Functional analysis of homeodomain-containing transcription factor Lbx1 in satellite cells of mouse skeletal muscle

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Summary

Satellite cells are usually mitotically quiescent muscle stem cells, located between the sarcolemma and the basement membrane of muscle fibers. When muscles are damaged, satellite cells become activated, proliferate and differentiate to form multinucleate myofibers. The molecular mechanisms underlying these processes are poorly understood. In the present study, we found that, following treatment with cardiotoxin, homeodomain-containing transcription factor Lbx1 was strongly expressed in the satellite cells of regenerating adult skeletal muscle. Our immunohistochemical and northern blot analyses indicate that Lbx1 is expressed in activated but not quiescent satellite cells. In vitro, this Lbx1 expression was gradually downregulated when satellite cells differentiate into mature myofibers. Transfection and forced expression of Lbx1 in satellite-cell-derived C2C12 myoblast cells resulted in severe depression of myogenic differentiation and incomplete myotube formation, concomitantly with the activation of the paired-box transcription factor Pax7 and depression of the myogenic regulatory factor MyoD. Moreover, knockdown of Lbx1 in in-vitro-cultured satellite cells resulted in downregulation of Pax7. These results suggest that Lbx1 plays important roles in differentiation and maintenance of satellite cells of mature myofibers, probably through the regulation of Pax7.

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Key words: Lbx1, Pax7, Satellite cells, Stem cells, Muscle

Introduction

Lbx1 is a homeodomain-containing transcription factor related to the *Drosophila* ladybird genes. In *Drosophila*, ladybird genes have been reported to be expressed in a specific subset of cardioblasts and in important components of the cardiogenic pathway required for the diversification of heart precursor cells (Jagla et al., 1997). In vertebrates, expression of Lbx1 has been described in the CNS and in muscle precursor cells (Jagla et al., 1995; Uchiyama et al., 2000).

Skeletal muscles in vertebrates consist predominantly of myofibers, syncytial cells with peripheral, postmitotic myonuclei, which are formed by the fusion of mononuclear precursor cells termed myoblasts. In mice, the first muscle mass to form under the dermomyotome is the myotome, which has an epaxial and a hypaxial component, subsequently integrated into the trunk musculature. The myoblasts produced from the epaxial lip generate epaxial muscles, such as deep back muscles, whereas those from the hypaxial lip give rise to hypaxial muscles of the body wall and limbs. *Lbx1* has been reported to be expressed specifically in particular myoblasts that delaminate from the hypaxial dermomyotome and undergo long-range migration to their target sites where they form distant muscle masses, such as those of the limbs, diaphragm and tongue. A *Lbx1*-null mutation caused a specific lack of limb musculature attributed to migration defects, whereas hypaxial body wall muscles underwent normal development (Schafer and Braun, 1999; Gross et al., 2000; Brohmann et al., 2000). In *Lbx1*-mutant embryos, muscle progenitor cells delaminated from the dermomyotome, but remained in the proximity of the somite. These results suggested that murine Lbx1 controls the expression of genes essential for recognition or interpretation of cues that guide migrating muscle precursor cells and maintain their migratory potential (Brohmann et al., 2000). Targets of Lbx1 have not yet been identified in muscle progenitor cells.

The broad outline of the origin of embryonic skeletal muscle is well established, whereas it is less clear what happens later in the perinatal period. The first muscle fibers that appear are known as primary fibers – approximately embryonic day (E) 11-14 in mouse limbs – around which secondary fibers form at the time when innervation begins to be established (~E14-16) (Ontell and Kozeka, 1984). Whereas most secondary myoblasts differentiate to form secondary myofibers that contribute to myofiber formation and muscle growth during the perinatal period, some remain undifferentiated as satellite cells in adult muscles. The satellite cells are mononucleated muscle stem cells located between the sarcolemma and the basement membrane of terminally differentiated muscle fibers. They account for 2-5% of sublaminal nuclei in adult muscle, and are considered to be reserve cells that contribute to muscle

regeneration (Bischoff and Heintz, 1994). Normally, satellite cells are mitotically quiescent and replicate very slowly to replenish the satellite cell pool (Schultz, 1996; Decary et al., 1997). However, when muscles are damaged, they become activated and undergo multiple rounds of cell division before they delaminate from the myofibers to migrate to the damage sites. Eventually they differentiate terminally to form multinucleate myofibers either de novo or from preexisting muscle fibers (Seale and Rudnicki, 2000). Although there is debate about the origin of secondary myoblasts and satellite cells (reviewed by Charge and Rudnicki, 2004), recently conducted chick-quail grafting experiments strongly suggest that most, if not all, of these cells derive from somites (Gros et al., 2005; Schienda et al., 2006).

In the present study, we focused our attention on the physiological roles of Lbx1 that might play a role in postnatal growth, repair and maintenance of muscular tissues. Although Jagla et al. reported the expression of Lbx1 in adult skeletal muscles (Jagla et al., 1995), its physiological function remains poorly understood. We describe the strong expression of *Lbx1* in satellite cells of regenerating adult skeletal muscles induced by cardiotoxin treatment. By contrast, we could not detect *Lbx1* expression in mature quiescent satellite cells. We also detected *Lbx1* expression in cultivated satellite cells isolated from single myofibers. This *Lbx1* expression in satellite cells was gradually downregulated in vitro, when cells were induced to differentiate into mature myofibers. To gain insights into the function of Lbx1 in the satellite cells, we performed gain-of-function-like experiments using the C2C12 myoblast cell line, which derives from murine satellite cells and has the ability to differentiate into myofibers in vitro. We found that forced expression of *Lbx1* in C2C12 cells resulted in severe depression of myogenic differentiation and incomplete myotube formation. In such differentiation-restrained C2C12 cells expression of *Pax7* was maintained at a high level, whereas that of MyoD was depressed. Additionally, knockdown of Lbx1 caused primary cultured satellite cells to decrease their *Pax7* expression. These results are discussed in relation to the physiological functions of Lbx1 in satellite cells of adult skeletal muscle.

Results

Lbx1 protein expression is induced in regenerating muscle

To ascertain whether Lbx1 was expressed in muscle tissues of adult mice, immunohistochemical analysis was carried out. The tibialis anterior (TA) muscles were isolated, cryosectioned, fixed in 4% PFA for 10 minutes and immunostained with anti-Lbx1 antibody highly specific for Lbx1 protein (Mizuhara et al., 2005). Migrating myoblasts were clearly immunostained using this antibody, without any nonspecific stainings on the cryosections of E10.5 mouse embryos (data not shown). Fig. 1A shows the results of TA muscles from 8-week-old ICR mice using anti-Lbx1 antibody. Neither multi-nuclei of mature myofibers nor nuclei of mononucleated cells around the myofibers, including quiescent satellite cells, stained positive

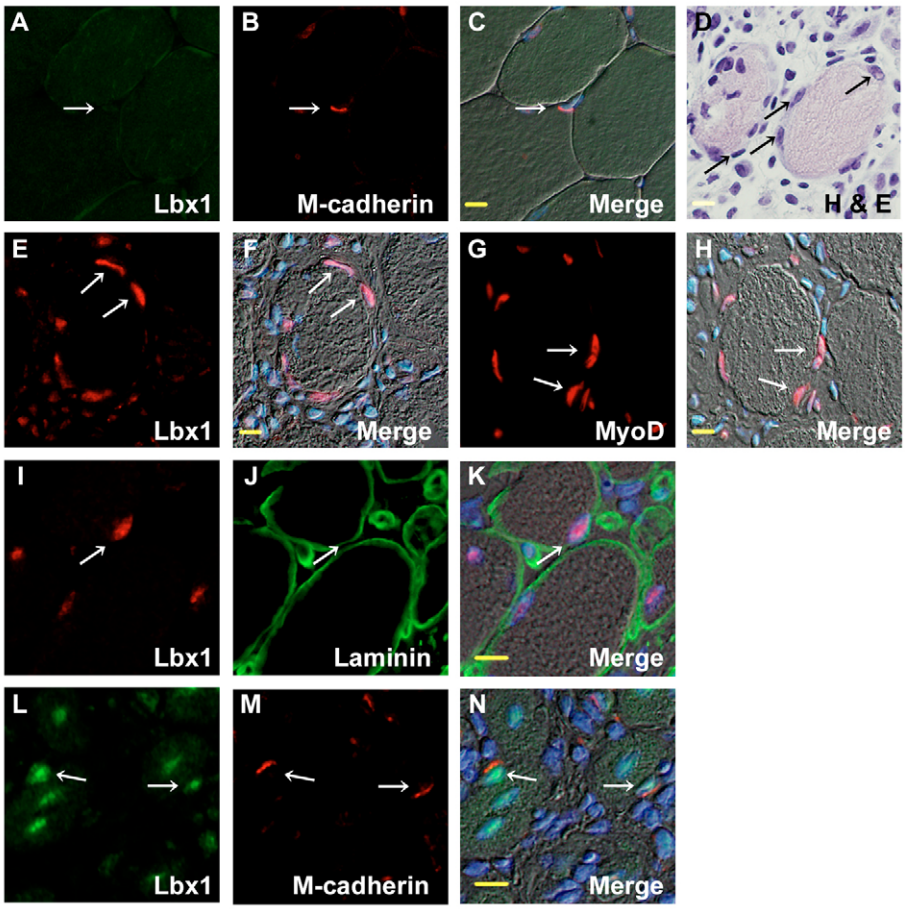

Fig. 1. Lbx1 expression in regenerating skeletal muscles of adult mice. (A-C) Transverse sections of uninjured TA muscles taken from 8-week-old ICR mice immunostained for (A) Lbx1 and (B) M-cadherin and counterstained with Hoechst dye 33342. Note M-cadherin-positive satellite cells do not express Lbx1 (arrows). (E-N) Transverse sections of regenerating TA muscles taken from cardio-toxin-treated 8-week-old mice stained with antibodies against (E,I,L) Lbx1, (J) laminin, (M) M-cadherin and (G) MyoD, and also counter-stained with Hoechst dye 33342. Note most of the cells attached to myofibers are MyoD positive (G, arrows) and also Lbx1 positive (E, arrows). Note that Lbx1-positive cells are located beneath the basal lamina, which is laminin-positive (I-K, arrows). M-cadherin positive cells attached to centrally nucleated regenerating muscles also express Lbx1 (L-N, arrows). (D) Hematoxylin-eosin staining. Several mononucleated cells are seen attached to myofibers (arrows). Bars, 10 μm.

for Lbx1. However, in TA muscles of younger mice (3 weeks old), some Lbx1-positive nuclei were observed (data not shown). To ascertain that Lbx1 was not expressed in quiescent satellite cells, we double-immunostained the skeletal muscles with anti-Lbx1 antibody and anti-M-cadherin antibody, because M-cadherin is known to be expressed in satellite cells (Cornelison and Wold, 1997; Ishido et al., 2006). Careful observations failed to detect any Lbx1 expression in the M-cadherin positive cells (Fig. 1A-C), indicating that Lbx1 was not expressed in quiescent satellite cells.

In the next experiment, we sought to examine the expression of Lbx1 in the activated satellite cells that appeared in regenerating muscle tissues. To induce muscle regeneration, cardiotoxin was injected into a hind limb muscle and the muscle then allowed to heal for 4 days. The results of immunostaining the regenerating TA muscles usoing anti-Lbx1 antibody of are shown in Fig. 1E. By contrast to normal quiescent muscles, a number of mononucleated cells were observed in the regenerating muscles (Fig. 1D). Lbx1-positive signals were observed in the nuclei of mononucleated cells and in centrally located nuclei of young myotubes. The muscle-fiber-attached Lbx1-positive mononucleated cells were located between the sarcolemma and the basement membrane of muscle fibers (Fig. 1I,J,K), and were found to express M-cadherin (Fig. 1L,M,N) – a well-known satellite cell marker, and MyoD (Fig. 1G,H) – which is reported to express in activated satellite cells (Cooper et al., 1999). From these results, we concluded that Lbx1 was expressed in activated satellite cells but not in quiescent ones.

Lbx1 expression in satellite cells isolated from myofibers

To determine in more detail the expression of *Lbx1* in satellite cells, northern blot analysis was performed using satellite cells freshly isolated from single myofibers (see Materials and Methods). Briefly, extensor digitorum longus (EDL) muscles were taken from 3-week-old ICR mice and single myofibers recovered after collagenase treatment, followed by cultivation in satellite-cell growth medium for up to 2-4 days. A number of sphere-shaped cells were observed to attach to myofibers, but they quickly detached and migrated onto the bottom of the culture vessels. These cells continued to proliferate and maintained their spherical morphology for 2 weeks when cultured at a low-density in satellite-cell growth medium, although some flat spindle-shaped cells occasionally appeared (Fig. 2A). We observed that most of the sphere-shaped cells quickly converted to flat spindle-shaped cells when cultured for 1day at high density (Fig. 2B) and then fused to each other to form myotubes. Eventually, after 5 days, they developed into contractible mature myotubes when serum-starved (Fig. 2C).

Results of the northern blot experiments are shown in Fig. 2D, and it can be seen that sphere-shaped satellite cells strongly expressed *Lbx1* mRNA. This *Lbx1* expression gradually decreased according to the myogenic differentiation of the cells in vitro, i.e. their conversion to flat spindle-shaped cells, followed by myotube formation (Fig. 2D). This expression pattern was similar to that of *Pax7*, a well-known transcription factor, which is expressed specifically in undifferentiated satellite cells (Fig. 2D). By contrast, myogenin – the key myogenic transcription factor expressed in a later stage of myogenesis – was induced in the flat spindle-shaped cells (Fig. 2D) and upregulated during myogenic differentiation (Fig. 2D).

To obtain more details regarding Lbx1 expression, immunohistochemical analysis was conducted. The majority of the sphere-shaped cells expressed Pax7 (Fig. 3A), MyoD (Fig. 3B), Myf5 (data not shown) and Lbx1 (Fig. 3A-C), but did not express myogenin (Fig. 3C). In the occasionally appearing flat spindle-shaped cells, the expression of Pax7, MyoD, Myf5 and Lbx1 (Fig. 3A-C and data not shown) was detected. Additionally, some cells were also found to express myogenin. The levels of Lbx1 in these myogenin-expressing flat spindle-shaped cells was usually rather low (Fig. 4A).

Next, we examined Lbx1 expression in differentiated myotubes induced by cultivating in differentiation medium. In the multinucleate myotubes formed in vitro, expressions of myogenin, myoD, and Myf5 were detected (Fig. 4B and data not shown), but the expression of Pax7 was not (Fig. 4C). Lbx1 expression was also detected in the myotubes, but was apparently lower than that observed in sphere-shaped cells (Fig. 3A-C). In differentiation medium, most cells differentiated to form multinucleate myotubes, but some cells remained as mononucleated cells. Interestingly, these mononucleated cells were observed to express Pax7 but not Lbx1 (Fig. 4C).

We also examined the expression of Lbx1 in satellite cells isolated from muscles other than EDL, including soleus, tongue, diaphragm and trunk muscles. Sphere-shaped cells were prepared from these muscles by essentially the same method as that for EDL. These cells were Pax7- and MyoD-positive, indicating that they were activated satellite cells. We observed that all of these cells express Lbx1 (Fig. 3D,E and

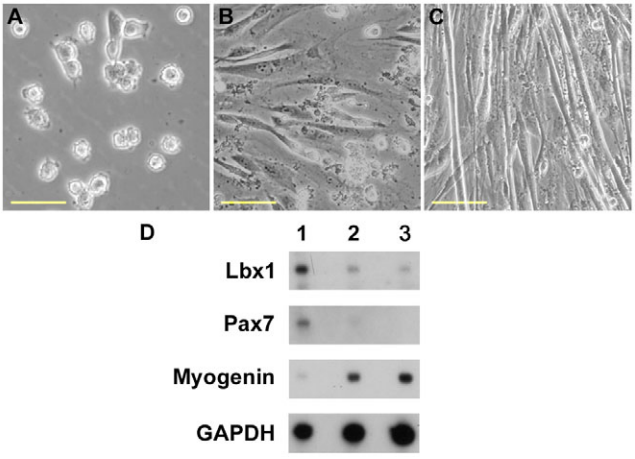

Fig. 2. Northern blot analysis of *Lbx1* expression in satellite cells during differentiation in vitro. (A-C) Differentiation of satellite cells in vitro. (A) Sphere-shaped cells after cultivation on a collagen-coated dish for 2 weeks at low density. (B) When cells were cultured at high density, most of the sphere-shaped cells differentiate into flat spindle-shaped cells within 1 day. (C) When cultured in differentiation medium for 5 days, most of the flat spindle-shaped cells eventually fuse to form mature myofibers. Bars, 25 μm. (D) Northern blot analysis. mRNAs were isolated from sphere-shaped cells shown in A (lane 1), flat spindle-shaped cells shown in B (lane 2) and myofibers shown in C (lane 3), and hybridized with probes against *Lbx1*, *Pax7*, *Myog* (myogenin) and *Gapdh*. *Lbx1* and *Pax7* were expressed at high levels in sphere-shaped cells (lane 1), and the expressions decreased during myogenic differentiation in vitro (lanes 2 and 3). By contrast, *Myog* expression was not detected in sphere-shaped cells and it increased during differentiation.

data not shown). From the above results, we concluded that Lbx1 is strongly expressed in sphere-shaped activated satellite cells in various muscle tissues and that this activity gradually decreased in accord with their myogenic differentiation.

Ectopic expression of Lbx1 in C2C12 cells prevents myotube formation

To gain insights into the function of Lbx1 during muscle regeneration, we tested what happened if Lbx1 was expressed constitutively throughout myogenic differentiation, because Lbx1 expressed strongly in sphere-shaped satellite cells and was gradually downregulated during their differentiation. For that, we transfected the Lbx1-expressing vector construct pCX-Lbx1 into C2C12 myoblast cells. Because of their potential to differentiate into myotubes in vitro, C2C12 cells are widely used for studying the mechanisms of myogenic differentiation. Another reason why we chose C2C12 cells for this experiment is that these cells do not express endogeneous Lbx1, although they are originally derived from a murine satellite cell. As shown in Fig. 5A, we could not detected any Lbx1 in C2C12 cells.

C2C12 cells were co-transfected with either pCX-Lbx1 and pEGFP-C2 or with pCX-EGFP and EGFP-C2 (as control), and then cultures in medium containing G418 (Gibco). After 7 days in culture, G418-resistant C2C12 cells were harvested and exogenous expression of Lbx1 was determined. As shown in Fig. 5A, strong expression of Lbx1 was confirmed in pCX-Lbx1- transfected C2C12 cells. Lbx1-expressing C2C12 cells were morphologically normal (Fig. 5B) and proliferated at the same rate as mock-transfected control cells (Fig. 5F) when cultured in C2C12-cell growth medium. Thus, no apparent differences were detected between pCX-Lbx1-transfected and mock-transfected control cells. However, one remarkable effect due to the ectopic expression of Lbx1 was observed when cells were induced to differentiate after 5 days following serum withdrawal. Whereas mock-transfected control C2C12 cells differentiated into multinuclear myotubes, Lbx1-expressing C2C12 cells could not form mature myotubes, and most cells remained mononucleated myoblasts (Fig. 5B). Even if myotubes were formed, the average myotube length was extremely short, suggesting that myoblast fusion was impaired. To verify this, we counted the nuclei of those myotubes stained with antibody against myosin heavy chain (MHC) and counter-stained with

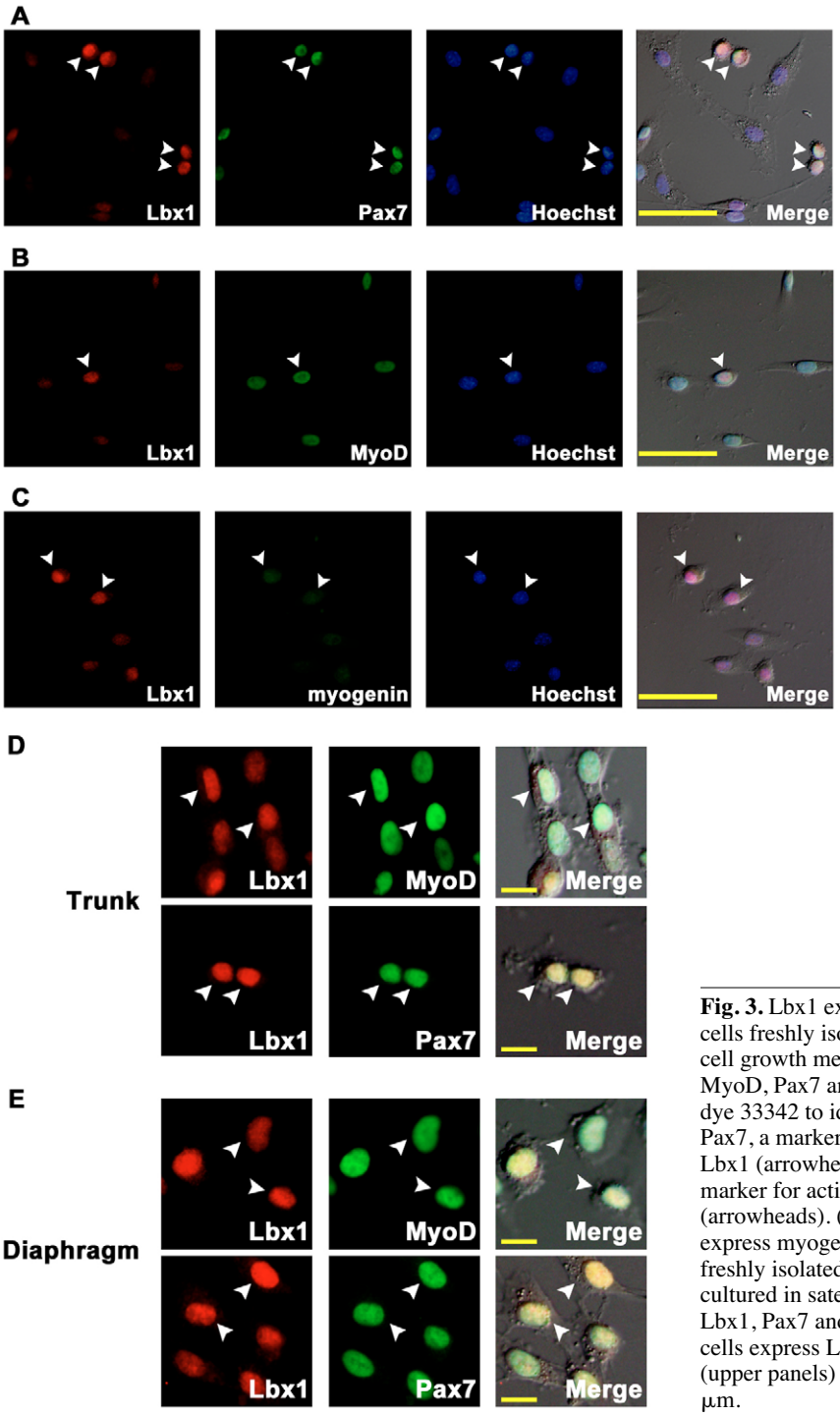

Fig. 3. Lbx1 expression in activated satellite cells. (A-C) Satellite cells freshly isolated from EDL muscles were cultured in satellite cell growth medium for 4 days and then immunostained for Lbx1, MyoD, Pax7 and myogenin, and also counter-stained with Hoechst dye 33342 to identify nuclei. (A) Sphere-shaped cells expressing Pax7, a marker for activated satellite cells, also show high levels of Lbx1 (arrowheads). (B) Sphere-shaped cells expressing MyoD, a marker for activated satellite cells, show high levels of Lbx1 (arrowheads). (C) Sphere-shaped cells that express Lbx1 do not express myogenin (arrowheads). Bars, 50 μm. (D,E) Satellite cells freshly isolated from (D) trunk or (E) diaphragm muscles were cultured in satellite cell growth medium, and then immunostained for Lbx1, Pax7 and MyoD. Note that MyoD- and Pax7-positive satellite cells express Lbx1. Arrowheads indicate $Lbx1^+/MyoD^+$ satellite cells (upper panels) or $Lbx1^+/Pax7^+$ satellite cells (lower panels). Bars, 10 μm.

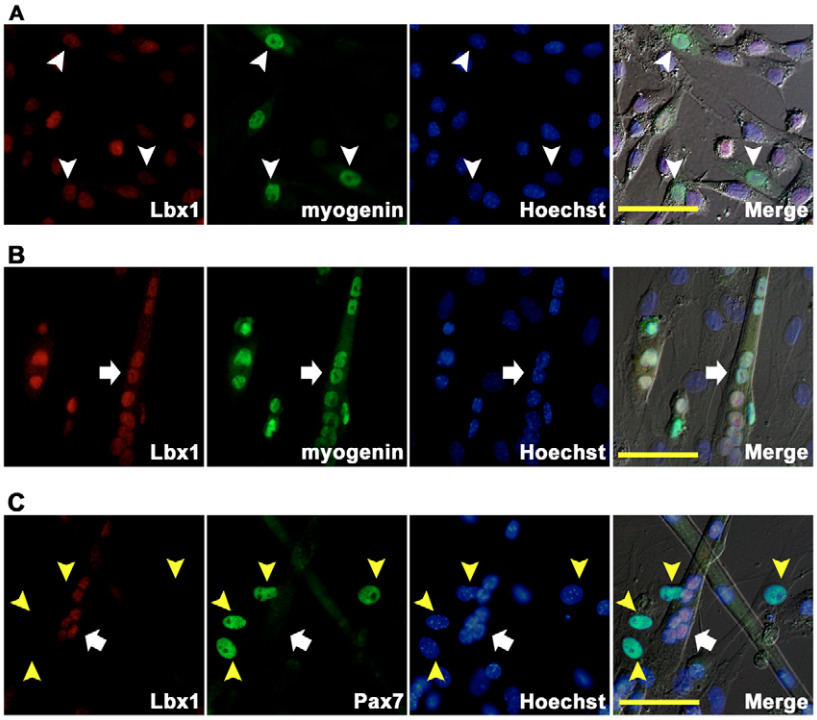

Fig. 4. Lbx1 expression in differentiating myoblasts and myotubes. Satellite cells were induced to differentiate in vitro following the method described in Materials and Methods, and then immunostained for Lbx1, myogenin and Pax7, and also counter-stained with Hoechst dye 33342 to identify nuclei. (A) Primary satellite cells were induced to differentiate into flat spindle-shaped cells by cultivating them at high density. Only some of the actively proliferating flat spindle-shaped cells express myogenin. Such myogenin-expressing cells (arrowheads) also express Lbx1, but at rather low levels. (B,C) Primary satellite cells were induced to differentiate into myotubes by cultivating them in differentiation medium for 5 days. The developing myotubes express both myogenin and Lbx1 but do not express Pax7 (arrows). The level of Lbx1 expression is rather low. Note that Pax7-expressing mononucleated cells (arrowheads) do not express Lbx1. Bar, 50 μm.

Hoechst dye 33342 (Fig. 5C). Myotubes formed from mock-transfected control cells contained 6.9±1.5 nuclei per one myotube (n=2002), whereas those from Lbx1-expressing C2C12 cells contained only 1.7±0.2 nuclei (n=644) (Fig. 5E).

Lbx1 expression did not seem to affect apoptosis in the C2C12 cells during differentiation in culture because we could not detect any significant difference in the rates of nuclear condensed cells between Lbx1- and mock-transfected cells (Fig. 5G). Sarcomere assembly is an important step in a latter stage of myogenesis (Schwander et al., 2003). Therefore, we examined the ultrastructure of myotubes formed by Lbx1-expressing C2C12 cells using electron microscopy. As shown in Fig. 5D, myotubes of sarcomeres from Lbx1-expressing C2C12 cells were partially assembled but severely disorganized and Z-bands could hardly be detected. These results suggest that, in Lbx1-expressing C2C12 cells, sarcomere assembly is initiated but not completed or cytoskeletal organization cannot be maintained.

Effect of continuous Lbx1 expression on myogenic gene regulation

To determine the mechanisms that lead to the abnormal myotube formation due to continuous expression of Lbx1, we examined by northern blotting the expression of various genes (MHC, MyoD, Myf5, myogenin, NCAM, α7 integrin, α5 integrin and *Pax7*) known to be related to myogenesis, and their respective protein levels by western blotting. Control and Lbx1-expressing C2C12 cells were induced to differentiate in differentiation medium for 5 days, then harvested and used for analysis as described in Materials and Methods. The results are shown in Fig. 6A,B. Western and northern blot analyses revealed that expression levels of MHC, myogenin, NCAM and α7 integrin were all upregulated, whereas *Pax7* and *Myf5* were downregulated according to the myogenic differentiation of control C2C12 cells, agreeing well with previous reports (Miller, 1990; Charlton et al., 2000; Yao et al., 1996; Kitzmann et al., 1998). By comparing the expressions of these genes in control and Lbx1-expressing C2C12 cells, we found that continuous expression of *Lbx1* affected the expressions of *Pax7* and MyoD. Expressions of other genes examined were not effectively influenced by expression of *Lbx1*.

Pax7, a paired-box transcription factor expressed specifically in undifferentiated satellite cells, was expressed in C2C12 cells in growth medium at a high level. This expression decreased during myogenic differentiation. By contrast, in Lbx1-expressing C2C12 cells, Pax7 expression increased when cells were induced to differentiate (Fig. 6A). To obtain more details of Pax7 expression in C2C12 cells, we estimated the ratio of Pax7-expressing cells to total cells by using anti-Pax7 antibody and Hoechst dye 33342. When cultured in C2C12 cell growth medium, the ratio of Pax7-expressing cells to the number of total cells was not significantly different between control and Lbx1-expressing cells. In control C2C12 cells, the percentage of Pax7-expressing cells was 26±5% and in Lbx1-expressing cells 28±5% (a ratio of approximately 1:1). When cultured in differentiation medium, the percentage of of Pax7-expressing cells was 24±6% in control C2C12 cells and 54±9% in Lbx1-expressing C2C12 cells (a ratio of approximately 1:2.25) (Fig. 6G). From these results, Lbx1 expression seemed to prevent downregulation of Pax7 during in vitro differentiation of C2C12 cells.

MyoD, a myogenic transcription factor, is one of the essential genes for myogenic differentiation and it is known that this gene is expressed in activated satellite cells (Cooper et al., 1999). As shown in Fig. 6B, MyoD is strongly expressed in control cells during myogenic differentiation, but considerably depressed by expression of *Lbx1* during differentiation. However, the percentage of MyoD-positive cells to total cells was not significantly different between

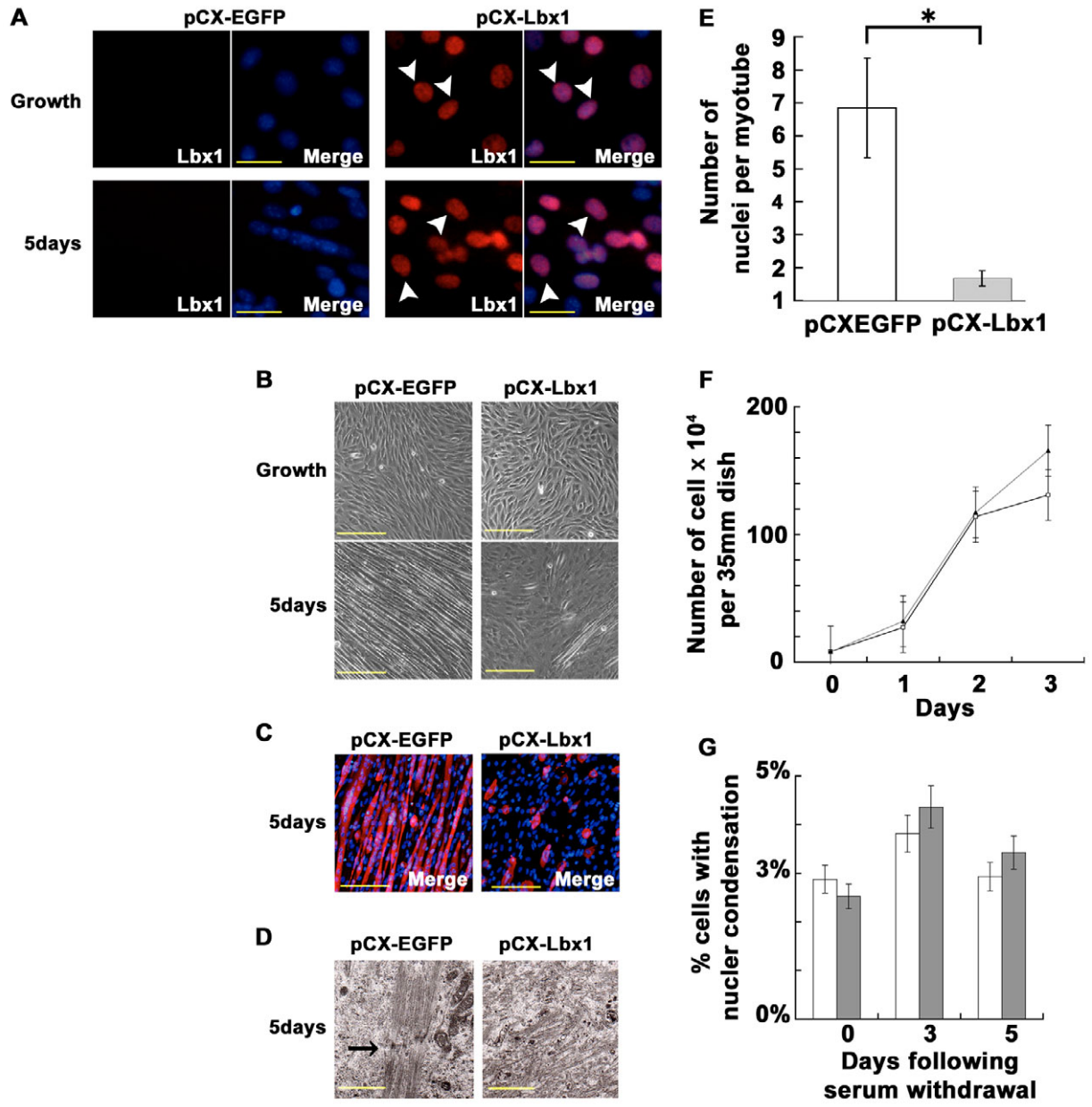

Fig. 5. Transient expression of Lbx1 in transfected C2C12 cells prevents myotube formation. (A) Immunochemical detection of Lbx1 protein in C2C12 cells. In control cells (pCX-EGFP), no Lbx1 can be detected. In cells transfected with the pCX-Lbx1 construct, Lbx1 is expressed in cells cultured in growth medium and 5 days after serum withdrawal (arrowheads). Bars, 25 μm. (B) Phase-contrast microscopy of Lbx1-expressing and control C2C12 cells. C2C12 cells were transfected with either pCXLbx1 or pCXEGFP (control). Both cells are morphologically indistinguishable when cultured in C2C12 growth medium (Upper panels). When induced to differentiate by serum withdrawal, Lbx1-expressing C2C12 cells exhibit abnormal myotube formation; numerous cells fail to fuse and remain as mononucleated cells. Even if myotubes are formed, they are extremely short, whereas control cells differentiated into myotubes after 5 days under serum-free conditions (Lower panels). Bars, 100 μm. (C) Immunochemical analysis. To observe details of forming myotubes, PCX-EGFP-transfected (control) and pCX-Lbx1-transfected cells were immunostained for myosin heavy chain (MHC, red) and counterstained with Hoechst dye 33342 (blue). Bars, 100 μm. (D) Ultrastructural analysis of myotubes formed in vitro. The sarcomeric organization with Z bands (arrow) is evident in pCX-EGFP-transfected cells. In C2C12 cells transiently expressing Lbx1 (pCX-Lbx1), muscle fibers do not contain a rudimentary sarcomeric organization with Z bands. Bars, 1 μm. (E) Number of nuclei of in-vitro-formed myotubes. At 120 hours after serum withdrawal, the average number of nuclei in MHC-positive myotubes was counted. The myotubes formed from control cells (pCX-EGFP) contain 6.9±1.5 nuclei (n=2002), whereas those from Lbx1-expressing C2C12 cells (pCX-Lbx1) contain only 1.7±0.2 nuclei (n=644). This difference was statistically significant as calculated using Student's t-test (P<0.05). (F) Results of cell counting. No significant differences were found in the numbers of cells of Lbx1-expressing C2C12 cells and control cells comparing 35-mm culture dishes. □, Lbx1-expressing cells; ▲, control cells. (G) Rate of cells with nuclear condensation. Lbx1-expressing C2C12 and mock-transfected control cells were stained with Hoechst dye 33342, and the number of cells with nuclear condensation was counted. The numbers of cells examined were as follows. White bars (Control): D0, n=3908; D3, n=9847; D5, n=10,763. Gray bars (Lbx1-transfected): D0, n=3501; D3, n=6180; D5, n=6005.

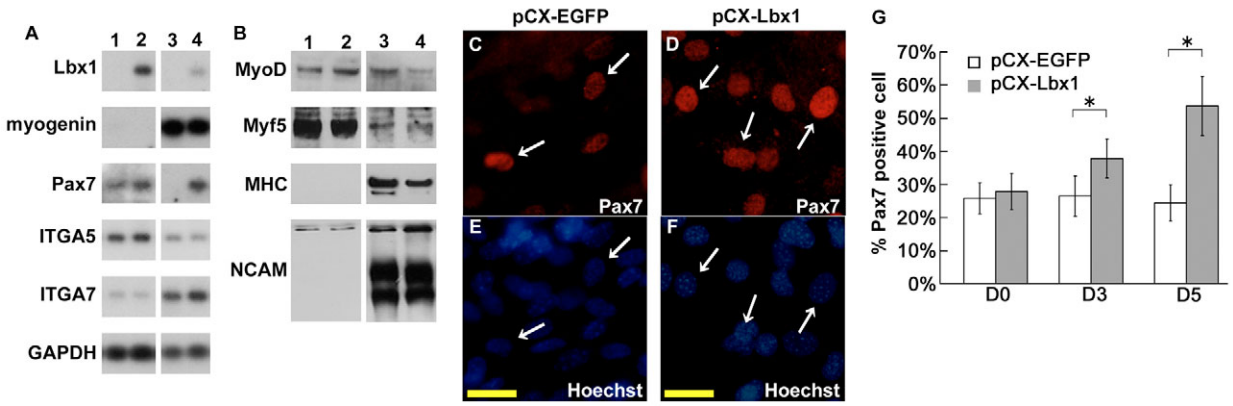

Fig. 6. Transient expression of Lbx1 in transfected C2C12 cells results in upregulation of Pax7 and downregulation of MyoD during myogenic differentiation. (A) Northern blot analysis. (Lanes 1, 2) mRNAs extracted from (lane 1) mock-transfected and (lane 2) Lbx1-expressing C2C12 cells cultured in growth medium. (Lanes 3, 4) mRNA extracted from (lane 3) mock-transfected and (lane 4) Lbx1-expressing differentiated cells grown in differentiation medium for 5 days. mRNAs were hybridized with ^{32}P-labeled probes against *Lbx1*, *Myog* (myogenin), *Pax7*, α5 integrin (*Itga5*), α7 integrin (*Itga7*) and *Gapdh*. Note that *Lbx1* expression is not detected in control cells, whereas it is expressed at a high level in Lbx1-expressing C2C12 cells. (B) Western blot analysis. (Lane 1, 2) Total proteins extracted from (lane 1) mock-transfected and (lane 2) Lbx1-expressing C2C12 cells cultured in growth medium. (Lanes 3, 4) Total proteins extracted from (lane 3) mock-transfected and (lane 4) Lbx1-expressing differentiated C2C12 cells by cultivating in differentiation medium for 5 days. Extracted proteins were electrophoresed and probed with antibodies against Myf5, MyoD, MHC and NCAM. MyoD is strongly expressed in control cells throughout differentiation (lanes 1, 3), but is downregulated in Lbx1-expressing cells during differentiation (lanes 2, 4). (C-F) Immunochemical analysis. Pax7-expressing cells were identified in differentiated C2C12 cells by immunostaining using the anti-Pax7 antibody (C,D). Cells were counterstained with Hoechst dye 33342 to identify nuclei (E,F). In mock-transfected control cells (pCX-EGFP), few Pax7-expressing cells were detected (arrows). By contrast, most of the Lbx1-expressing cells (pCX-Lbx1) express Pax7 strongly (arrows). Bars, 25 μm. (G) Pax7-positive cells (identified by immunostaining using the anti-Pax7 antibody) were counted during differentiation. D0, D3 and D5, day 0, 3 and 5 after serum withdrawal, respectively. White bars, mock-transfected C2C12 cells (control); gray bars, pCX-Lbx1-transfected C2C12 cells. Control: D0, n=3067; D3, n=8643; D5, n=10681. Lbx1 transfected: D0, n=2571; D3, n=5949; D5, n=4371. *P<0.05, statistically significant difference calculated using Student's t-test.

control and Lbx1-expressing cells (supplementary material Fig. S1).

Effect of Lbx1 knockdown on Pax7 expression in activated satellite cells

To confirm the effect of Lbx1 on Pax7 expression during myogenic differentiation in vitro, we conducted Lbx1 knockdown experiments by transfecting freshly isolated satellite cells with Lbx1 siRNA. As we were not be able to distinguish between the loss of Lbx1 occurring during normal differentiation or losses as a result of siRNA transfection, the ability of the siRNA to reduce Lbx1 was initially tested in C2C12 cells ectopically expressing Lbx1. Lbx1-expressing C2C12 cells were transfected with either Lbx1 siRNA or control siRNA. To successfully detect transfected cells, pCX-EGFP was co-transfected with each siRNA. As shown in Fig. 7A, Lbx1 expression in the C2C12 cells became undetectable or severely decreased by transfecting Lbx1 siRNA. Such

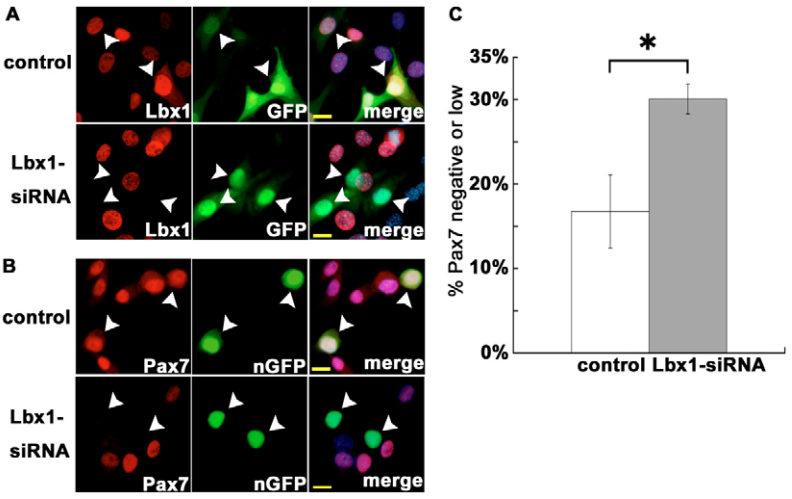

Fig. 7. Effect of siRNA targeting Lbx1 in primary satellite cells. (A) C2C12 cells transiently expressing Lbx1 were transfected either with siRNA targeting a specific region within *Lbx1* or control siRNA. pCX-EGFP was co-transfected with each siRNA. After 48 hours, the cells were immunostained with anti-Lbx1 antibody. Note that Lbx1 expression becomes undetectable in cells transfected with Lbx1 siRNA (arrowheads). Bars 10 μm. (B) Primary satellite cells were co-transfected with Lbx1 siRNA and pCX-nlsEGFP or control siRNA and pCX-nlsEGFP (control). After 24 hours, cells were immunostained using anti-Pax7 antibody. Pax7 could not be detected in cells transfected with Lbx1 siRNA, but was present in those transfected with control siRNA (arrowheads). Bars, 10 μm. (C) Effect of Pax7 knockdown in the satellite cells. Pax7-negative satellite cells were counted in cells used for B. Control (white bars), n=490; Lbx1-siRNA transfected (gray bars), n=444. *P<0.05, statistically significant difference calculated using Student's t-test.

suppression of Lbx1 expression was not observed by transfecting control siRNA. These data confirm that this siRNA is capable of reducing or eliminating Lbx1 protein expression.

Freshly isolated satellite cells were transfected with either Lbx1 siRNA or control siRNA. To detect successfully transfected cells, pCX-nlsEGFP was co-transfected with each siRNA. After 48 hours in culture, we immunostained the cells for Pax7 and counted those cells whose Pax7 expression was severely repressed (Fig. 7B). When Lbx1 siRNA was transfected, 30.1±1.8% of the GFP-positive cells were found to be Pax7 negative; whereas, 16.8±4.3% of the GFP-positive cells were Pax7 negative when control siRNA was transfected. This result suggested that knockdown of Lbx1 led to a downregulation of Pax7 in the satellite cells (Fig. 7C).

Discussion

Adult skeletal muscle exhibits a remarkable ability to regenerate in response to injury. This ability is mostly attributed to satellite cells (Schultz, 1996; Decary et al., 1997). Although the essential role of satellite cells in muscle regeneration has been well documented, molecular events occurring in the satellite cells are not yet fully understood; that is, apart from the crucial roles played by the myogenic regulatory factors (MRFs) in the activation and differentiation of satellite cells. Here, we demonstrated that Lbx1 is expressed in activated satellite cells. Our conclusion was based on the following observations. (1) Lbx1 protein could be detected immunohistochemically in regenerating muscle tissue after cardiotoxin injection, but was not detected in quiescent skeletal muscles of 8-week old mice. Absence of Lbx1 in the quiescent satellite cells was further confirmed by immunohistochemistry. (2) The expression of *Lbx1* was identified in primary cultured satellite cells isolated from single myofibers by northern blotting and immunohistochemistry. (3) The highest level of Lbx1 expression was observed in sphere-shaped satellite cells, which were presumed to be the activated satellite cells, because Pax7 (Fig. 3A), MyoD (Fig. 3B) and Myf5 (data not shown) were expressed in these cells at high level. Interestingly, we detected Lbx1 expression in satellite cells of all the type of muscles examined, including limb (EDL, soleus), diaphragm, tongue and trunk muscles. Trunk muscles are known to be derived from non-migratory muscle precursor cells that do not express Lbx1 during embryogenesis. However, the satellite cells attached to the trunk muscles were confirmed to express Lbx1 (Fig. 3E).

The strong expression of Lbx1 observed in activated satellite cells gradually decreased during their myogenic differentiation in vitro. In mouse embryos, Lbx1 is expressed specifically in migrating myoblasts derived from hypaxial myotomes (Brohmann et al., 2000). From analyses of *Lbx1*-null mice, Lbx1 expressed in migratory myoblasts has been suggested to regulate the genes involved in guiding the myoblasts to the proper regions. By analogy, it is tempting to speculate that Lbx1 expressed in activated satellite cells also plays a crucial role in guidance of the satellite cells during muscle regeneration. We also examined to ascertain whether α7 integrin, α5 integrin or β1 integrin, all known to be involved in myoblast migration, are also regulated by Lbx1 in satellite cells; however, a positive correlation of the expression of these genes with *Lbx1* expression was not detected in cultured satellite cells (Fig. 6A and data not shown). Thus, we currently have no data supporting this idea, although the possibility has not yet been excluded. To clarify this, production of satellite-cell-specific Lbx1 knockout mice may be necessary; indeed, this line of experimentation is now under investigation in our laboratory.

Embryonic myogenesis is regulated by MRFs, including MyoD, Myf5, myogenin and MRF4 (Sabourin and Rudnicki, 2000). MyoD and Myf5 are involved in the establishment of the skeletal muscle lineage (Rudnicki et al., 1993), whereas myogenin is required for terminal differentiation (Hasty et al., 1993; Nabeshima et al., 1993). Although quiescent satellite cells do not have any detectable levels of MRFs, they become activated in response to muscle injury and express MyoD as well as Myf5 (Cornelison and Wold, 1997; Cooper et al., 1999). Since MyoD-null mutant mice fail to repair damaged muscle, MyoD is apparently important for muscle regeneration (Megeney et al., 1996). Myogenin is expressed at a later stage, presumably associated with fusion and terminal differentiation (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994). Thus, the regulatory genes essential for muscle regeneration seem to be similar to those deployed in the embryo. In the present study, we have shown that ectopic expression of Lbx1 strongly prevents myogenic differentiation of C2C12 in vitro. Under low-serum conditions, control C2C12 cells could differentiate efficiently and fuse to each other to form myotubes. However, the majority of the C2C12 cells that ectopically express Lbx1 failed to fuse to form myotubes but remained as mononucleated cells. The myotubes that somehow formed were very short, with only a few myonuclei (Fig. 5C,E). Moreover, the assembly of sarcomeres of such myotubes was extremely disorganized and sarcomeric Z bands were not detected (Fig. 5D). These results strongly suggest some molecular lineage between *Lbx1* and myogenic regulatory genes, although satellite cells conducted myogenic differentiation mainly with rather low expression of *Lbx1* under natural conditions.

A number of genes have already been reported to be involved in regulating the various steps involved in muscle generation and regeneration (Knapp et al., 2006; Parker et al., 2003). We examined such genes to see whether their expressions were influenced by Lbx1 (Fig. 6A,B). As a result, we found that expressions of Pax7 and MyoD were influenced by ectopic expression of Lbx1. Control C2C12 cells in proliferating cultures expressed MyoD and Pax7, although Pax7 was heterogeneously expressed among the cultured cells. When induced to differentiate, MyoD was maintained at a high level but Pax7 decreased, in agreement with previously reported results (Seale et al., 2000; Miller, 1990; Sabourin et al., 1999). By contrast, when transfected C2C12 cells that expressed Lbx1 were induced to differentiate, the expression of MyoD was suppressed. The expression of Pax7 was maintained at a high level and the population of Pax7 expressing cells was increased (Fig. 6G). MyoD is known to be essential for satellite-cell differentiation (Sabourin et al., 1999; Cornelison et al., 2000); therefore, this suppression of MyoD might be the cause of the failure of Lbx1-expressing C2C12 cells to complete myogenic differentiation. However, the possibility that Lbx1 directly prevents satellite cells from myogenic differentiation has not been excluded.

A particularly noteworthy finding in this study is that, *Lbx1* expression in C2C12 cells leads to retention of a high level of *Pax7* expression (Fig. 6) and, *Lbx1*-knockdown in satellite cells results in decreased Pax7 expression (Fig. 7). Pax7, a member of the paired-box transcription factor family, has been regarded as a key player in muscle regeneration because it is specifically expressed in satellite cells, and satellite cells are absent in *Pax7*-mutant mice. Pax7 is expressed in quiescent satellite cells as well as in activated satellite cells where Pax7 and MyoD are co-expressed. Pax7 is then rapidly downregulated during terminal differentiation. Previous analyses of *Pax7*-null mice have implicated Pax7 in lineage determination, especially in the specification of myogenic progenitors to the satellite cell lineage (Seale et al., 2000). However, several lines of evidence indicate a role for Pax7 in facilitating satellite cell self-renewal and maintaining proliferation (Oustanina et al., 2004). In this study, we have shown that Lbx1 is expressed in activated satellite cells, but not in quiescent ones, which strongly suggests that Lbx1 plays a role in activated satellite cells. Because expression of *Lbx1* in C2C12 cells resulted in maintaining Pax7 at a high level and reducing MyoD expression, the most plausible speculation regarding the function of Lbx1 might be that Lbx1 maintains the proliferation of activated satellite cells before they are committed to terminal differentiation.

Recently, two reports have described that Lbx1 plays a crucial role in myogenic cell proliferation in chicken and in *Xenopus laevis* (Mennerich and Braun, 2001; Martin and Harland, 2006), shedding new light on the myogenic function of Lbx1. In chick embryos, ectopic expression of *Lbx1* strongly stimulates myogenesis, monitored by MyoD and myogenin expression in somites and limbs in vivo. These data indicate that a crucial function of chick Lbx1 is to enlarge the number of muscle cell, thereby increasing the bias for myogenic differentiation and enabling myogenic cells to respond to environmental cues (Mennerich and Braun, 2001). In *Xenopus laevis*, knockdown of *Lbx1* has been shown to cause a specific reduction of body-wall and hypoglossal muscles originating from somites. It has been claimed that a primary function of *Xenopus* Lbx1 in hypaxial muscle development is to repress MyoD, in contrast to chick Lbx1, which allows myoblasts to proliferate before the eventual onset of terminal differentiation (Martin and Harland, 2006). Thus, in both of these species, *Lbx1* expression results in an increase in the population of myogenic cells, although modes of action of Lbx1 appear to differ from each other. If our speculation that Lbx1 promotes proliferation of activated satellite cells before they are committed to terminal differentiation is found to be correct, we will have gained a much better understanding of the function of Lbx1 in myogenesis of vertebrates.

Materials and Methods

Mice

Institute of Cancer Research (ICR) mice were purchased from CLEA Japan. The Institutional Animal care and Use Committee of Kitasato University approved all experimental protocols. Care was taken to minimize the number of animals used, as well as any pain and suffering.

Cell line

The murine C2C12 myoblast cell line was purchased from American Type Culture Collection (Manassas, VA). The cell line was maintained in C2C12-cell growth medium, consisting of 90% Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). Cell were grown under a 10% CO_2 atmosphere at 37°C on tissue culture dishes coated with gelatin (Sigma). To induce differentiation, C2C12 cells were grown to 90% confluence and then cultivated in differentiation medium (98% DMEM and 2% horse serum).

Preparation of satellite cells and primary cultures

Satellite cells were prepared by a method that was essentially the same as that described by Hashimoto et al. (Hashimoto et al., 2004). Briefly, muscles of 3 or 12 week old ICR mice were digested with 2.5% collagenase type I (Sigma) in DMEM for approximately 1 hour to yield single intact fibers. This was followed by cultivating in satellite cell growth medium (80% DMEM and 20% FBS supplemented with 2 ng/ml basic fibroblast growth factor, 2.5% chick embryo extract and 100 U/ml mouse leukemia inhibitory factor) for 48 hours on matrigel-coated 24-well plates (Becton, Dickinson and Company). Sphere-shaped cells were selected, recovered and cultured at low density (between 1×10^5 and 2.5×10^5 cells in a collagen-I-coated 10-cm diameter culture dish). When these cells were grown at high density ($>5\times10^5$ cells per culture dish), they quickly differentiated into flat spindle-like cells. To induce further differentiation, flat spindle-like cells were cultured confluently in differentiation medium. Within 5 days in culture, they fused to each other and formed mature myotubes.

Plasmid construct and transfections

A 4.0 kbp *Apa*I-*Not*I mouse genomic fragment covering the complete Lbx1 open reading frame was subcloned with an *Apa*I-*Hin*dIII linker into the *Hin*dIII and *Not*I sites of a pBluescriptII-KS (Stratagene). Then, a 1.6-kbp *Sal*I-*Eco*RI fragment, containing the chicken β-actin promoter and the CMV-enhancer region of pCX-EGFP (kindly provided by M. Okabe, Osaka University, Japan) was inserted into the Lbx1-containing pBluescript vector. The resulting plasmid construct pCX-Lbx1 was co-transfected with pEGFP-C2 (Clontech) in C2C12 cells.

For transfection experiments, C2C12 cells were plated in C2C12 cell growth medium at a density of 3×10^4 cells per 35-mm plate. Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) and cultivation in C2C12 growth medium containing 2 mg/ml of the antibiotic G418 (Gibco).

RNA interference experiments

Freshly isolated satellite cells were transfected with the Stealth™ siRNA duplex oligoribonuleotides (Invitrogen) targeting Lbx1. The sequence of the Lbx1 siRNA used was 5′-UUGAGGAUGUCCUCGAUGCUGAACG-3′. To detect transfected cells, this siRNA was co-transfected with pCX-EGFP or pCX-nls-EGFP.

Cardiotoxin injection and tissue preparation

Cardiotoxin (0.1 ml of 10 mM solulution in PBS; Sigma) was injected directly into the right tibialis anterior (TA) muscle of 8-week-old ICR mice with a 27-gauge needle under ether anesthesia. The cardiotoxin-injected TA muscles (right) and non-injected contralateral TA muscles (left) of those mice were taken for analysis at 4 days after injection.

Immunohistochemical analysis

Primary antibodies used were rabbit anti-Lbx1 (kindly provided by Y. Ono, Kan Research Institute, Inc. Kyoto), mouse anti-Pax7 and mouse anti-myogenin (Developmental Studies Hybridoma Bank), mouse anti-MyoD (Pharmingen), goat anti-M-cadherin, rabbit anti-MyoD and rabbit anti-Myf5 (Santa Cruz), rabbit anti-laminin (Chemicon) and mouse anti-MHC (kindly provided by T. Obinata). Secondary antibodies used were Alexa-Fluor-568 goat anti-rabbit, Alexa-Fluor-488 goat anti-rabbit, Alexa-Fluor-568 goat anti-mouse and Alexa-Fluor-488 goat anti-mouse (Molecular Probes), and Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch). Primary antibodies were diluted 1:500 (rabbit anti-Lbx1, rabbit anti-MyoD, mouse anti-MyoD, rabbit anti-Myf5), 1:200 (mouse anti-MHC) or 1:5000 (mouse anti-Pax7, mouse anti-myogenin) with Can Get Signal solution 1 (Nakarai Tesque, Inc., Kyoto, Japan). Secondary antibodies were diluted 1:2000 with Can Get Signal solution 2, expect for Cy3-conjugated donkey anti-goat IgG (diluted with 0.3% Triton X-100 in PBS). Skeletal muscles, cryosections of those or cultured cells were fixed in 4% PFA for 10 minutes, incubated with primary antibodies for 1 hour, followed by incubation with secondary antibodies for 1 hour and then counterstained with Hoechst dye 33342. For co-immunostaining with anti-M-cadherin, fixed samples were incubated with primary antibodies for 1 hour followed by incubation with Cy3-conjugated donkey anti-goat IgG diluted with 0.3% Triton X-100 in PBS. Samples were then incubated with another secondary antibody diluted with Can Get Signal solution 2 and counterstained with Hoechst dye 33342. For co-immunostaining with anti-laminin antibody, we pre-incubated anti-laminin antibody with Alexa-Fluor-488-conjugated Fab fragment using Zenon™ rabbit IgG labeling kits (Molecular Probes).

RNA isolation and northern blot analyses

Total RNA was isolated from cell culture using RNeasy (Qiagen) followed by isolation of poly (A+) RNA using Dynabead oligonucleotides (dT) 25 (Dynal Biotech). mRNA samples (0.34 μg per lane) were electrophoresed using 1% agarose gel containing 1× MOPS, 7% formaldehyde and then transferred to

Hybond-XL membrane (Amersham) in 20×SSC (3 M NaCl, and 30 mM sodium citrate) overnight. Hybridization was carried out at 65°C in hybridization buffer. The cDNA probes were ^{32}P-labeled using the Megaprime DNA labeling system (Amersham). The 1-335 bp and 442-862 bp fragments of the *Lbx1* cDNA, the 388-695 bp fragment of myogenin cDNA, the 324-1801 bp and 2420-3627 bp fragments of *Pax7* cDNA, the 3318-3490 bp fragment of α5 integrin cDNA, and the 3056-3457 bp fragment of α7 integrin cDNA were used to generate ^{32}P-labeled probes. Integrity of the RNA and comparable amounts of RNA were tested by rehybridization using a probe against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot analyses

Protein extracts from C2C12 cells were resolved on NuPAGE 10% Tris-acetate gel (Invitrogen) and then electrophoretically transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal antibodies against MyoD (Santa Cruz) and NCAM (Chemicom), and mouse monoclonal antibody against MHC. Bound antibodies were detected with the following secondary antibodies, peroxidase-conjugated donkey anti-goat IgG, anti-mouse IgG, and anti-rabbit IgG (Jackson ImmunoResearch). Equal loading of protein extracts was assessed using Coomassie brilliant blue (CBB) staining.

Electron microscopy

Cells growing on 35-mm permanox dishes were fixed with 2.5% glutaraldehyde. Areas rich in folding intermediates were selected and marked on the dishes. Samples were dehydrated through a serial dilution of graded alcohol, embedded in Epon and sectioned with a diamond knife (Srikakulam and Winkelmann, 2004). Thin sections were stained in uranyl acetate and lead citrate, and examined by a JEOL JEM1010 electron microscope operated at 80 kV.

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References

Bischoff, R. and Heintz, C. (1994). Enhancement of skeletal muscle regeneration. *Dev. Dyn.* **201**, 41-54.

Brohmann, H., Jagla, K. and Birchmeier, C. (2000). The role of Lbx1in migration of muscle precursor cells. *Development* **127**, 437-445.

Charge, S. B. and Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* **84**, 209-238.

Charlton, C. A., Mohler, W. A. and Blau, H. M. (2000). Neural cell adhesion molecule (NCAM) and myoblast fusion. *Dev. Biol.* **221**, 112-119.

Cooper, R. N., Tajbakhsh, S., Mouly, V., Cossu, G., Buckingham, M. and Butler-Browne, G. S. (1999). In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. *J. Cell Sci.* **112**, 2895-2901.

Cornelison, D. D. and Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev. Biol.* **191**, 270-283.

Cornelison, D. D. W., Olwin, B. B., Rudnicki, M. A. and Wold, B. J. (2000). MyoD –/– satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Dev. Biol.* **224**, 122-137.

Decary, S., Mouly, V., Hamida, C. B., Sautet, A., Barbet, J. P. and Butler-Browne, G. S. (1997). Replicative potential and telomere length in human skeletal muscle: implications for satellite cell-mediated gene therapy. *Hum. Gene Ther.* **8**, 1429-1438.

Gros, J., Manceau, M., Thomé, V. and Marcelle, C. (2005). A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature* **435**, 954-958.

Gross, M. K., Moran-Rivard, L., Velasquez, T., Nakatsu, M. N., Jagla, K. and Goulding, M. (2000). Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. *Development* **127**, 413-424.

Hashimoto, N., Murase, T., Kondo, S., Okuda, A. and Inagawa-Ogashiwa, M. (2004). Muscle reconstitution by muscle satellite cell descendants with stem cell-like properties. *Development* **131**, 5481-5490.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506.

Ishido, M., Uda, M., Masuhara, M. and Kami, K. (2006). Alterations of M-cadherin, neural cell adhesion molecule and b-catenin expression in satellite cells during overload-induced skeletal muscle hypertrophy. *Acta Physiol.* **187**, 407-418.

Jagla, K., Dolle, P., Mattei, M. G., Jagla, T., Schuhbaur, B., Dretzen, G., Bellard, F. and Bellard, M. (1995). Mouse Lbx1 and human LBX1 define a novel mammalian homeobox gene family related to the *Drosophila* lady bird genes. *Mech. Dev.* **53**, 345-356.

Jagla, K., Frasch, M., Jagla, T., Dretzen, G., Bellard, F. and Bellard, M. (1997). ladybird,a new component of the cardiogenic pathway in *Drosophila* required for diversification of heart precursors. *Development* **124**, 3471-3479.

Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N. J. C. and Fernandez, A. (1998). The muscle regulatory factors MyoD and Myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J. Cell Biol.* **142**, 1447-1459.

Knapp, J. R., Davie, J. K., Myer, A., Meadows, E., Olson, E. N. and Klein, W. H. (2006). Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size. *Development* **133**, 601-610.

Martin, B. L. and Harland, R. M. (2006). A novel role for lbx1 in Xenopus hypaxial myogenesis. *Development* **133**, 195-208.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E. and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.* **10**, 1173-1183.

Mennerich, D. and Braun, T. (2001). Activation of myogenesis by the homeobox gene Lbx1 requires cell proliferation. *EMBO J.* **20**, 7174-7183.

Miller, J. M. (1990). Myogenic programs of mouse muscle cell lines: expression of myosin heavy chain isoforms, MyoD1, and myogenin. *J. Cell Biol.* **111**, 1149-1159.

Mizuhara, E., Nakatani, T., Minaki, Y., Sakamoto, Y. and Ono, Y. (2005). Corl1, a novel neuronal lineage-specific transcriptional corepressor for the homeodomain transcription factor Lbx1. *J. Biol. Chem.* **280**, 3645-3655.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532-535.

Ontell, M. and Kozeka, K. (1984). The organogenesis of murine striated muscle: a cytoarchitectural study. *Am. J. Anat.* **171**, 133-148.

Oustanina, S., Hause, G. and Braun, T. (2004). Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. *EMBO J.* **23**, 3430-3439.

Parker, M. H., Seale, P. and Rudnicki, M. A. (2003). Looking back to the embryo: defining transcriptional networks in adult myogenesis. *Nat. Rev. Genet.* **4**, 497-507.

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H. and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Sabourin, L. A. and Rudnicki, M. A. (2000). The molecular regulation of myogenesis. *Clin. Genet.* **57**, 16-25.

Sabourin, L. A., Girgis-Gabardo, A., Seale, P., Asakura, A. and Rudnicki, M. A. (1999). Reduced differentiation potential of primary MyoD –/– myogenic cells derived from adult skeletal muscle. *J. Cell Biol.* **144**, 631-643.

Schafer, K. and Braun, T. (1999). Early specification of limb muscle precursor cells by the homeobox gene Lbx1h. *Nat. Genet.* **23**, 213-216.

Schienda, J., Engleka, K. A., Jun, S., Hansen, M. S., Epstein, J. A., Tabin, C. J., Kunkel, L. M. and Kardon, G. (2006). Somitic origin of limb muscle satellite and side population cells. *Proc. Natl. Acad. Sci. USA* **103**, 945-950.

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. *Dev. Biol.* **175**, 84-94.

Schwander, M., Leu, M., Stumm, M., Dorchies, O. M., Ruegg, U. T., Schittny, J. and Muller, U. (2003). Integrins regulate myoblast fusion and sarcomere assembly. *Dev. Cell* **4**, 673-685.

Seale, P. and Rudnicki, M. A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev. Biol.* **218**, 115-124.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777-786.

Smith, T. H., Kachinsky, A. M. and Miller, J. B. (1994). Somite subdomains, muscle cell origins, and the four muscle regulatory factor proteins. *J. Cell Biol.* **127**, 95-105.

Srikakulam, R. and Winkelmann, D. A. (2004). Chaperone-mediated folding and assembly of myosin in striated muscle *J. Cell Sci.* **117**, 641-652.

Uchiyama, K., Ishikawa, A. and Hanaoka, K. (2000). Expression of Lbx1 involved in the hypaxial musculature formation of the mouse embryo. *J. Exp. Zool.* **286**, 270-279.

Yablonka-Reuveni, Z. and Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev. Biol.* **164**, 588-603.

Yao, C. C., Ziober, B. L., Sutherland, A. E., Mendrick, D. L. and Kramer, R. N. (1996). Laminins promote the locomotion of skeletal myoblasts via the alpha 7 integrin receptor. *J. Cell Sci.* **109**, 3139-3150.