CXCR4 and *Gab1* cooperate to control the development of migrating muscle progenitor cells

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Long-range migrating progenitor cells generate hypaxial muscle, for instance the muscle of the limbs, hypoglossal cord, and diaphragm. We show here that migrating muscle progenitors express the chemokine receptor *CXCR4*. The corresponding ligand, *SDF1*, is expressed in limb and branchial arch mesenchyme; i.e., along the routes and at the targets of the migratory cells. Ectopic application of *SDF1* in the chick limb attracts muscle progenitor cells. In *CXCR4* mutant mice, the number of muscle progenitors that colonize the anlage of the tongue and the dorsal limb was reduced. Changes in the distribution of the muscle progenitor cells were accompanied by increased apoptosis, indicating that *CXCR4* signals provide not only attractive cues but also control survival. *Gab1* encodes an adaptor protein that transduces signals elicited by tyrosine kinase receptors, for instance the c-Met receptor, and plays a role in the migration of muscle progenitor cells. We found that *CXCR4* and *Gab1* interact genetically. For instance, muscle progenitors do not reach the anlage of the tongue in *CXCR4;Gab1* double mutants; this target is colonized in either of the single mutants. Our analysis reveals a role of *SDF1/CXCR4* signaling in the development of migrating muscle progenitors and shows that a threshold number of progenitor cells is required to generate muscle of appropriate size.

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Skeletal muscle of vertebrate embryos originates from the dermomyotome, a derivative of the somite. On particular axial levels, cells of the ventral dermomyotomal lip lose their epithelial morphology, delaminate, and migrate as single cells over long distances using stereotypic routes. These migrating progenitor cells generate hypaxial muscles of the extremities, tongue, and diaphragm (Chevallier et al. 1977; Christ et al. 1977; Christ and Ordahl 1995).

Various genes are essential for the development of migrating hypaxial progenitors; these function in the specification of muscle progenitor cells, their delamination from the dermomyotome, and their subsequent migration, survival, proliferation, and differentiation. *Pax3* is required for the correct establishment of the progenitor pool in the ventral dermomyotome. Consequently, the development of all hypaxial muscle is impaired in *Pax3* mutant mice (Franz et al. 1993; Bober et al. 1994; Tajbakhsh

et al. 1997). The tyrosine kinase receptor c-Met and its ligand, scatter factor/hepatocyte growth factor (SF/HGF), are essential for the delamination of the progenitors that are destined to migrate, and all muscle groups that derive from migrating progenitors are absent in *c-Met* or *SF/HGF* mutant mice (Bladt et al. 1995; Dietrich et al. 1999). *Six1* controls the proliferation of muscle progenitor cells and forms a complex with *Eya1* and *Dach*. This complex permits the expression of *c-myc*, a key molecule in the control of proliferation. Specific limb muscle groups are reduced in size in *Six1* mutant mice, whereas *Six1;Eya1* double mutants show a complete absence of limb musculature (Li et al. 2003). Migration of muscle progenitor cells is a complex process and requires signals that allow the cells to remain motile and find their targets. *Gab1* encodes an adaptor molecule that transmits c-Met signals and its mutation impairs but does not completely abolish delamination of muscle progenitors (Sachs et al. 2000). A detailed analysis of *Gab1* mutant mice indicates that c-Met signals mediated by *Gab1* are essential not only for delamination, but also for migration and survival of muscle progenitor cells (Sachs et al. 2000; M. Strehle and C. Birchmeier, unpubl.). *Lbx1* encodes a

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homeodomain transcription factor that is expressed exclusively in migrating progenitor cells that will form hypaxial muscle. Inactivation of *Lbx1* severely impairs migration of those progenitors that move to the limbs while other populations of migrating muscle progenitors find their targets (Schafer and Braun 1999; Brohmann et al. 2000; Gross et al. 2000). This indicates that different subpopulations of migrating muscle progenitors encounter and respond to distinct guidance cues during migration. In the limbs of chick embryos, muscle progenitors that express the EphA4 receptor are repelled from ectopically applied ephrinA5, suggesting that EphA4/ephrin-A5 signals prevent their entry into inappropriate domains (Swartz et al. 2001). Progenitor cells that reach their targets continue to proliferate and start to express myogenic regulatory factors like *MyoD*, *Myf5*, *myogenin*, and *MRF4* that determine their terminal differentiation program (Arnold and Braun 2000; Perry and Rudnick 2000; Buckingham 2001).

Chemokine receptors and their ligands regulate migration of cells in the developing and adult organism. This role of chemokine receptors first became apparent in the hematopoietic system, and the analysis of mutant mice demonstrated that various migration events of lymphoid cells are controlled by chemokine receptors (for review, see Müller et al. 2003). SDF1, the ligand of the CXCR4 receptor, acts as a potent chemoattractant for cultured B lymphocytes, monocytes, and CD34-positive hematopoietic progenitor cells (Nagasawa et al. 1994; Bleul et al. 1996). The analysis of *CXCR4* or *SDF1* mutant mice has, however, revealed numerous developmental functions in cell lineages other than hematopoietic cells (Nagasawa et al. 1996; Ma et al. 1998; Zou et al. 1998). Mutations of *CXCR4* or *SDF1* affect the migration of cerebellar granule cells and of hippocampal and cortical neuronal progenitors (for review, see Lazarini et al. 2003). Furthermore, *CXCR4*/SDF1 signaling is important for the migration of primordial germ cells, a function that is conserved in fish, birds, and mammals (Doitsidou et al. 2002; Knaut et al. 2003; Molyneaux et al. 2003; Stebler et al. 2004). Finally, chemokine signals also control the migration of malignant cancer cells. Metastasis of human breast cancer cells to particular preferred sites correlates with the expression of *CXCR4* in tumor cells, and with the expression of *SDF1* in the organ invaded by the metastatic cells (Müller et al. 2001). In addition to the regulation of various migration processes, *CXCR4*/SDF1 also controls growth and survival of different cell types (Zou et al. 1998; Bagri et al. 2002; Molyneaux et al. 2003). *CXCR4* is expressed in cell lines derived from muscle satellite cells, the stem cells of the adult skeletal muscle. The ability of SDF1 to attract and stimulate the activation of cultured satellite cells suggested possible functions of *CXCR4*/SDF1 in muscle cells (Ratajczak et al. 2003).

We used microarray technology to determine the expression profile of genes in migrating muscle progenitors. Among the expressed genes, we identified the chemokine receptor *CXCR4*. *SDF1*, which encodes the corresponding ligand, is expressed in the mesenchyme of

the limb and the first branchial arch, which represent targets of the migrating cells. Application of SDF1 into the limb of chick embryos directs the muscle progenitor cells toward the ectopic source of the factor and inhibits their differentiation. Analysis of *CXCR4* mutant mice demonstrates changes in the distribution of migrating muscle progenitors. Furthermore, we observe a genetic interaction between *CXCR4* and *Gab1*. For example, the anlage of the tongue is reached in *CXCR4* and *Gab1* mutant mice, but migrating muscle progenitor cells do not arrive at this site in the double-mutant mice. This genetic interaction might reflect a cross-talk between signaling cascades employed by G-protein-coupled receptors (GPCRs) and tyrosine kinases.

Results

The Lbx1^{GFP} allele allows the sorting of muscle progenitor cells for gene expression profiling

Previous studies showed that *Lbx1* is expressed during development of long-range migrating hypaxial cells, but not in other types of muscle progenitor cells (Jagla et al. 1995). To allow the isolation of muscle progenitor cells by cell sorting, we generated a mutant *Lbx1* allele, in which *GFP* was fused to the initiation codon by homologous recombination in ES cells (Fig. 1A). The mutant ES cells were then used to generate an *Lbx1^{GFP}* mouse strain (Fig. 1B; for additional information, see Materials and Methods). GFP protein was produced from the *Lbx1^{GFP}* allele and was located in migrating muscle progenitors of the dorsal and ventral limb in a pattern similar to the endogenous *Lbx1* protein (Fig. 1C).

GFP⁺ muscle progenitors were isolated from the embryonic limb bud. For this, the forelimbs of embryonic day 10.5 (E10.5) embryos were dissected, cells were dissociated, and GFP-positive cells were isolated by flow cytometry. Probes were generated from RNA obtained from these cells and used for hybridization on MG U74A/B/Cv2 Affymetrix GeneChips. Genes encoding cell surface molecules that might control cell migration were identified, and in situ hybridization was used to analyze their expression pattern in the embryo. Among the genes identified (Table 1) was the chemokine receptor *CXCR4*. *CXCR4* and its ligand SDF1 are critical for migration, proliferation, and survival of various cell types (Cyster 2003; Lazarini et al. 2003; Raz 2003).

Expression of CXCR4 and SDF1 in mouse and chick embryos

We examined expression of *CXCR4* and *SDF1* in the limbs of mouse and chick embryos. Indeed, *CXCR4* is expressed in the limb buds. The distribution of *CXCR4*-expressing cells resembles, but is not entirely identical to, that of *Pax3*- or *Lbx1*-expressing muscle progenitors (Figs. 2A,C,E, 3; Supplementary Fig. 1). A stream of *CXCR4*⁺ cells is also observed along the hypoglossal chord and in the first branchial arch; i.e., along the route

Figure 1. Generation of the *Lbx1*^{GFP} mutant allele. (A) Schematic representation of the targeting vector, the wild-type *Lbx1* locus, and the mutated *Lbx1* allele before and after removal of the neomycin (neo) cassette. The *Lbx1* gene has two exons (yellow boxes); the first was interrupted by the insertion of a *Gap43-GFP* cassette (green box). In addition, a frame-shift mutation (indicated by a black line) was introduced into the BglIII site of exon 2 that encodes the *Lbx1* homeodomain. Neomycin (neo) and thymidine kinase (tk) cassettes present in the targeting vector were used for positive and negative selection. The positions of the probes, A and B, used for Southern analysis are shown by black and red bars, respectively. The predicted fragment sizes obtained after HindIII (H) and BamHI (B) digestion of genomic DNA are indicated. In addition, the following restriction enzyme sites are indicated: ClaI (C), Sse8387I (S), and NheI (Nh). (B) Southern blot analysis of HindIII-digested genomic DNA from wild-type and *Lbx1*^{GFP/+} ES cells using probe A for hybridization (left) and BamHI-digested genomic DNA from wild-type, *Lbx1*^{GFP/+}, and *Lbx1*^{GFP/GFP} F1 animals using probe B for hybridization (right). (C) Immunohistological analysis of a forelimb section of an *Lbx1*^{GFP/+} embryo at E10 stained with anti-Lbx1 (red) and anti-GFP (green) antibodies. Bar, 125 μ m.

and at the target of those migrating muscle progenitor cells that will generate the intrinsic tongue muscle (Fig. 2G). However, *CXCR4* expression in muscle progenitors that migrate to the anlage of the diaphragm is very low (data not shown). *SDF1* expression can be detected in the limb bud mesenchyme, and the pattern of expression is dynamic during development (Fig. 2B,D,F; Supplementary Fig. 1). At the time muscle progenitor cells delaminate, *SDF1* is expressed in the proximal limb bud (Supplementary Fig. 1A). After the cells enter the limb, *SDF1* expression is also observed further distally in the mesenchyme (Fig. 2B,D,F; Supplementary Fig. 1B,C). Hence, migrating muscle progenitor cells in the limb are found in the vicinity of *SDF1*-expressing cells. Few muscle progenitors reach the limb of *Lbx1*^{-/-} embryos (Schafer and Braun 1999; Brohmann et al. 2000; Gross et

al. 2000). A comparison of the control and *Lbx1*^{-/-} embryos demonstrates very similar expression patterns of *SDF1*, indicating that muscle progenitors do not express *SDF1* (Supplementary Fig. 1C,D). *SDF1* transcripts are also present in the floor of the first branchial arch (Fig. 2H). High expression of *SDF1* is thus observed at the target of the migrating cells that move to the anlage of the tongue.

We analyzed the distribution of *Lbx1* and *CXCR4* by immunohistochemistry to identify if *CXCR4* and *Lbx1* are expressed in identical cell populations. We observed that all *CXCR4*⁺ cells in the premuscle masses of the limbs are also *Lbx1*⁺, demonstrating that *CXCR4* is present in migrating muscle progenitors (Fig. 3A–D).

Table 1. Selected genes encoding integral membrane proteins that are expressed in *Lbx1*⁺ muscle progenitor cells, as determined by microarray analysis

Affy ID	GenBank ID	Gene	Detection p-value
98169_s_at	AU020229	Fzd3	0.0005
139426_r_at	AW228933	Fgfr1	0.003
93090_at	M23362	Fgfr2	0.006
108468_at	AW121015	Bmpr1a	0.002
95117_at	U04710	Igf2r	0.004
106644_at	AW047110	Tgfb1	0.0004
104188_at	AI853703	Notch2	0.0004
102250_at	AF053005	Il27ra	0.002
102794_at	Z80112	CXCR4	0.002
93430_at	AF000236	Cmkor1	0.006
114749_at	AW107659	Gpr23	0.0003
104673_at	X65138	Epha4	0.02
98446_s_at	U06834	Ephb4	0.0002
160857_at	U30244	Efnb2	0.0004
95387_f_at	AA266467	Sema4b	0.0004
117151_at	AI838057	c-Met	0.0007
160480_at	X82288	Ptprs	0.0002
160760_at	L10106	Ptprk	0.006
97750_at	X06406	Lamr1	0.0002
101585_at	AF042491	Pgrmc1	0.0002
98094_f_at	AI843627	Amfr	0.002
103958_g_at	X57349	Trfr	0.002
103783_at	AI648965	Xpr1	0.004
102852_at	M31131	Cdh2	0.0002
100006_at	D21253	Cdh11	0.0008
129896_at	AW125163	Pcdh17	0.014
166351_f_at	AV245394	Pcdh18	0.001
100124_r_at	X15202	Itgb1	0.0002
95292_at	AA189389	Itga4	0.010
94117_f_at	AF026465	Punc	0.0002
100153_at	X15052	Ncam	0.0002
100977_at	AA691492	Glycam1	0.001
92558_at	M84487	Vcam1	0.002
93604_f_at	AF061260	Igsf4	0.0002
92270_at	AI847616	Maged3	0.002
93389_at	AF039663	Prom	0.0006

Genes were annotated using the Affymetric Netaffy, Ensemble, and NCBI databases. The average detection p-value as calculated by Affymetrix MAS 5.0 software is given. Displayed are genes with a p-value for expression ≤ 0.02 . Genes whose expression in migrating muscle progenitor cells was previously reported or verified by in situ hybridization are indicated in bold.

Figure 2. Expression of *CXCR4* and *SDF1* in mouse and chick embryos. (A–F,H) In situ hybridization of chick embryos at HH25 (A,B) and mouse embryos at E10.25 (C–F) using probes specific for *CXCR4* (A,C,E) and *SDF1* (B,D,F). (G,H) Consecutive sections, displayed as mirror images, through the first branchial arch of wild-type mouse embryos at E10.25 were stained with antibodies against CXCR4 (red) and Lbx1 (green) (G), or hybridized with an *SDF1*-specific probe (H). CXCR4 and Lbx1 were coexpressed in muscle progenitors migrating toward the tongue anlage (arrowhead in G), whereas *SDF1* transcripts were detected in the mesenchyme of the first branchial arch (arrows in H). Bars: A,B, 500 μ m; C–H, 250 μ m.

However, not every Lbx1⁺ cell expresses CXCR4. Lbx1 appears in progenitors prior to their delamination from the ventrolateral dermomyotome, but CXCR4 protein is not detectable in the ventral dermomyotomal lip, in delaminating Lbx1⁺ cells, or in the myotome (Fig. 3A; data not shown). CXCR4 and Lbx1 are coexpressed only after the migrating progenitor cells have moved away from the dermomyotome. CXCR4 protein is present in cells that have already entered the forelimb at E10.0–E10.25, but present only in a subpopulation of the Lbx1⁺ cells that is located close to the ectoderm (Fig. 3A–C). Between E10.25 and E11.0, the number of CXCR4⁺ cells increases, but a significant number of Lbx1⁺ cells remain negative for CXCR4 (Fig. 3B,C). All Pax3⁺ cells in the limb coexpress Lbx1 (see also Gross et al. 2000; data not shown). Accordingly, Pax3⁺/CXCR4⁺ and Pax3⁺/CXCR4[−] cells exist in the limb. Similarly, the in situ hybridiza-

tion patterns of CXCR4 and Pax3 are similar, but not identical, indicating that not all Pax3⁺ cells coexpress CXCR4 (Supplementary Fig. 1F,I,G,J). We also examined CXCR4 and Lbx1 expression in those muscle progenitors that migrate to the first branchial arch. Again, we observed that most, but not all, Lbx1⁺ cells coexpress CXCR4 (Fig. 2G).

Examination of CXCR4 and MyoD by immunohistology reveals that the CXCR4⁺ and the MyoD⁺ cell populations are distinct at E10.25, E10.5, and E11.25 (Fig. 3E,F; data not shown). In contrast, MyoD⁺/Lbx1⁺ or MyoD⁺/Pax3⁺ cells are frequently detected. In the developing limb, CXCR4⁺ cells locate closer to the ectoderm than MyoD⁺ cells. These data indicate that migrating muscle progenitors do not express CXCR4

Figure 3. Expression of CXCR4, Lbx1, and MyoD in limb muscle progenitors. (A–D) Sections of the forelimb of E10.25 (A,D), E10.75 (B), and E11.25 (C) mouse embryos stained with anti-CXCR4 (red) and anti-Lbx1 (green) antibodies. CXCR4⁺ and Lbx1⁺ cells are present in the muscle masses, and analysis at high magnification demonstrates that they are present in the same cells. (D) Note that not every Lbx1⁺ cell expressed CXCR4, but all CXCR4⁺ muscle progenitor cells were also positive for Lbx1. (A) In addition, CXCR4 was also present in limb endothelial cells (arrowhead). (E,F) Section of the forelimb of mouse embryo at E10.25 (E) and E11.25 (F) stained with anti-CXCR4 (red) and anti-MyoD (green) antibodies. CXCR4⁺/MyoD⁺ double-positive cells were very rare. (G) Schematic representation of gene expression in developing muscle progenitors. CXCR4 expression is induced after muscle progenitors have delaminated and have reached the limb, and is extinguished prior to their differentiation. Bars: A–C,E–F, 250 μ m; D, 30 μ m.

at the time they are specified in dermomyotome. CXCR4⁺ cells coexpress Lbx1 and Pax3, i.e., proteins known to be expressed in migrating progenitors, but not the muscle differentiation factor MyoD. Thus, CXCR4 is down-regulated in muscle progenitors prior to their differentiation (for summary, see Fig. 3G).

Ectopic application of SDF1 in the chick limb attracts muscle progenitor cells and delays their differentiation

To determine if CXCR4⁺ muscle progenitor cells respond to SDF1, we applied SDF1 to ectopic positions within the chick limb. For this, COS1 cells were transiently transfected with an SDF1-expression construct or with a control construct. Western blotting was used to verify that SDF1 protein was secreted from transfected, but not from control, COS1 cells (Fig. 4L). The COS1 cells were harvested 36 h after transfection and implanted into the right forelimb bud of chick embryos (Hamburger-Hamilton stage 19–20; HH19–20); the untreated left forelimb served as a control. Embryos that had received an implant were examined by in situ hybridization (HH24–25). In the limb of those embryos that received an implant of SDF1-expressing COS1 cells, CXCR4-positive muscle progenitors accumulated at ectopic positions (16 out of 18 cases examined) (Fig. 4A–C,J,K). For instance, when the implant was located in the proximal portion of the dorsal limb, we observed that CXCR4⁺ cells assembled close to the implant (Fig. 4B,K). After implanting SDF1-expressing cells, a substantial redistribution of muscle progenitor cells was also observed by in situ hybridization with a *Pax3* probe (11 out of 17 cases examined) (Fig. 4D–F). The change in the distribution of Pax3⁺ cells was less pronounced than the one observed for CXCR4⁺ cells, indicating that not all Pax3⁺ cells responded to SDF1. This is in accordance with the existence of a Pax3⁺/CXCR4[−] cell population. Dorsal or ventral CXCR4⁺ or Pax3⁺ cells assembled close to the implant, but such cells rarely moved around the implant into the central limb bud mesenchyme. None of the embryos that received an implant of COS1 cells transfected with the control plasmid showed ectopically positioned muscle progenitors ($n = 5$). These data indicate that an ectopic source of SDF1 attracts CXCR4⁺ muscle progenitor cells.

To test the effect of ectopic SDF1 on differentiation, chicken embryos with implants were hybridized with *MyoD* and *Myf5* probes. Hybridization signals for *MyoD* (6 out of 6 cases examined) or *Myf5* (5 of 5 cases examined) were lower in limbs that had received an implant of SDF1-producing cells than in the untreated, contra-lateral limbs (Fig. 4G–I; data not shown). In contrast, no change in *MyoD* and *Myf5* expression was observed in embryos containing the control implant (5 out of 5 cases examined; data not shown). These data show that ectopic SDF1 not only attracts muscle progenitor cells, but also suppresses their differentiation.

Figure 4. Muscle progenitors are attracted by an ectopic source of SDF1. COS1 cells cotransfected with SDF1 and GFP expression plasmids were implanted into the right wing bud of chick embryos at HH19–20. The distribution of muscle progenitor cells was analyzed at HH25 in the untreated (A,D,G) and treated (B,E,H) contra-lateral limb by in situ hybridization using *chCXCR4*-specific (A,B), *chPax3*-specific (D,E), and *chMyoD*-specific (G,H) probes. (C,F,I) The positions of the GFP positive implants are shown and are also indicated by arrows. Note the aberrant position of the CXCR4⁺ and Pax3⁺ progenitor cells and the reduction of the MyoD signal in the limb implanted with SDF1-expressing cells. (J,K) COS1 cells transfected with GFP expression plasmid only (J) or COS1 cells cotransfected with SDF1 and GFP expression plasmids (K) were implanted into the limb bud, and the distribution of muscle progenitors was analyzed on sections after in situ hybridization using *chCXCR4*. The position of the implant is indicated. (L) Western blot analysis of supernatant from COS1 cells transfected with a plasmid encoding SDF1 (right lane); as a control, a plasmid encoding GFP was transfected (left lane). Bars, 500 μ m.

Changes in the distribution and number of muscle progenitor cells in CXCR4^{−/−} embryos

We examined the distribution of hypaxial muscle progenitor cells in mouse embryos that carry a mutation in the *CXCR4* gene. The *CXCR4* mutant allele was described previously (Ma et al. 1998). Antibodies directed against Lbx1 and MyoD were used to visualize the muscle progenitor cell population that moves to the anlage of the tongue in the first branchial arch of *CXCR4*^{+/−} and *CXCR4*^{−/−} embryos. In control embryos at E10.75, a stream of muscle progenitors along the hypoglossal cord could be observed, and a large number of muscle progenitor cells had reached the floor of the first branchial arch. The Lbx1⁺ or MyoD⁺ cell population that had reached

Figure 5. Migration of muscle progenitors along the hypoglossal cord. Sections of the first branchial arch of *CXCR4*^{+/−} (A), *CXCR4*^{−/−} (B), *Gab1*^{−/−} (C), and *CXCR4*^{−/−}*Gab1*^{−/−} (D) embryos at E10.75 were analyzed with anti-Lbx1 (red) and anti-MyoD (green) antibodies to identify muscle progenitor cells. In control embryos, muscle progenitors were observed along the hypoglossal cord (arrow) and colonized the mesenchyme of the first branchial arch, the target (arrowhead). Note the reduction in the numbers of muscle progenitors in the first branchial arch of *CXCR4*^{−/−} and *Gab1*^{−/−} embryos, and their absence in *CXCR4*^{−/−}*Gab1*^{−/−} embryos. Bar, 250 μ m.

the floor of the first branchial arch was reduced in number in *CXCR4*^{−/−} embryos compared with controls (Fig. 5A,B). We also analyzed the effect of the *CXCR4* mutation in a *Gab1*^{−/−} genetic background. In the *Gab1* mutant embryos, the number of Lbx1⁺ or MyoD⁺ cells in the first branchial arch was lower than in control mice (Fig. 5A,C). In *CXCR4*^{−/−}*Gab1*^{−/−} embryos, the migrating muscle progenitor cells did not reach this target, and Lbx1⁺ or MyoD⁺ cells were not detectable in the floor of the first branchial arch (Fig. 5D). Also at E11.5, we found no Lbx1⁺ or MyoD⁺ cells in the branchial arch of *CXCR4*^{−/−}*Gab1*^{−/−} embryos (data not shown). Thus, in the *CXCR4*;*Gab1* double mutants, the migrating cells are not only delayed, but they do not reach this target. The complex morphology made it difficult to count the cells that migrate along the hypoglossal cord or reach the branchial arch. Therefore, quantification was performed for cells migrating into the limb.

We examined the distribution and determined absolute numbers of muscle progenitor cells in the limbs of *CXCR4*^{−/−} and control mice. For this, the limb was divided into four domains, dorsal proximal, dorsal distal, ventral proximal, and ventral distal, and the number of Lbx1⁺ cells was determined in each domain (Fig. 6I). In *CXCR4*^{−/−} mice, we observed changes in the distribution of progenitor cells in the dorsal limb; i.e., reduction in the numbers of Lbx1⁺ cells at E10.75 (Fig. 6A,B,E, domains I and II). The reduction in cell numbers was more

pronounced in the distal (35%) than in the proximal (25%) domain of the dorsal limb (Fig. 6E). Similarly, the number of MyoD⁺ cells was reduced in the dorsal limb, and the distal domain was more strongly affected than the proximal one (data not shown). However, the differentiation rate (number of MyoD⁺ cells/number of Lbx1⁺ cells) was not markedly altered (control: $59 \pm 3\%$; *CXCR4*^{−/−}: $50 \pm 4\%$, p value = 0.04). Proliferation of muscle progenitors was analyzed using BrdU labeling showing no significant change in the proliferation rate of progenitors located in the dorsal or ventral limbs of *CXCR4*^{−/−} and control embryos at E10.75 (control: $58 \pm 5\%$; *CXCR4*^{−/−}: $61 \pm 5\%$, p value = 0.18). Cell death was analyzed by TUNEL staining and was rarely observed in Lbx1⁺ nuclei, indicating that the nuclear proteins are not properly maintained in apoptotic nuclei. We therefore counted all apoptotic nuclei in the area occupied by muscle progenitor cells and observed a significant increase in apoptosis in the proximal domain of the dorsal limb in *CXCR4*^{−/−} compared with control embryos (Fig. 6F; for further detail, see Materials and Methods). In conclusion, these data show that muscle progenitor cells in the dorsal limb of *CXCR4*^{−/−} mutant embryos are not correctly distributed and their survival is impaired.

We then analyzed the effect of the *CXCR4* mutation in the *Gab1*^{−/−} background. For this analysis, *CXCR4*^{−/−}*Gab1*^{−/−} and *CXCR4*^{+/+}*Gab1*^{−/−} embryos were compared (Fig. 6C,D). In the dorsal and ventral limbs, the numbers of Lbx1⁺ and MyoD⁺ cells were further reduced in *CXCR4*^{−/−}*Gab1*^{−/−} compared with *CXCR4*^{+/+}*Gab1*^{−/−} embryos (Fig. 6G). The number of apoptotic cells in the limb areas occupied by muscle progenitor cells was increased in *CXCR4*^{−/−}*Gab1*^{−/−} mutants compared with *CXCR4*^{+/+}*Gab1*^{−/−} mice (Fig. 6H). We detected no significant differences in proliferation of Lbx1⁺ muscle progenitor cells in the limb of control, *CXCR4*^{+/+}*Gab1*^{−/−}, or *CXCR4*^{−/−}*Gab1*^{−/−} mice (proliferation rate: control, $58 \pm 5\%$; *CXCR4*^{+/+}*Gab1*^{−/−}, $56 \pm 4\%$; *CXCR4*^{−/−}*Gab1*^{−/−}, $53 \pm 5\%$). A small reduction in the differentiation rate was observed in *CXCR4*^{+/+}*Gab1*^{−/−} and *CXCR4*^{−/−}*Gab1*^{−/−} mice (control: $59 \pm 3\%$; *CXCR4*^{+/+}*Gab1*^{−/−}: $50 \pm 8\%$; *CXCR4*^{−/−}*Gab1*^{−/−}: $44 \pm 5\%$).

To assess whether the changes in progenitor numbers affected the generation of differentiated skeletal muscle, we compared tongue and limb muscle in control and mutant E13.5 embryos using anti-myosin and anti-MyoD antibodies (Fig. 7). In the tongue, several muscle groups are present at this stage. The extrinsic tongue muscle and proximal component of intrinsic tongue muscle derive from the head mesenchyme, whereas the distal component of the intrinsic tongue muscle is formed mainly by long-range migrating cells that derive from occipital somites (Huang et al. 1999; in Fig. 7A, intrinsic and extrinsic muscle are indicated by an arrow and arrowhead, respectively). No major difference was observable when the tongue muscles of *CXCR4*^{−/−} and control mice were compared (Fig. 7A,B). However, when we compared the tongue muscle in *CXCR4*^{−/−}*Gab1*^{−/−} and *CXCR4*^{+/+}*Gab1*^{−/−} animals, major

Figure 6. Distribution of muscle progenitor cells in the limb. (A–D) Sections of forelimbs of *CXCR4*^{+/+} (A), *CXCR4*^{-/-} (B), *Gab1*^{-/-} (C), and *CXCR4*^{-/-}*Gab1*^{-/-} (D) embryos at E10.75 were analyzed with anti-Lbx1 (red) and anti-MyoD (green) antibodies. (E–H) Quantification of the numbers of Lbx1⁺ and TUNEL⁺ cells located in distinct domains of forelimbs of embryos with the genotypes *CXCR4*^{+/+} (blue bars) and *CXCR4*^{-/-} (green bars) (E,F); and *Gab1*^{-/-} (orange bars) and *CXCR4*^{-/-}*Gab1*^{-/-} (yellow bars) (G,H). For this, consecutive sections of E10.75 embryos were analyzed by immunohistochemistry, and the Lbx1⁺ or TUNEL⁺ cell numbers were counted on every third section in the different limb domains; (*) *p* value < 0.007, *n* = 5; (**) *p* value < 0.005, *n* = 4 (see also Material and Methods for further details). (I) Schematic drawing of a developing limb and the four domains defined therein: dorsal proximal (I), dorsal distal (II), ventral proximal (III), and ventral distal (IV). Bar, 250 μ m.

differences were noted (Fig. 7C,D). In *CXCR4*^{+/+}*Gab1*^{-/-} mice, the intrinsic tongue muscle was small but muscle tissue was observable in the proximal and distal tongue. In *CXCR4*^{-/-}*Gab1*^{-/-} mice, only a fragment of the intrinsic tongue muscle was present in the proximal tongue, and the overall size of the tongue was very small (Fig. 7C,D). The appearance of the tongue muscle of *CXCR4*^{-/-}*Gab1*^{-/-} embryos is comparable to that observed in *c-Met*^{-/-} or *SF/HGF*^{-/-} mutants; i.e., comparable to the morphology of a muscle that completely lacks the contribution of migrating muscle progenitor cells (data not shown). Comparison of the size and distribution of muscle groups in the fore- or hindlimbs revealed occasionally minor, but no reproducible, differences between *CXCR4*^{+/+} and *CXCR4*^{-/-} embryos at E13.5 (Fig. 7E,F). Thus, the moderate reduction in the number of progenitors at early developmental stages was subsequently compensated for and did not affect the final muscle size. The limb muscle of *CXCR4*^{+/+}*Gab1*^{-/-} mutant mice was, however, affected (Fig. 7G). Thus, the reduction in the number of progenitor cells observed at earlier stages of *Gab1* mutants was not compensated. When we compared the limb muscle groups in *CXCR4*^{+/+}*Gab1*^{-/-} and *CXCR4*^{-/-}*Gab1*^{-/-} mice, additional major differences were observed. In the proximal portion of the lower forelimb, particular extensor muscles of the dorsal limb (for instance *extensor carpi radialis*, *extensor digitorum communis*, *extensor digitorum lateralis*) were absent in *CXCR4*^{-/-}*Gab1*^{-/-} mice (Fig. 7H). In *CXCR4*^{+/+}*Gab1*^{-/-} mice, these were observable but were reduced in size compared with control mice (Fig. 7G). Flexor muscles in the ventral limb were still present, but significantly smaller in *CXCR4*^{-/-}*Gab1*^{-/-} than in *CXCR4*^{+/+}*Gab1*^{-/-} mice (Fig. 7G,H). This indicates that compensatory mechanisms operate only if a threshold number of muscle progenitor cells reaches the limb.

Discussion

Cell sorting allowed us to determine the gene expression profile of embryonic muscle progenitor cells obtained from limb buds. Among the expressed genes, we identified the chemokine receptor *CXCR4*. The corresponding ligand, *SDF1*, is expressed in the mesenchyme of the limbs and the first branchial arch. In *CXCR4*^{-/-} mice, a reduction in the number of muscle progenitor cells was observed at particular target sites of migration. Furthermore, we observed a genetic interaction between *CXCR4* and *Gab1*. Thus, the chemokine receptor *CXCR4* that uses G-proteins for signaling and *Gab1* that transduces signals of tyrosine kinases, for instance of the c-Met receptor, elicit similar cellular responses in vivo, which might reflect cross-talk between such signaling systems. We conclude that the signals provided by *CXCR4* and *Gab1* control migration and survival of muscle progenitor cells.

CXCR4 is transiently expressed during migration of muscle progenitor cells

We show here that the chemokine receptor *CXCR4* is expressed only transiently in migrating muscle progenitor cells of chick and mouse. *CXCR4* protein in muscle progenitors is present only after delamination from the dermomyotome and is observed on forelimb levels only in those muscle progenitor cells that had already entered the limb. *CXCR4* expression in progenitor cells is thus observed after the onset of *Pax3* or *Lbx1* expression. In *Lbx1* mutant mice, *CXCR4* was not expressed in the muscle progenitor cells on limb levels; *CXCR4* expression was, however, observed in the progenitors of the hypoglossal stream of *Lbx1* mutant mice (E. Vasyutina and C. Birchmeier, unpubl.). Thus, *Lbx1* might participate in controlling *CXCR4* expression. *Pax3*⁺ or *Lbx1*⁺

Figure 7. Differentiated muscle groups of the limb and tongue. (A–D) Sections of the tongue of *CXCR4*^{+/+} (A), *CXCR4*^{-/-} (B), *Gab1*^{-/-} (C), and *CXCR4*^{-/-}*Gab1*^{-/-} (D) embryos at E13.5 stained with antibodies to myosin (green) and MyoD (red). (A) The intrinsic and extrinsic tongue muscles are indicated by an arrow and arrowhead, respectively. (E–H) Transverse sections through the proximal part of the lower forelimb of *CXCR4*^{+/+} (E), *CXCR4*^{-/-} (F), *Gab1*^{-/-} (G), and *CXCR4*^{-/-}*Gab1*^{-/-} (H) embryos at E13.5 stained with antibodies to myosin (green) and MyoD (red). (E) Indicated are extensor (ex) and flexor (fl) muscles; arrowhead and arrow point toward the radius and ulna, respectively. Bars, 250 μ m.

muscle progenitor cells in the limb are heterogeneous with respect to *CXCR4* expression. However, *CXCR4* is exclusively expressed in MyoD-negative cells, and thus only in undifferentiated progenitor cells. Therefore, *CXCR4* is expressed during migration of the muscle progenitor cells. Endogenous *SDF1* expression is observed in the mesenchyme of the limb close to the positions occupied by progenitor cells. Along the route of muscle progenitor cells that migrate into the head, expression of *SDF1* is found at the migration target; i.e., in the floor of the first branchial arch. The distribution of the endogenous *SDF1* indicates that migrating muscle progenitors can encounter the factor during migration.

Ectopic application of SDF1 demonstrated that Pax3⁺ and CXCR4⁺ muscle progenitor cells migrate toward the SDF1 source. The redistribution of CXCR4⁺ cells was more pronounced than the redistribution of Pax3⁺ cells, in accordance with the existence of two Pax3⁺ cell populations, one population that expresses CXCR4 and responds to SDF1, and a second population that is CXCR4-negative and nonresponsive. These gain-of-function experiments indicate that SDF1 can provide directional cues for migrating muscle progenitor cells. Moreover, a loss-of-function mutation of *CXCR4* in mice causes a change in the distribution of muscle progenitor cells in the dorsal forelimbs at E10.75. A reduction in numbers of progenitor cells is most pronounced in the distal limb of *CXCR4* mutant mice. Increased apoptosis of limb cells was not detected in this distal domain, but was observed proximally. Thus, CXCR4 provides signals that control distribution and survival of migrating muscle progenitor cells. However, the highest concentration of SDF1 mRNA is observed in the central and distal limb bud at E11. The distribution of the protein is unknown, as antibodies that allow the detection of SDF1 within the tissue are not available. Muscle progenitors migrate distally, but not into the central domain. This indicates that the distribution of SDF1 cannot account for all aspects of the migratory behavior of the cells, raising the

possibility that more than a single factor guides the migration. Such additional factors, for example, members of the ephrin family, could repulse muscle progenitor cells from the center of the limb (Swartz et al. 2001). Despite the significant reduction in the number of muscle progenitor cells of *CXCR4* mutant mice at E10.75, reproducible changes in the size of muscle groups were not observed at E13.5, indicating that changes in progenitor numbers can be compensated at subsequent developmental stages.

We observed a genetic interaction between *CXCR4* and *Gab1*. *Gab1* is an important c-Met signal transduction molecule, which binds and recruits other signal transduction components to activated c-Met (for review, see Birchmeier et al. 2003). In *Gab1* mutant mice, muscle progenitor cells reach the first branchial arch and the forelimb in reduced numbers. The deficit in progenitor numbers cannot be compensated at later developmental stages and is reflected in a change in size (or even the absence of) particular muscle groups (this study; Sachs et al. 2000). In *CXCR4*^{-/-}*Gab1*^{-/-} mice, a further decrease in the number of progenitor cells that reach the forelimb is observed that is more pronounced than in either of the single-mutant mice. The deficit is not compensated for and causes substantial changes in the size and distribution of muscle groups in *CXCR4*^{-/-}*Gab1*^{-/-} mice compared with *CXCR4*^{+/+}*Gab1*^{-/-} mice. Furthermore, migrating muscle progenitor cells do not reach the floor of the first branchial arch in *CXCR4*^{-/-}*Gab1*^{-/-} double-mutant mice; this target is colonized in either of the single-mutant mice. The consequence is a severe deficit in development of the intrinsic tongue muscle in the double-mutant mice. Increased apoptosis is observed in the hypoglossal cord and limbs, indicating that impaired cell migration and survival contribute to the changes in the distribution of progenitor cells.

The reduction in the numbers of distal muscle progenitor cells in the dorsal limb of *CXCR4* mutants is compensated for at subsequent stages, since we did not

observe reproducible changes in the size of differentiated limb muscles. In contrast, the reduction in the numbers of dorsal progenitors in *Gab1*^{-/-} or *CXCR4*^{-/-}*Gab1*^{-/-} double-mutant mice was more pronounced and resulted in a smaller size or even an absence of limb muscles. In none of these mutant strains did we observe a change in the proliferation rate of muscle progenitors at E10.75; i.e., at the developmental stage at which the progenitor numbers were counted. Compensatory mechanisms might prolong the proliferative phase of the progenitors at subsequent stages. Our data indicate that compensation is only possible if a threshold number of progenitor cells reaches the limb. We attempted to estimate a critical number of progenitor cells, which ensures the generation of muscle of normal size. For this, the reduction in progenitor cell numbers caused by mutation of *CXCR4* or *Gab1* in particular limb domains (see Fig. 6I) were compared with resulting abnormalities of differentiated muscle groups observed at later development stages (E13.5). A 35% reduction in progenitors of the dorsal distal limb domain of *CXCR4*^{-/-} mice did not affect the size of extensor muscle. The 60% reduction in the progenitor numbers in the ventral proximal limb domain of *Gab1*^{-/-} mice results in only a small size reduction of flexor muscles. This indicates that the critical number corresponds to about half of the number of progenitors present in the limb of wild-type animals. A balance between proliferation and differentiation controls the muscle progenitor pool and the size of the differentiated muscle (Amthor et al. 1999). Compensatory proliferation of muscle progenitors might be only possible during a limited time period in which a favorable environment is provided in the developing limb.

A genetic interaction of Gab1 and CXCR4

Tyrosine kinase receptors and chemokine receptors are two important classes of molecules implicated in the regulation of cell migration, and can also affect proliferation and cell death. *Gab1* is an adaptor molecule that functions in the signal transduction of c-Met and of other tyrosine kinases (Gu and Neel 2003). The phenotypes observed in the developing muscle of the *Gab1* and *CXCR4* mutations are similar: Both mutations affect the distribution and the survival of muscle progenitor cells, albeit to a different extent. In addition, a genetic interaction between the two loci can be observed: The muscle lineage of *CXCR4*;*Gab1* double-mutant mice is more strongly affected than in single *CXCR4* or *Gab1* mutants.

Cell motility relies on the ordered disruption and reformation of cell adhesion sites, as well as on cytoskeletal dynamics (Ridley et al. 2004). *Gab1* and *CXCR4* signaling might have distinct endpoints, for instance one in the control of cell matrix attachment and the other in the control of actin polymerization, but might nevertheless cooperate to regulate cell migration. Alternatively, the genetic cooperation of *Gab1* and *CXCR4* might reflect the fact that both employ similar signaling cascades. *Gab1* contains multiple docking sites for SH2 domain containing proteins like Shp2, the p85 subunit of

PI3kinase, Src, Crk, and PLC γ (for review, see Birnmeier et al. 2003; Gu and Neel 2003). Tyrosine phosphorylation of *Gab1* results in the activation of Ras/MAPK, PI3K/Akt, and PLC γ /PKC signaling pathways that regulate cell motility, proliferation, and survival. Chemokine receptors such as *CXCR4* use G-proteins to transmit signals in the cytoplasm. Classical GPCR signaling involves activation of second messenger-regulated serine/threonine kinases or ion channels (for review, see Mellado et al. 2001). More recently, GPCRs were also found to stimulate tyrosine phosphorylation cascades (Daub et al. 1996). The molecular mechanisms responsible for this depend on the exact cell type. Signals provided by GPCR can induce the activity of metalloproteinases, which cause cell surface shedding of ligands of tyrosine kinase receptors like HB-EGF, and thus activate the signaling cascades downstream of the corresponding tyrosine kinase receptors (Prenzel et al. 1999; Yan et al. 2002). G- α_i , which is used by *CXCR4* to transmit signals, directly binds and activates the c-Src tyrosine kinase, which in turn activates the Ras/MAPK and PI3K/Akt pathways (Ma et al. 2000). Experiments performed on cultured cells indicate that signaling molecules shared by tyrosine kinase and chemokine receptors regulate cell migration. For instance, SF/HGF and SDF1 cooperate to elicit chemotaxis in cultured rhabdomyosarcoma cells, and PI3K/Akt activities are required for this response (Jankowski et al. 2003). c-Kit and *CXCR4* signals attract hematopoietic progenitor cells, act synergistically, and both receptors rely on MAPK activity to transduce their chemotactic signals (Dutt et al. 1998). The genetic interaction that we observe for *Gab1* and *CXCR4* might reflect the fact that the signal transduction cascades used by these molecules converge on identical effectors to control migration and survival of muscle progenitor cells.

Materials and methods

Generation of an Lbx1^{GFP} mutant allele

The *Lbx1* targeting vector was assembled using the pTV vector (Riethmacher et al. 1995), which contains a *neomycin* gene flanked by loxP sites as well as a *thymidine kinase* gene to allow positive and negative selection, respectively. *Lbx1* genomic sequences were isolated from the 129Sv library. The *Gap43-GFP* cassette (provided by U. Mueller, Friedrich Miescher Institute, Basel, Switzerland) was fused to the initiation codon of *Lbx1*; a frameshift mutation was introduced into the BglII site in the second exon of *Lbx1*. In addition, the targeting vector contains 3.9 kb upstream and 6.1 kb downstream homologous sequences of the *Lbx1* locus. The linearized targeting vector was introduced into E14.1 embryonic stem (ES) cells by electroporation. ES colonies were selected with G418 and gancyclovir and screened for homologous recombination events using Southern blot hybridization. The *neomycin* resistance cassette was removed by transient expression of *Cre* in ES cells. *Lbx1^{GFP}* ES cells were injected into C57B6 blastocysts to generate a mouse strain that carries the *Lbx1^{GFP}* allele. *CXCR4* mutant mice were obtained from the Jackson Laboratory and are described in Ma et al. (1998). The generation of the *Gab1* mutant mice was detailed previously (Sachs et al. 2000).

FACS sorting, cRNA probe preparation and hybridization of Affymetrix GeneChips

The forelimbs of *Lbx1*^{+/GFP} embryos were dissected, the cells were dissociated by treatment with 0.02% Trypsin (PAN Bio-tech GmbH) for 10 min, and the GFP-positive cells were isolated using fluorescence-activated cell sorting (FACS). Cell sorting was performed on a FACS Vantage SE (Becton-Dickinson) using an argon ion laser (488 nm) for excitation. Total RNA from sorted cells was used for preparation of biotinylated cRNA probes according to the Affymetrix protocol. The probes of three independent cell pools were hybridized on MG U74A/B/Cv2 Affymetrix GeneChips. The results on the expression of a selected set of genes that encode surface molecules are indicated in Table 1. Four probes for *SDF1* are present on the MG U74A/B/Cv2 Affymetrix GeneChips, 162234_f_at, 100112_at, 105704_at, and 160511_at. The hybridization signals of three of these, 162234_f_at, 105704_at, and 160511_at were called absent; signals of 100112_at were called present, but the intensity of the signal was low (*p* value = 0.006; hybridization signal intensity = 330 ± 65).

Cell transfection and implantation in ovo

Coding sequences of chicken *SDF1* (Stebler et al. 2004) or *EGFP* (BD Biosciences Clontech) were inserted into the pcDNA3.1(-) expression vector (Invitrogen). COS1 cells were cotransfected with 10 µg of pcDNA-SDF1 and/or pcDNA-EGFP, using Lipofectamine2000 (GIBCO-BRL). Transfection efficiency was estimated by GFP expression. Thirty-six hours after transfection, cell aggregates were implanted into the right limb of chick embryos (HH19–20). At HH24–25, embryos in which we detected GFP-expressing cells in the limb were analyzed.

We analyzed the secreted SDF1 protein from transfected COS1 cells by Western blot. For cotransfection, 10 µg of each plasmid (pcDNA-SDF1 and pcDNA-EGFP) was used; for a control, 10 µg pcDNA-EGFP was transfected. Cells were grown for 36 h in a serum-containing medium, which was followed by a 24 h culture in a serum-depleted medium. The supernatants were then collected, precipitated with 20% (w/v) trichloroacetic acid, and dissolved in SDS-containing loading buffer. The samples were analyzed for the presence of SDF1 by Western blot analysis using goat anti-human SDF1 antibody (Sigma).

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization analysis of mouse and chick embryos was performed as described (Wilkinson 1992). The following RNA probes were used for in situ hybridization: A 945-bp fragment encoding mouse *CXCR4*; a fragment (660 bp) that encompasses the 3' coding sequence and 3' UTR of the mouse *SDF1* gene; chicken *Pax3* (Goulding et al. 1994); chicken *MyoD* (Lin et al. 1989); chicken *Myf5* (Dechesne et al. 1994); chicken *CXCR4* and *SDF1* (Stebler et al. 2004). RNA transcripts were synthesized using a digoxigenin (DIG)-labeling kit (Roche Mannheim) according to the manufacturer's protocol.

Immunofluorescence staining was performed on 12-µm cryosections of embryonic tissue that had been fixed with 4% paraformaldehyde for 2 h. The following antibodies were used: monoclonal mouse anti-skeletal fast myosin (Sigma); rabbit anti-mouse MyoD (Santa Cruz); rabbit anti-GFP (Molecular Probes); guinea pig anti-mouse Lbx1 (Müller et al. 2002); rabbit anti-mouse CXCR4 (Stumm et al. 2002); and secondary antibodies conjugated with Cy2, Cy3, or Cy5 (Jackson ImmunoResearch Laboratories). To visualize CXCR4 protein, the signal obtained after incubation with a biotinylated goat anti-rabbit antibody

(KPL) was amplified using the Cy3-TSA Fluorescence System (PerkinElmer Life Sciences).

To assess the proliferation rate, BrdU (75 µg/g of body weight; Sigma) was injected intraperitoneally into the dams 2 h prior to the dissection of the embryos; incorporated BrdU was detected using mouse anti-BrdU antibodies (Sigma). Cell death was determined by TUNEL staining using an Apop-Tag fluorescein in situ apoptosis detection kit (Intergen). To determine the number of Lbx1⁺ cells in the limb bud, consecutive sections of stage and size-matched embryos (E10.75) were analyzed by immunohistochemistry, and the number of Lbx1⁺, BrdU⁺/Lbx1⁺ double-positive, and MyoD⁺ cells were counted on every third section in different limb domains. Additionally, TUNEL⁺ cells that were also Lbx1⁺ or positioned close to Lbx1⁺ cells were counted. The proliferation rate was determined as the number of BrdU⁺/Lbx1⁺ cells divided by the total number of Lbx1⁺ cells. Similarly, the differentiation rate was determined as the number of MyoD⁺ cells divided by the total number of Lbx1⁺ cells.

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