs and shoulder/girdle), diaphragm and hypoglossal cord, respectively.

Mice deficient for the c-Met tyrosine kinase receptor or its ligand, SF/HGF, lack all muscle groups that derive from migratory precursor cells of the dermomyotome (Bladt et al., 1995; Maina et al., 1996). In these mutants, myogenic precursors fail to delaminate from the lateral dermomyotome but retain their epithelial organisation. Earlier development and specification of migratory precursors, like Lbx1 induction, occur appropriately (Dietrich et al., 1999). SF/HGF is expressed close to all somites that form migratory cells, suggesting that SF/HGF-driven activation of c-Met disperses and releases the cells from the dermomyotome (Bladt et al., 1995; Dietrich et al., 1999). Indeed, ectopic application of SF/HGF to the flank of the chick induces delamination and emigration of cells from the lateral dermomyotome, which otherwise never releases migratory precursors (Brand-Saberi et al., 1996b; Heymann et al., 1996).

Migratory muscle precursors are also absent in the limbs of Splotch mice that carry mutations in Pax3. In Splotch mutants, c-Met expression is barely detectable in the lateral dermomyotome and Lbx1 expression is lacking in the trunk. In addition, upregulated expression from the mutant Pax3 gene in the lateral dermomyotome is not observed. Genetically, Pax3 can therefore be placed upstream of c-Met and Lbx1, and it is also required for regulation of its own transcription (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; Mennerich et al., 1998; Tremblay et al., 1998; Dietrich et al., 1999; Li et al., 1999). The gene encoding the homeobox-containing factor Mox2 is expressed in migratory myogenic precursor cells after they exit the dermomyotome, and in limb mesenchyme. Specific muscle groups are absent, small or aberrantly shaped in the limbs of Mox2 mutant mice. Mox2 appears to participate in the regulation of Pax3 and c-Met, since the corresponding transcripts are reduced in the limbs of Mox2 mutant mice (Mankoo et al., 1999).

We introduced a targeted mutation into the mouse Lbx1 gene, using embryonal stem (ES) cell technology. Lbx1 mutant mice lack extensor muscle groups in the limbs, whereas flexor muscles are present but reduced in size. Hypaxial muscle precursor cells delaminate but do not migrate appropriately. Thus, muscle precursors do not reach the dorsal field of the forelimb bud, and are distributed ventrally in an abnormal manner. Precursors that migrate from occipital somites to the floor of the branchial arch find their route, but fewer cells reach the distal target; as a consequence, the lingual muscle is hypoplastic at birth. We conclude that Lbx1 determines lineage-specific properties of the migrating myogenic precursors and might be essential for the recognition of cues that guide the cells and that maintain their migratory potential.

### MATERIALS AND METHODS

#### Generation of Lbx1 mutant mice

The Lbx1 targeting vector was constructed from genomic DNA isolated from a mouse 129Sv library. To follow the expression of Lbx1, lacZ was fused to a NorI site located 180 bp downstream of the ATG in exon 1 (for details of the genomic structure of Lbx1 see Jagla et al., 1995). A neor(¢) cassette was inserted 3’ of the lacZ, and all Lbx1 coding sequences downstream of the NorI site were removed. In addition, the thymidine kinase gene was inserted into the targeting vector to allow for negative selection in ES cells. E14.1 cells, a subclone of the E14 cell line, were used to introduce the targeting vector by electroporation. ES cell colonies that had inserted the targeting vector into their genome were selected with G418 and gancyclovir, and analysed for homologous recombination events by Southern hybridisation. We injected blastocysts and identified chimera that transmitted the mutant Lbx1 gene as reported (Bladt et al., 1995). Three independently generated ES cell colonies gave rise to germline chimeras. The majority of the phenotype analysis was performed with mutant mice generated from one ES subclone, and verified in mice generated from the two independent ES cell subclones. Routine genotyping was performed by PCR; occasionally, genotypes were verified by Southern hybridisation.

#### In situ hybridisation analysis

To visualise expression from the mutant Lbx1-lacZ fusion gene with high sensitivity, in situ hybridisation with a lacZ probe was used; staining for the enzymatic β-galactosidase activity demonstrated similar patterns, but relatively lower signal intensity, possibly due to reduced stability of the fusion protein. RNA probes for in situ hybridisation were prepared as previously described: 1.1 kb lacZ fragment; mouse c-Met 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., 1994); a mouse Lbx1 fragment (520 bp) that corresponds to the 3’ coding region including the homeobox (Jagla et al., 1995); myogenin (Sassoon et al., 1989) and myf5 cDNA (Ott et al., 1991). Labelled RNA transcripts were synthesised either with T3 or T7 RNA polymerase using digoxigenin (DIG)-labelling (DIG-RNA labelling kit, Boehringer Mannheim). Whole-mount in situ hybridisation was performed as described (Wilkinson, 1992). All probes reproducible hybridisation patterns when used in antisense orientation, whereas transcripts in sense orientation revealed no specific hybridisation patterns.

#### Histological analysis

Embryos were fixed overnight in 4% PFA in PBS, dehydrated and embedded in hydroxyethylmethacrylate (Technovit 7100 resin, Kulzer GmbH, Wehrheim, Germany). Serial sections (5-6 μm) were cut and counterstained with Delafield’s Hematoxylin and Eosin. For vibratome sectioning, stained embryos were embedded in 20% gelatine in PBS and refixed in 4% PFA in PBS overnight. Sections were cut to 35 μm. For immunohistochemical analysis, tissues from E18 embryos were embedded in OCT compound (Sakura). Cryosections (12 μm) were fixed in 4% PFA, blocked with 10% goat serum (GS) in PBS containing 0.1% Triton X-100 and incubated with monoclonal anti-myosin antibody (clone MY-32, Sigma); as secondary antibody, a Cy2-conjugated anti-mouse IgG (Dianova) was used.

### RESULTS

#### Generation of Lbx1 mutant mice

In order to mutate the Lbx1 gene, a targeting vector was constructed in which lacZ was fused in frame to coding
sequences in exon 1. The mutant allele encodes a Lbx1-β-galactosidase fusion protein that contains the first 60 amino acids of Lbx1 but lacks all C-terminal sequences, including the homeodomain (Fig. 1A). The targeting vector was introduced by homologous recombination into ES cells, and the cells were used to generate a mouse strain that carries the mutant Lbx1 gene (Fig. 1B). Lbx1<sup>+/−</sup> animals were viable and fertile, but Lbx1<sup>−/−</sup> animals died at birth and did not expand their lungs. Although the overall appearance of mutants was normal, limbs were notably thin, the forelimbs were bent in an abnormal position and the animals did not move their limbs. Lbx1 expression provides an excellent tool for identification of migrating muscle precursor cells. Expression from the wild-type and mutant Lbx1 genes were compared, to assess whether lacZ transcripts generated from the mutant Lbx1 gene can be used for the identification of migrating precursors. Indeed, lacZ expression from the mutant allele in Lbx1<sup>+/−</sup> embryos closely reproduced the expression pattern of the wild-type gene (Fig. 1C, and data not shown).

**Hypaxial muscle precursor cells delaminate but migrate abnormally in Lbx1 mutant embryos**

We examined the formation of migrating hypaxial muscle precursor cells in heterozygous and homozygous Lbx1 mutant embryos. At E9.75, transcription from the mutant Lbx1 gene was induced on the correct axial levels, i.e. lacZ expression was detected in the lateral dermomyotome of occipital and cervical somites, as well as in somites located on the limb levels (Fig. 2A,B). Delaminated cells moved from occipital somites towards the floor of the first branchial arch, forming the hypoglossal stream. However, the stream in control embryos extended further towards the floor of the first branchial arch than in Lbx1<sup>+/−</sup> embryos (arrows in Fig. 2C,D). A similar distribution of cells in this stream was observed when Pax3 was used as probe (not shown).

On the forelimb level, cells that express lacZ or Pax3 had moved away from the lateral lip of the dermomyotome, but their distribution was abnormal in the Lbx1<sup>+/−</sup> embryos (Fig. 2A-F). This was particularly notable when the embryos were inspected from their dorsal end. Whereas precursor cells that express lacZ or Pax3 had entered the forelimb buds of control embryos, precursor cells in the limbs were absent in Lbx1<sup>+/−</sup> embryos (Fig. 2E,F). This was further analysed on transverse sections: in control embryos at E9.75, Pax3-, c-Met- or β-gal-positive migrating precursor cells had delaminated from the lateral dermomyotomal lip and moved into the forelimb (Fig. 3A,C and data not shown). In Lbx1 mutants, Pax3-, c-Met- or β-gal-positive cells moved from the lip of the dermomyotome in a ventral direction but did not enter the limb bud at this stage (Fig. 3B,D and data not shown). At E9.75, the delaminated cells do not express myf5 (not shown).

**Differentiating muscle cells are found at ectopic sites in Lbx1 mutant embryos at E10.75**

Myogenic determination factors are expressed at the onset of muscle differentiation. Myf5 is the first of these factors expressed in this migratory lineage of the mouse, whereas others appear later (Sassoon et al., 1989; Bober et al., 1991; Fig. 1A). The structure of the targeting vector (top), the genomic structure of the wild-type Lbx1 locus (middle) and of the targeted Lbx1 allele (bottom) are depicted schematically. Exons 1 and 2 of Lbx1 are shown by black boxes. In the targeting vector, lacZ sequences (shown as blue box) were fused to the ATG in exon 1 of Lbx1; the majority of the coding sequences, including those encoding the homeobox, were deleted. The neomycin (neo) and thymidine kinase (tk) cassettes are present in the targeting vector and were used for positive and negative selection in embryonic stem cells. The probe used for Southern hybridisation is shown, as are the sizes of predicted DNA fragments obtained after EcoI digestion of the wild-type and mutant alleles. NotI (N), NcoI (Nc), EcoRI (E) restriction sites and the initiation codon of the Lbx1 gene (ATG) are indicated. (B) Southern blot analysis of NotI-digested genomic DNA from wild-type (lane 1) and Lbx1<sup>+/−</sup> (lane 2) ES cells, and from wild-type (lane 3), Lbx1<sup>+/−</sup> (lane 4) and Lbx1<sup>−/−</sup> (lane 5) animals. (C) Wild-type (left) and Lbx1<sup>+/−</sup> (right) embryos at E9.75 were hybridised with a Lbx1 and a lacZ-specific probe, respectively. Bar 500 μm.
Hinterberger et al., 1991; Ott et al., 1991; Buckingham et al., 1992; Smith et al., 1994; Tajbakhsh and Buckingham, 1994).

In control embryos at E10.75, myf5 transcripts were observed in cells of the hypoglossal stream, the limb buds and in the myotomes of all somites (Fig. 4A). Myotomal expression of myf5 in \( \text{Lbx1}^-/- \) embryos was observed as it is in control embryos, and cells in the hypoglossal stream expressed the factor at this stage (Fig. 4B).

Myf5 expression in the limbs was however severely reduced in \( \text{Lbx1}^-/- \) mutants compared to control embryos (arrowheads in Fig. 4A,B).

The distribution of myogenic precursor cells was also analysed on sections. In control embryos, dispersed lacZ-positive precursors are present in the ventral and dorsal forelimb at E10.75 (Fig. 4E). In \( \text{Lbx1}^-/- \) mutants, migrating hypaxial muscle precursor cells are lacking in the dorsal forelimb (Fig. 4F, arrow). Dense groups of lacZ-positive precursors were observed in the proximal portion of the ventral limb and in the lateral plate mesoderm of \( \text{Lbx1}^-/- \) mutants (arrowheads in Fig. 4F and data not shown). Serial sections demonstrated that these dense groups were present exclusively ventroproximally in the anterior half of the forelimb and are thus distributed abnormally (Fig. 4F).

Differentiating cells that express myf5 are present in control embryos in the dorsal and ventral forelimb at E10.75. They are observable only ventroproximally in the anterior half of the forelimb in \( \text{Lbx1}^-/- \) mutant embryos, and are located thus at the same positions as lacZ-positive cells (Fig. 4C,D). myf5-positive cells are also present in the lateral plate mesoderm.

The density of myf5-positive cells in the lateral plate mesoderm is higher in the \( \text{Lbx1}^-/- \) mutants than in the controls (Fig. 4C,D). The positions at which these cells are observed in \( \text{Lbx1}^-/- \) embryos overlap with, but are not restricted to, the route employed by the stream of migrating cells that form the diaphragm muscle in control embryos. The increase in numbers of migrating or differentiating myogenic cells in the lateral plate mesoderm of \( \text{Lbx1}^-/- \) mutants interferes with a detailed analysis of this stream.

Sections of control embryos at E10.75 show that lacZ- or lacZ-positive cells are present in somites located occipitally, cervically, and on the levels of the forelimb and hindlimb (A,B). Delaminated cells that form the hypoglossal stream and migrate towards the floor of the first branchial arch are indicated by arrows (C,D). Note that at the forelimb level, precursor cells are distributed differently in control and \( \text{Lbx1}^-/+ \) embryos (arrowheads in C, D). Dorsal view of \( \text{Lbx1}^-/+ \) (E) and \( \text{Lbx1}^-/- \) (F) embryos; hypaxial muscle precursor cells are lacking in the forelimb buds of \( \text{Lbx1}^-/- \) embryos. Bars (A) 500 \( \mu \text{m} \), (C) 300 \( \mu \text{m} \), (E) 400 \( \mu \text{m} \).

Fig. 2. Formation and distribution of migrating hypaxial muscle precursor cells in \( \text{Lbx1}^-/- \) mutant embryos at E9.75. Hypaxial muscle precursor cells in \( \text{Lbx1}^-/+ \) (A,C,E) and \( \text{Lbx1}^-/- \) (B,D,F) embryos at E9.75 are visualised by in situ hybridisation using lacZ as probe. In \( \text{Lbx1}^-/+ \) and \( \text{Lbx1}^-/- \) embryos, the lacZ transcripts that derive from the mutant \( \text{Lbx1} \) gene are observed in somites located occipitally, cervically, and on the levels of the forelimb and hindlimb (A,B). Delaminated cells that form the hypoglossal stream and migrate towards the floor of the first branchial arch are indicated by arrows (C,D). Note that at the forelimb level, precursor cells are distributed differently in control and \( \text{Lbx1}^-/+ \) embryos (arrowheads in C, D). Dorsal view of \( \text{Lbx1}^-/+ \) (E) and \( \text{Lbx1}^-/- \) (F) embryos; hypaxial muscle precursor cells are lacking in the forelimb buds of \( \text{Lbx1}^-/- \) embryos. Bars (A) 500 \( \mu \text{m} \), (C) 300 \( \mu \text{m} \), (E) 400 \( \mu \text{m} \).

Fig. 3. Aberrant position of migrating muscle precursor cells in \( \text{Lbx1}^-/- \) mutants at E9.75. Sections through the forelimbs of \( \text{Lbx1}^-/+ \) and \( \text{Lbx1}^-/- \) embryos at E9.75 after in situ hybridisation with probes specific for Pax3 (A,B), c-Met (C,D). Note that in control but not in \( \text{Lbx1}^-/- \) embryos, Pax3- and c-Met-positive cells are distributed in the entire proximal forelimb. Dermomyotome (D). Bar 150 \( \mu \text{m} \).
myf5-positive cells of the hypoglossal stream have reached the anlage of the tongue in the floor of the first branchial arch (arrows in Fig. 5A and data not shown); both markers reveal a similar distribution of migratory cells. myogenin is also expressed by the migrating cells located along the route, but not all migratory precursors in the floor of the first branchial arch express myogenin in control embryos at this stage (arrows in Fig. 5D).

In Fig. 5A,C), in Lbx1−/− embryos, the density of precursors and differentiating cells detected with lacZ or myf5 as probes is reduced in the floor of the first branchial arch (arrow in Fig. 5B and data not shown). Myogenin-positive cells are detected along the route of the hypoglossal stream, but not in the floor of the first branchial arch of Lbx1−/− embryos (arrow in Fig. 5D).

We also examined the dermomyotome of control and Lbx1 mutants histologically. The dermomyotomes on forelimb and hindlimb levels appeared similar in length and overall morphology in control and Lbx1−/− embryos. As in control embryos, cells that delaminate from the lateral lip were observed in Lbx1−/− embryos (data not shown).

Distinct muscle groups in the limbs are lacking in Lbx1 mutant mice at E18.5

Lbx1−/− mice at E18.5 were analysed by dissection, histologically and by immunohistochemistry using an anti-myosin antibody to assess the presence and size of skeletal muscle groups that derive from migratory precursors (Fig. 6).
In \( Lbx1^{-/-} \) embryos, extensor muscles of the forelimb are lacking and flexor muscles are reduced in size (Fig. 6A-D). Remaining muscles of the upper hindlimb (\( M. \) vastus lateralis, \( M. \) caudofemoralis, \( M. \) semimembranosus, \( M. \) biceps femoris) are very small and the phenotype is thus more severe than in the forelimbs. The \( M. \) biceps femoris extends into the lower hindlimbs and is the only muscle observable on sections of the proximal upper (A,B) and lower forelimb (C,D), and of the proximal upper (E,F) and lower hindlimb (G,H). The arrowhead in F points towards body wall muscles. Histology of muscle fibers in the forelimb of control (I) and \( Lbx1^{-/-} \) embryos (J). Arrows in J point towards centrally located nuclei in myofibers of \( Lbx1 \) mutants. Bars (A) 500 \( \mu \)m, (I) 80 \( \mu \)m.

**Fig. 6.** Lack of distinct muscle groups in limbs of \( Lbx1^{-/-} \) embryos at E18.5. Immunohistochemical analysis of transverse sections of the forelimbs and hindlimbs of control (A,C,E,G) and \( Lbx1^{-/-} \) (B,D,F,H) embryos at E18.5, using a anti-myosin antibody. Shown are sections of the proximal upper (A,B) and lower forelimb (C,D), and of the proximal upper (E,F) and lower hindlimb (G,H). The arrowhead in F points towards body wall muscles. Histology of muscle fibers in the forelimb of control (I) and \( Lbx1^{-/-} \) embryos (J). Arrows in J point towards centrally located nuclei in myofibers of \( Lbx1 \) mutants. Bars (A) 500 \( \mu \)m, (I) 80 \( \mu \)m.

In \( Lbx1^{-/-} \) embryos, extensor muscles of the forelimb are lacking and flexor muscles are reduced in size (Fig. 6A-D). Remaining muscles of the upper hindlimb (\( M. \) vastus lateralis, \( M. \) caudofemoralis, \( M. \) semimembranosus, \( M. \) biceps femoris) are very small and the phenotype is thus more severe than in the forelimbs. The \( M. \) biceps femoris extends into the lower hindlimbs and is the only muscle observable on sections of the lower hindlimb (Fig. 6E-H). Muscles in the paws are completely absent. Histologically, the remaining muscle fibres in the limbs of \( Lbx1^{-/-} \) embryos display central nuclei, which are typically observed in myopathies of skeletal muscle (Fig. 6I,J). Internal lingual muscles are present in \( Lbx1 \) mutants, but are reduced in size compared to control embryos (Fig. 7). Shoulder muscles are little affected, and the diaphragm muscle is normal in size (data not shown).

**DISCUSSION**

We show here that particular skeletal muscle groups that derive from migrating precursors are absent or small in \( Lbx1 \) mutants, whereas others are normal or only mildly affected. Migrating precursor cells form and delaminate from the lateral dermomyotome, but these cells migrate in an aberrant manner. Most strongly affected are cells that move to the limbs. Precursor cells do not migrate to the dorsal target field in the forelimb and are abnormally distributed in the ventral limb field. Moreover, an increased density of precursor cells can be observed in the lateral plate mesoderm, indicating that cells are misrouted. Cells that move to the anlage of the tongue in the floor of the first branchial arch use the correct path in \( Lbx1^{-/-} \) embryos, but migrate at reduced speed, are delayed in reaching the target and arrive in reduced numbers. Thus, myogenic precursors require \( Lbx1 \) for appropriate migration.

**\( Lbx1, \) c-Met and migrating hypaxial muscle precursor cells**

A major finding that emerges from our analysis of \( Lbx1 \) and c-Met or SF/HGF mutant mice is that independent cues converge on migrating hypaxial muscle precursors (this study and Dietrich et al., 1999). Both the \( Lbx1 \) and c-Met mutations interfere with the development of migrating hypaxial muscle...
precursors, but affect this lineage at distinct stages. In c-Met or SF/HGF mutants, delamination of precursors does not occur and cells remain in the epithelial dermomyotome. c-Met activation that results in delamination requires SF/HGF, which is provided in a spatially restricted manner by the mesoderm. Although Lbx1 expression and c-Met activity are restricted to similar axial levels, they are controlled by independent mechanisms. Thus, Lbx1 is induced correctly in c-Met mutant embryos. Conversely, c-Met expression in migrating precursors and the c-Met-dependent delamination occur in mice deficient for Lbx1.

Lbx1 and differentiation of hypaxial muscle precursor cells

In development, Lbx1 expression is confined to those migrating myogenic precursor cells that delaminate from the ventrolateral dermomyotome; expression correlates thus with a delayed differentiation of muscle precursors typical for the migratory lineage. This is in contrast to the cells that leave the dermomyotome on other axial levels or at other positions; such cells enter the myotome and immediately express myogenic determination factors (Bober et al., 1991; Pownall and Emerson, 1992; Smith et al., 1994; Williams and Ordahl, 1994). Delaminated cells in Lbx1 mutants do not express myf5 immediately after they leave the lateral dermomyotome (i.e. at E9.75). This indicates that Lbx1 does not suppress differentiation of delaminated cells. However, the migrating cells can differentiate even when located ectopically, since myf5-positive cells can be observed at increased densities in the lateral plate mesoderm on forelimb levels, and proximally in the ventral forelimb at E10.75.

Migratory hypaxial muscle precursor cells, after transplantation from somites or from the limbs to ectopic sites, do generally differentiate. Similarly, manipulations that arrest migration of cells in the limb result in their differentiation at ectopic positions (Brand-Saberi et al., 1993, 1996a; Daston et al., 1996). The myogenic cells that differentiate at ectopic positions in the lateral plate mesoderm and in the proximal limb in Lbx1 mutants might thus arise by the same mechanisms as in such transplantation experiments. When and where hypaxial muscle precursor cells differentiate is finely regulated in the embryo, and is controlled by the presence of growth factors (BMP4, SF/HGF, FGFs) as well as by differentiation signals (Shh). In general, factors that promote growth also inhibit differentiation, whereas factors that induce differentiation cause a cessation of growth (Amthor et al., 1999; Scaal et al., 1999; Hannon et al., 1996; Itoh et al., 1996; Webb et al., 1997). Thus, a limiting amount of growth factors encountered by migratory precursors at the ectopic positions in Lbx1 mutants could cause their differentiation. In the hypoglossal stream, muscle precursor cells are found along the normal migratory route in Lbx1 mutants. However, the differentiation of these migrating cells is delayed.

Extensor muscle groups in the forelimbs of Lbx1 mutant mice are lacking. Flexor muscles form, but are reduced in size, and their size reduction is more pronounced in the distal than the proximal parts of the limbs. This might reflect the reduced numbers of precursor cells in the limbs at early stages, and the abnormal distribution of precursors restricted to the ventroproximal part, indicating that precursors in the ventral field reach distal positions later or even never at all. It is difficult to assess whether Lbx1 affects also muscle patterning, as was previously reported for Mox2. It should be noted that mutation of Mox2 does not notably affect the numbers of precursors in the limbs, but rather the presence or shape of particular muscle groups (Mankoo et al., 1999).

Lbx1 and directed migration of hypaxial muscle precursors

In Lbx1 mutants, muscle precursor cells move away from the dermomyotome from which they delamate but are not found at one of their targets, the dorsal limb field. Although impaired survival at this target could cause such a phenotype, this does not seem the mechanism responsible in Lbx1 mutants. We cannot at any stage in development detect precursor cells in the dorsal target field. Moreover, ectopic Lbx1-positive precursors and myf5-positive myogenic cells are present in the lateral plate mesoderm and in the ventral portion of the proximal forelimb bud of Lbx1 mutants. This indicates that some precursors were misrouted. At birth, ectopic muscle was, however, not observed in the body wall. Therefore, the ectopic muscle cells present at E10.75 are either regrouped or eliminated by programmed cell death at subsequent stages.

Theoretically, genes required for migration can affect four interrelated processes: (i) differentiation of the migrating cells, (ii) differentiation of the tissue through which the cells migrate, (iii) motility itself, i.e. the migratory potential of the cells, and (iv) the direction of migration.

Lbx1 is not expressed in tissues through which cells migrate and appears to function cell autonomously in migrating cells. Premature myogenic differentiation, as observed after application of Shh, interferes with the migratory ability of hypaxial muscle precursor cells (Amthor et al., 1999). This does not seem to be the mechanism responsible in Lbx1 mutants, since cells are first observed at aberrant positions and subsequently differentiate.

The overall distance covered by misrouted cells on the forelimb level in Lbx1 mutants is shorter than the distance covered by cells that correctly move in control embryos. Similarly, the distance covered by cells in the hypoglossal stream is reduced in Lbx1 mutants compared to control embryos. Although the speed of migration of the precursor cells is impaired, a complete loss of the migratory ability is not observed and cannot account for the inability of cells to move into the dorsal target field of the forelimb.

Our analysis of Lbx1 mutants indicates therefore that migrating muscle precursor cells are unable to recognise or to interprete cues that direct them, and implies a defective guidance mechanism. Directed migration requires the ability of cells to respond to cues, which can be either attractive or repulsive. Parallels between cell migration and the directed outgrowth of axons are apparent. During cell migration and axon outgrowth, cellular extensions that point in a favourable direction are selectively stabilised, whereas other extensions are made and then withdrawn. It is interesting to note that identical classes of molecules can provide cues during cell migration and axon guidance. Thus, ligands of the Eph-receptors, the ephrins, can repel migrating neural crest cells as well as axons (Muller et al., 1996; Robinson et al., 1997; Bruckner and Klein, 1998). Similarly, netrins, a class of surface or extracellular molecules recognised by the DCC receptor,
guide migrating myoblasts in *C. elegans* as they guide axons (Chen and Stern, 1998; Culotti and Merz, 1998).

Migration of myogenic precursors in the chick limb is inhibited by antibodies against fibronectin or N-cadherin (Brand-Saberi et al., 1993, 1996a). Like in other cell types, appropriate cell-matrix and cell-cell interactions are therefore important for the migration of hypaxial muscle precursors. The c-Met receptor and its ligand, SF/HGF, are essential for delamination of migrating hypaxial muscle precursors. Additional roles of this signalling system during migration are possible, since SF/HGF is expressed along the migratory routes and at the targets and can increase the motility of migrating cells (Bladt et al., 1995; Dietrich et al., 1999; Scaal et al., 1999). In *Lbx1* mutant mice, c-Met expression in migrating precursors is maintained, indicating that SF/HGF can be recognised during migration, although the expression of downstream components that function in signal processing might be affected. Members of the FGF family of growth factors are expressed in the limb, and their ectopic application increases the motility of precursor cells (Webb et al., 1997). Thus, the *Lbx1* mutation might interfere with the expression of molecules that are required for recognition or processing of guidance cues in hypaxial muscle precursor cells.

Not all streams of migrating myogenic precursors are equally affected in mice that lack *Lbx1*. Cells cannot migrate into the dorsal limb field; cells that move in the ventral limb field or in the hypoglossal stream migrate with reduced efficiency. Thus, changes in directed migration as well as reduced motility are observed. This suggests that not all streams of migrating cells use identical cues during directed migration. Indeed, the complexity of signals employed during cell migration in other systems suggests that a single guidance scheme for migration of all myogenic precursors may not exist.

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