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Activation of myogenesis by the homeobox gene *Lbx1* requires cell proliferation

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Myogenic differentiation can be initiated by a limited number of molecules. In this work, we analyzed the function of the homeobox gene *Lbx1* in chicken embryos and explant cultures. We demonstrate that overexpression of *Lbx1 in vivo* and *in vitro* leads to a strong activation of various muscle markers. We show that cell proliferation, which is strongly stimulated by *Lbx1* and *Pax3*, is required for *Lbx1*- or *Pax3*-dependent myogenic activation. Inhibition of cell proliferation prevents expression of muscle differentiation markers, while the activation of other putative downstream targets of *Pax3* and *Lbx1* is not affected. Our findings imply that a critical function of *Pax3* and *Lbx1* during muscle cell formation is the enlargement of muscle cell populations. The growth of the muscle precursor cell population may increase the bias for myogenic differentiation and thus enable myogenic cells to respond to environmental cues.

Keywords: homeobox gene/*Lbx1*/myogenesis/*Pax3*

Introduction

The majority of skeletal muscles are derived from transient structures of the mesoderm: the somites (Arnold and Braun, 2000). Somites can be subdivided into ventral and dorsal halves. Cells of the ventral half delaminate from the epithelial somite and form the sclerotome, which gives rise to the axial skeleton and ribs. The dorsal part of the somite remains epithelialized and forms the dermomyotome. Cells of the dermomyotome, which proliferate at a high rate, are the source of all epaxial and hypaxial muscle cells (Christ *et al*., 1977).

In contrast to the cells of the ventro-medial somite, where all the cells rapidly delaminate and are committed to the sclerotomal fate, muscle precursor cells are continuously recruited from the dorsal part of somite over many days. These muscle precursor cells are highly proliferative and responsive to permissive and instructive environmental cues. Although numerous studies have identified signals that emanate from the axial notochord, the neural tube (NT), the overlying lateral ectoderm and the lateral plate mesoderm (LPM), the exact nature and composition of these cues are still elusive.

A number of observations have been made that are not compatible with the view that instructive signals emanating from surrounding tissues at this relatively late stage of development determine the fate of muscle cells in the myotome and limb buds. Rather, they argue for a permissive role of signals that may act on cells that are already partially or completely committed to myogenesis before or during gastrulation. Such signals, which are generated during somitogenesis, might relieve the repression of muscle precursor cells imposed by surrounding tissues or cells (Cossu *et al*., 1996; George-Weinstein *et al*., 1996; Pourquie *et al*., 1996).

Another critical value in this context might be the control of cell proliferation and the resulting size of a potential 'colony' of muscle-forming cells. Gurdon has shown that a potential muscle cell must be within a group of cells of minimum size in order to respond to inductive signals or to differentiate autonomously. This cellular behavior, coined 'community effect' (Gurdon, 1988), was mainly based on transplantation experiments in *Xenopus* embryos where single cells failed to differentiate into muscle cells after transplantation, while groups of cells readily underwent myogenic differentiation. Interestingly, a few scattered Myf5-positive cells are present in NT, but normally do not undergo myogenic differentiation. However, when placed in culture, a small fraction of these cells can co-express muscle myosin heavy chain and neuronal β III tubulin within the same cell (Tajbakhsh *et al*., 1994).

Within the somite, two different myogenic cell populations have been identified by various means (Ordahl and Le Douarin, 1992). One cell population resides in the medial dermomyotome and generates epaxial muscles (such as intrinsic back muscles); the other originates from the lateral dermomyotome and produces hypaxial muscles of the body wall and limbs. *Pax3*, which is initially expressed throughout the entire paraxial mesoderm and later becomes restricted to the dermomyotome and hypaxial muscle precursor cells, is considered to be on top of the regulatory cascade leading to hypaxial muscle formation. Mutations in the *Pax3* locus in *splotch* mice result in an absence or reduction of all hypaxial muscles (Bober *et al*., 1994). Hypaxial muscle development is also impaired in mice lacking *Lbx1*. Targeted deletion of *Lbx1* leads to a failure of muscle precursor cells of the hindlimb and of extensor muscle precursor cells of the forelimb to migrate to their targets (Schäfer and Braun, 1999). In *splotch* mice, no *Lbx1* expression is detectable in somite or limb buds (Mennerich *et al*., 1998), while *Pax3* expression is normal in the remaining hypaxial muscle cells of *Lbx1* mutant mice.

Beside its role in hypaxial muscle development, *Pax3* appears to have a general function in muscle development. Generation of *Pax3–Myf5* double-mutant mice yielded mice without body muscles (Tajbakhsh *et al*., 1997), and ectopic expression of *Pax3* activates *MyoD* and *Myf5* in embryonic mesoderm and neural tissue (Maroto *et al*., 1997). These results imply that *Pax3* directly activates

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MyoD expression, while *Myf5* is located in a different myogenic pathway. However, alternative explanations are feasible and a detailed model for this hypothesis is required.

In this work, we investigate the function of the transcription factors *Lbx1* and *Pax3* during myogenic differentiation in chicken embryos and explant cultures. We demonstrate that ectopic expression of Lbx1 *in ovo* leads to a strong activation of muscle cell formation in somites and limbs, but not in other ectopic locations. Ectopic expression of *Lbx1* in explant cultures derived from several tissues induces various muscle cell markers. We show that cell proliferation, which is strongly stimulated both by *Lbx1* and *Pax3*, is required for *Lbx1*- or *Pax3*-dependent myogenic activation. Inhibition of cell proliferation abrogates activation of the myogenic program, while the regulatory feedback loop initiated by *Pax3* and *Lbx1* is still active. Our results might provide an explanation of how *Pax3* and *Lbx1* induce myogenesis by amplification of the myogenic precursor cell pool with an increasing potential for myogenic differentiation, and emphasize the critical role of the size of cell populations biased for differentiation.

Results

Ectopic expression of Lbx1 enhances the expression of the myogenic factors MyoD and myogenin

To analyze the effects of *Lbx1* on myogenic differentiation, a RCAS(A)-based *Lbx1* retrovirus was injected into somites I–IV or VI–X of HH10 chicken embryos. Three days after retroviral infection, at HH25, embryos were fixed and subjected to whole-mount *in situ* hybridization (Figure 1). The contralateral uninfected half of the embryo served as a negative control. As expected, the retrovirus generated a high level of expression of *Lbx1* mRNA. No differences in expression levels of ectopic *Lbx1* were discernible within infected tissues (red staining in Figure 1A–F). Likewise, a control virus encoding human alkaline phosphatase (hAP) yielded homogenously infected tissue, as indicated by staining for the enzymatic activity of the ectopically expressed hAP enzyme (red staining in Figure 1I–L).

At the same time, myogenesis was evaluated in injected embryos by monitoring expression of *MyoD* and *myogenin* using two-color whole-mount *in situ* hybridization (dark stainings in Figure 1). Infection of paraxial mesoderm at varying axial positions led to high level expression of *MyoD* (Figure 1A–D) and myogenin (Figure 1E–H). Interestingly, enhanced expression was restricted to the segmented mesoderm. The segmental upregulation was detected for both *MyoD* (Figure 1A–D) and *myogenin* (Figure 1E–H). No significant activation of expression was found outside the somites, although the areas of enhanced expression did not completely co-localize with the endogenous expression pattern of these genes. This is particulary evident in Figure 1D and H, where *MyoD* and *myogenin* expression is present in the dermomyotome, which does not normally express either of these molecules. No up-regulation of *MyoD* or *myogenin* was observed in areas without forced expression of *Lbx1*.

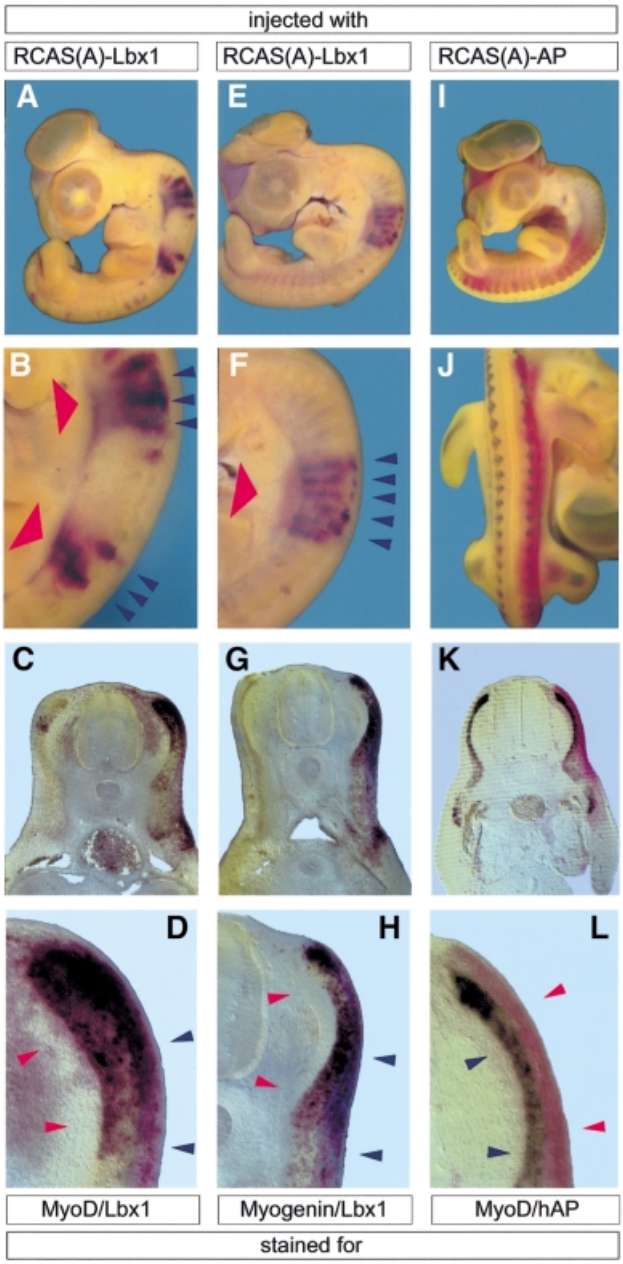

Fig. 1. Forced expression of Lbx1 stimulates myogenesis in the paraxial segmented mesoderm *in vivo*. HH25 chicken embryos were subjected to whole-mount *in situ* hybridization after injection of RCAS(A)–Lbx1 (**A**–**H**) and RCAS(A)–hAP (**I**–**L**) into somites I–IV or VI–X of HH10 embryos and 3 days of incubation. Depending on the injection procedure, which involved several injections per embryo, either two separate sets (A and B), a single block of somites (E and F) or most somites of one half (I and J) were infected. Two-color whole-mount *in situ* hybridization of *MyoD* (dark purple staining in A–D) and *Lbx1* (red staining in A–H) and of *myogenin* (dark purple staining in E–H) and *Lbx1*. Embryos in I–L were hybridized with a *MyoD* antisense probe (dark purple staining) and stained for hAP activity (red staining). Whole-mount preparations (A, B, E, F, I and J) and vibratome sections (C, D, G, H, K and L) are shown. Forced expression of *Lbx1* (indicated by bold red arrowheads) resulted in a strong up-regulation of *MyoD* (A–D) and myogenin (E–H) in somites (blue arrowheads in B, D, F and H), while the expression of hAP did not result in an up-regulation of *MyoD* (I–L) and other myogenic markers. A shorter staining time in RCAS(A)–Lbx1-injected embryos allowed only the detection of stimulated *MyoD* and *myogenin* expression, while uninjected halves showed only a very weak *MyoD* (C) and myogenin (G) signal.

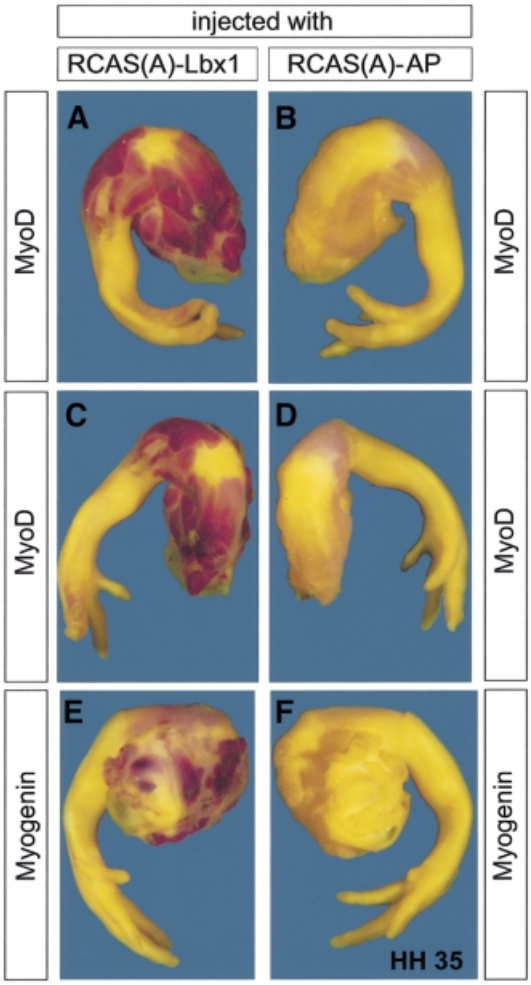

Fig. 2. Forced expression of *Lbx1* stimulates myogenesis in the leg *in vivo*. HH35 chicken embryo wings were subjected to whole-mount *in situ* hybridization after injection of RCAS(A)–Lbx1 (**A**, **C** and **E**) and RCAS(A)–hAP (**B**, **D** and **F**) into leg buds of HH24 embryos and 4 days of incubation. Whole-mount *in situ* hybridizations with *MyoD* (red staining in A–D) and *myogenin* probes are shown (E and F). Forced expression of *Lbx1* resulted in a strong up-regulation of *MyoD* (A and C) and *myogenin* (E) in presumptive muscles of the legs, while the expression of *hAP* did not result in an up-regulation of *MyoD* (B and D) and *myogenin* (F). Staining reactions for *MyoD* and *myogenin* were allowed to develop longer in RCAS(A)–hAP-injected embryos (B, D and F) to allow visualization of unstimulated *MyoD* and *myogenin* in the legs.

In contrast, virus-mediated expression of *hAP* did not lead to up-regulation of *MyoD* (Figure 1I–L) or myogenin (not shown) in the myotome or ectopic expression in the dermomyotome. Only the normal expression pattern and signal strength of *MyoD* within the myotome were visible (Figure 1I–L).

Lbx1 is mainly expressed in hypaxial muscle precursor cells that give rise to limb muscles. We therefore injected the Lbx1 retrovirus into leg buds of HH24 embryos and stained for an enhanced or ectopic expression of *myogenin* and *MyoD* at HH35 4 days later using whole-mount *in situ* hybridization. Although the size and density of the leg tissue prevented a complete penetration of the probe, we detected a striking up-regulation of *MyoD* (Figure 2A and C) and *myogenin* (Figure 2E) in prospective muscle tissue throughout the legs. No ectopic expression of *MyoD* and

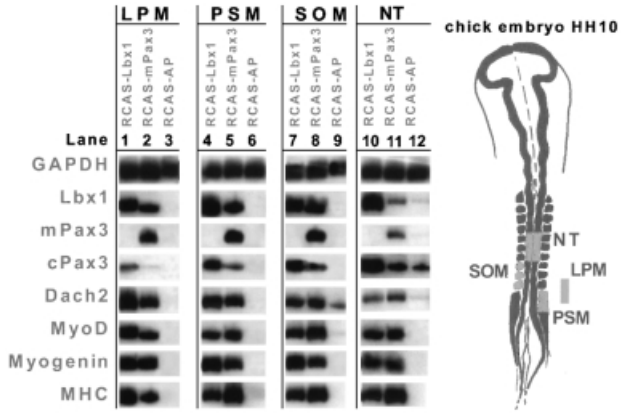

Fig. 3. Forced *Lbx1* and *Pax3* expression induces myogenesis in various parts of the mesoderm and NT. Tissues [LPM, lanes 1–3; PSM, lanes 4–6; somites (SOM), lanes 7–9; NT, lanes 10–12] were dissected from HH10 embryos (indicated by the yellow color), separated from surrounding tissues and cultured in collagen gels. RNA was isolated 5 days after infection with RCAS(A)–Lbx1 (lanes 1, 4, 7 and 10), RCAS(A)–Pax3 (lanes 2, 5, 8 and 11) and RCAS(A)–hAP (lanes 3, 6, 9 and 12), and analyzed by RT–PCR using various primer pairs. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MHC, embryonic myosin heavy chain. Exogenous expression of *Pax3* was monitored using primers specific for the mouse cDNA (mPAX3). Both *Lbx1* and *Pax3*, but not the control virus, were able to induce myogenic markers including MHC. Low level endogenous *Dach2* expression was detectable in isolated SOM (lane 9), but strongly up-regulated by *Lbx1* (lane 7) and *Pax3* (lane 8).

myogenin was found in epidermis and cartilage in these tissues. Similar to the situation in somites, we did not observe an enhancement of *MyoD* and *myogenin* expression when a RCAS(A)–hAP retrovirus (Figure 2B, D and F) or Pax3-encoding retrovirus was used (data not shown).

While the activation of ectopic and enhanced expression of myogenic factors was consistently observed in a large number of injected embryos (n = 42), we were never able to obtain similar effects with a Pax3-expressing retrovirus (n = 49). This inability of *Pax3* to ectopically induce myogenesis *in ovo* in the context of an intact embryo is in full agreement with previous reports by Maroto *et al*. (1997) and Heanue *et al*. (1999).

Expression of Lbx1 or Pax3 activates myogenesis in embryonic mesoderm in the absence of inducing tissues and in neural tube explants

In vivo inducing and repressing signals act together to ensure that initiation of myogenesis occurs at a defined location within the embryo. In order to analyze whether forced expression of *Lbx1* and *Pax3* activated myogenesis in mesodermal fragments cultured in the absence of inducing or repressing tissues, we isolated three different mesodermal regions [paraxial pre-segmented mesoderm (PSM), lateral plate mesoderm (LPM) and somites] from chicken embryos at HH10 (Figure 3). Explants were infected with retroviruses encoding *Lbx1*, *Pax3* or *hAP*, cultured in a collagen matrix for 5 days and analyzed by RT–PCR for expression of the myogenic markers *MyoD*, *myogenin* and *embMyHC*. Infection of all three types of mesodermal tissues with RCAS–mPax3 led to high level expression of *MyoD*, *myogenin* and *embMyHC* (Maroto *et al*., 1997) as well as to activation of *Dach2* (Heanue *et al*., 1999) and *Lbx1* mRNA (Figure 3, lanes 2, 5 and 8).

In contrast, a control infection with RCAS–hAP did not result in an up-regulation of any of these markers (Figure 3, lanes 3, 6 and 9). Uninfected and mock-infected somites, which were cultured in the absence of muscle-inducing tissues, lost expression of *Pax3* within 12 h and failed to undergo myogenic differentiation (Figure 3). Likewise, expression of *Pax3* in uninfected and mock-infected PSM was not sustained in the absence of muscle-inducing tissues and myogenesis did not occur (Figure 3).

Infection of somites, PSM and LPM with RCAS–Lbx1 resulted in a strong activation of the myogenic markers *MyoD*, *myogenin* and *embMyHC* as well as of *Pax3* and *Dach2* (Figure 3, lanes 1, 4 and 7). It is interesting to note that the forced expression of Lbx1 yielded a robust up-regulation of *Pax3* in all three mesodermal tissue fragments despite the fact that *Pax3* is genetically upstream of *Lbx1* (Mennerich *et al*., 1998).

It has been reasoned that either the lack of a co-factor or the presence of a repressor might be responsible for the lack of myogenesis in the neural tube, which can be overcome by forced expression of *Pax3* (Maroto *et al*., 1997). To test whether Lbx1 is capable of generating signals sufficient to alleviate the repression of muscle cell formation in this tissue, fragments of NT, isolated at the level of somites IV–VI from HH10 embryos, were infected with RCAS–Lbx1, RCAS–Pax3 and RCAS–hAP. Analysis of myogenic regulators such as *MyoD*, *myogenin* and *Dach2*, as well as terminal differentiation markers such as *embMyHC*, revealed a strong initiation of myogenesis (Figure 3, lanes 10 and 11). Owing to the endogenous expression of *Lbx1*, *Pax3* and *Dach2* mRNAs in NT, which were not lost in explanted NT fragments as in embryonic mesoderm separated from surrounding tissues, basal levels of these molecules were found in RCAS–hAP and uninfected tissue (Figure 3, lane 12). *Dach2* was up-regulated above the basal level by RCAS–Lbx1 and RCAS–Pax3. *Pax3* expression was virtually unaffected by its own forced expression, but strongly induced by RCAS–Lbx1. Likewise, *Lbx1* was induced by *Pax3* in NT.

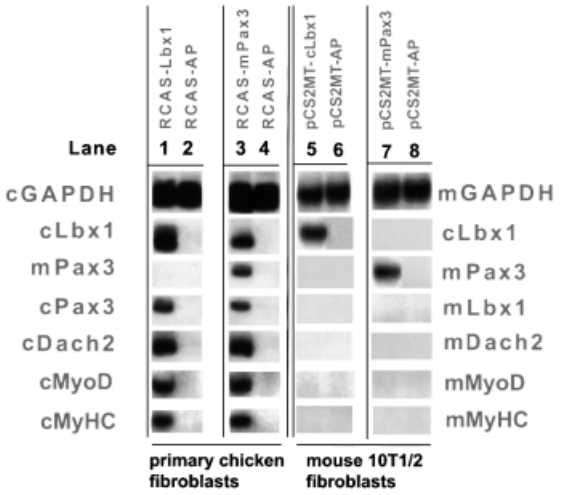

Fig. 4. Activation of myogenesis by forced expression of *Lbx1* and *Pax3* depends on the cell type. *Lbx1* (lanes 1 and 5), hAP (lanes 2, 4, 6 and 8) and *Pax3* (lanes 3 and 7) were expressed in primary chicken cells (lanes 1–4) and 10T1/2 mouse fibroblasts (lanes 5–8) using either a RCAS-based retrovirus (lanes 1–4) or a CMV promoter-based expression construct (lanes 5–8). After 3 days in differentiation medium, RNA was isolated and RT–PCR analysis was performed using primers specific for the chicken and the mouse mRNAs, respectively. Components of the *Pax3–Lbx1–Dach2* regulatory loop as well as *MyoD* and *MyHC* were readily activated in primary chicken cells by *Lbx1* (lane 1) and *Pax3* (lane 3). In contrast, forced expression of *Lbx1* or *Pax3* did not result in activation of *Pax3*, *Dach2* and endogenous *Lbx1* or of *MyoD* and *MyHC* in 10T1/2 cells (lane 5) despite a strong expression of exogenous *cLbx1* or *mPax3* in these cells.

Lbx1, Pax3 and Dach2 participate in a positive regulatory feedback loop to activate the myogenic differentiation program

The partially overlapping expression pattern of *Lbx1*, *Pax3* and *Dach2* and the analysis of *Pax3* and *Lbx1* mutant mice suggested that these molecules might act in a common regulatory pathway (Bober *et al*., 1994; Mennerich *et al*., 1998; Heanue *et al*., 1999; Schäfer and Braun, 1999). In addition, it has been shown that *Pax3* and *Dach2* positively regulate each other in tissue culture explants (Heanue *et al*., 1999). To analyze whether Pax3, Lbx1 and Dach2 participate in a positive regulatory loop in various cellular backgrounds, we expressed Lbx1 and *Pax3* in chicken dermal fibroblasts and murine 10T1/2 fibroblasts. Infection of chicken dermal fibroblasts with RCAS–Lbx1 induced expression of *Pax3*, *Dach2*, *MyoD* and *MyHC*. In contrast, transfection of murine 10T1/2 fibroblasts with an *Lbx1* expression plasmid did not result in enhanced expression of *Pax3*, *Dach2*, *MyoD* and *MyHC* (Figure 4, lanes 1 and 5). Similarly, *Lbx1*, *Dach2*, *MyoD*, *MyHC* and the endogenous *Pax3* gene were induced after infection of chicken dermal fibroblasts with RCAS–Pax3, while expression of *Pax3* in murine 10T1/2 fibroblasts by a cytomegalovirus (CMV) promotor-driven expression plasmid did not generate a detectable expression level of these genes (Figure 4, lanes 3 and 7). Similar expression levels of the ectopically expressed genes were reached in chicken dermal fibroblasts and 10T1/2 fibroblasts irrespective of the use of viral or plasmid-based expression systems (Figure 4, lanes 3 and 7), ruling out the possibility that the different cellular response is due to a different concentration of exogenous *Lbx1* and *Pax3* within the cells. As expected, infection of dermal fibroblasts with a control RCAS–hAP retrovirus did not initiate myogenesis (Figure 4, lanes 2 and 4).

Use of the murine *Pax3* cDNA allowed us to detect an up-regulation of the endogenous *cPax3* gene. Since *Pax3* also induces *Lbx1* and *Dach2*, it remains unclear whether *Pax3* acts in an auto-regulatory loop to stimulate its own expression or whether *Lbx1* and *Dach2* mediate this activation.

In conclusion, our experiments demonstrate that a positive regulatory feedback loop operates between *Lbx1*, *Pax3* and *Dach2* in certain cell types, such as primary dermal fibroblasts, while other cells like 10T1/2 fibroblasts are refractory to this regulatory loop.

Lbx1 and Pax3 enhance cell proliferation in embryonic mesoderm and NT explants

Lbx1 and *Pax3* may directly regulate the transcription of myogenic regulatory genes, thereby inducing myogenesis. Alternatively, the role of *Lbx1* and *Pax3* in muscle cell formation might be more complicated, involving regulation of cell proliferation and generation of a commit-

ment for myogenesis. To gain insight into the cellular mechanisms initiated by *Lbx1* and *Pax3*, we investigated whether one or both genes may directly stimulate cell proliferation.

Tissue fragments derived from NT, unsegmented PSM, LPM and somites were infected with RCAS–Lbx1, RCAS–Pax3 and RCAS–hAP. The rate of cell proliferation within explants was determined after 5 days using an antibody against proliferating cell nuclear antigen (PCNA) that marks cells undergoing mitosis. Forced expression of *Lbx1* and *Pax3* generated a strong increase in the rate of cell proliferation compared with the RCAS–hAP control virus in all tissues tested (Figure 5A). The strongest increase was observed in explanted somites infected with RCAS–Pax3 (n = 5) and RCAS–Lbx1 (n = 8), where the ratio of proliferating cells compared with RCAS(A)–hAP and uninfected controls was raised 18-fold for *Lbx1* and 22-fold for *Pax3*. In other tissues, the increase was less dramatic but still significant; in NT explants, *Pax3* increased the proliferation rate 10.5-fold and *Lbx1* 5-fold. In LPM, the situation was nearly reversed; a 9-fold increase in the proliferation rate was detected after infection with RCAS(A)–Lbx1 and a 5-fold increase after infection with RCAS(A)–Pax3. The weakest increase was found in PSM, with an ~4-fold increase for both *Lbx1* and *Pax3*. Although *Pax3* generated the strongest response in somites, *Lbx1* is not necessarily less effective at stimulating cell proliferation, as clearly indicated by infection of the LPM, which repetitively gave stronger responses for *Lbx1* (n = 8) than for *Pax3* (n = 9). We also investigated the number of proliferating cells at various time points after infection with RCAS–Lbx1. As shown in Figure 5B, the number of proliferating cells was relatively high immediately after explantation (day 0). After infection with RCAS–Lbx1 and RCAS–hAP at day 0, the number initially fell (day 1). In cultures infected with the RCAS–hAP control virus, the reduction of proliferating cells continued during the course of the experiment. In contrast, we observed a strong increase in proliferating cells at day 3 and day 5 in cultures infected with RCAS–Lbx1. The number of proliferating cells clearly exceeded the amount of dividing cells that was initially present in explants, suggesting that *Lbx1* recruits resting cells into the cell cycle. Although we did not trace the cells, which were induced to divide, the parallel increase in the expression of myogenic markers in infected cultures allows the conclusion that *Lbx1* and *Pax3* may increase the pool of muscle precursor cells and at the same time preserve or even improve their potential for myogenic differentiation.

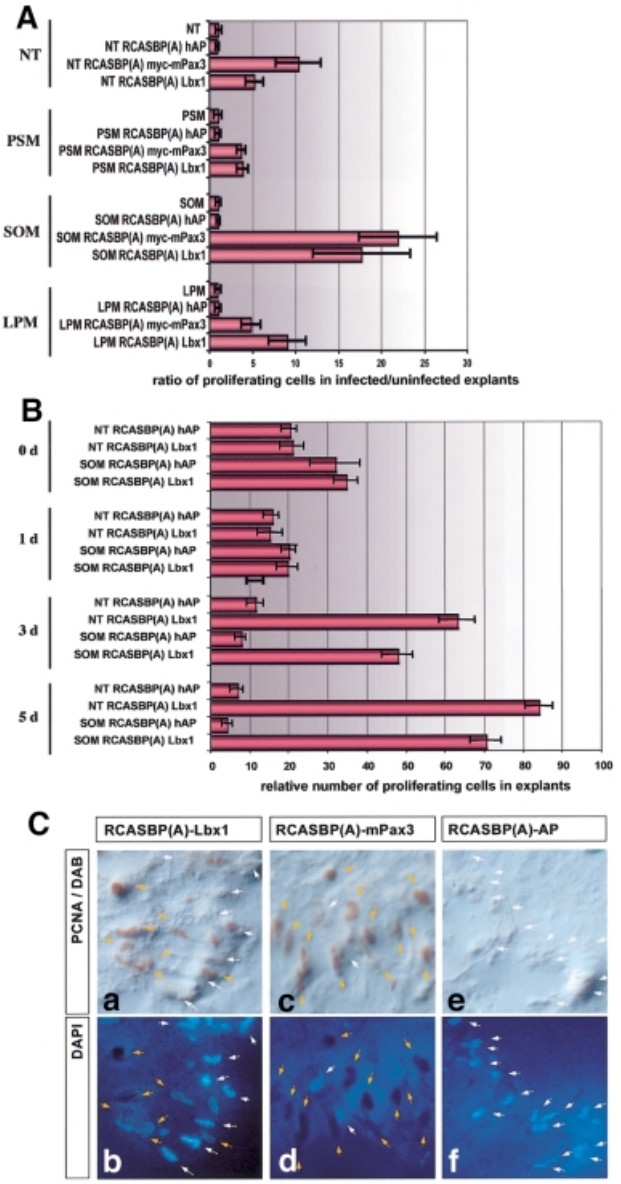

Fig. 5. Forced expression of *Lbx1* and *Pax3* strongly stimulates cell proliferation in embryonic mesoderm and NT explants. (**A**) Tissue fragments derived from NT (columns 1–4), PSM (columns 5–8), SOM (columns 9–12) and LPM (columns 13–16) were infected with RCAS–Lbx1, RCAS–Pax3 and RCAS–hAP viruses. Proliferating cells were identified by staining for PCNA antigen. Values on the *x*-axis indicate *x*-fold induction of the number of proliferating cells in infected versus uninfected explants. (**B**) Tissue fragments derived from SOM or NT were infected with either RCAS–hAP or RCAS–Lbx1 and analyzed for the presence of proliferating cells at different time points after explantation. Stimulation of proliferation by *Lbx1* became apparent 3 days after infection and exceeded the relatively high proliferation rate in cultures without *Lbx1* at day 0. Values on the *x*-axis indicate the number of proliferating cells relative to the absolute number of cells in explants. (**C**) Representative example for *Lbx1*- and *Pax3*-induced cell proliferation. SOM explants were immunostained with a PCNA antibody (a, c and e) 5 days after infection with RCAS–Lbx1 (a and b), RCAS–Pax3 (c and d) and RCAS–hAP (e and f) viruses. Nuclear DNA was visualized by DAPI staining (b, d and f). Yellow arrows mark PCNA-positive cells; white arrows mark non-proliferating cells. In explants infected with RCAS–hAP, very few proliferating cells were seen, and in RCAS–Lbx1 and RCAS–Pax3 infected explants numerous PCNA-positive, proliferating cells were detectable.

Cell proliferation is required for Lbx1- and Pax3-mediated activation of myogenesis but not for Dach2 activation

If the hypothesis is correct that a pivotal function of *Lbx1* and *Pax3* in the initiation of myogenesis is the increase in the pool of muscle precursor cells by stimulation of cell division, inhibition of cell proliferation should result in a repression of myogenesis. To test this postulate, somites I–IV from HH10 embryos were treated with mitomycin C for 6 h, 2 days after infection with RCAS(A)–Lbx1, RCAS(A)–Pax3 or RCAS(A)–hAP.

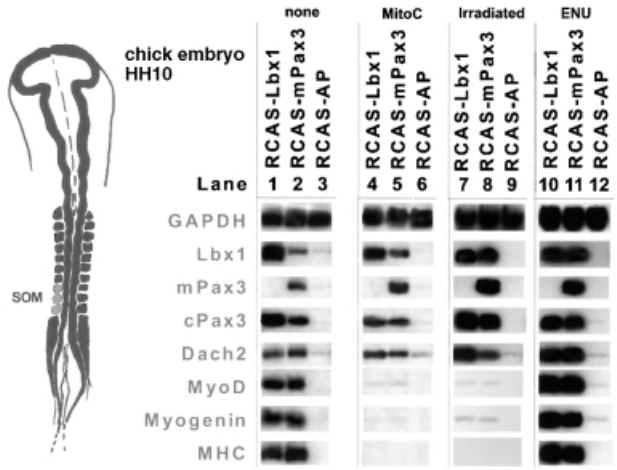

Fig. 6. Cell proliferation is required for *Lbx1*- and *Pax3*-mediated activation of myogenesis but not for *Dach2* activation. Somites were dissected from HH10 embryos, separated from surrounding tissues and cultured in collagen gels. Tissues were infected with RCAS(A)–Lbx1 (lanes 1, 4, 7 and 10), RCAS(A)–Pax3 (lanes 2, 5, 8 and 11) and RCAS(A)–hAP (lanes 3, 6, 9 and 12) and cultured for 2 days. One set of tissues was left without additives (lanes 1–3), the other set was treated with mitomycin C (lanes 4–6) or irradiated (lanes 7–9) to inhibit cell proliferation. Another set of tissues was incubated with ENU (lanes 10–12). Four days after treatment, tissue explants were analyzed by RT–PCR. In cultures treated with mitomycin C or subjected to irradiation, expression of *Lbx1* (lanes 4 and 7) and *Pax3* (lanes 5 and 8) did not result in an up-regulation of *MyoD*, *myogenin* and *MyHC*, while treatment with ENU did not affect activation of myogenic markers (lanes 10 and 11).

Four days later, tissue explants were fixed and stained with an antibody against PCNA to confirm the lack of dividing cells. We next monitored changes in the expression of myogenic markers and the *Pax3–Lbx1–Dach2* regulatory loop by RT–PCR and immunohistochemical staining (Figure 6 and data not shown). Apparently, inhibition of cell proliferation resulted in a virtually complete lack of activation of the myogenic markers *MyoD*, *myogenin* and *myosin heavy chain* (Figure 6, lanes 4–6) despite a strong retrovirus-mediated expression of *Lbx1* and *Pax3*. In contrast, the mutual stimulation of *Lbx1* and *Pax3* expression and the up-regulation of *Dach2* mRNA occurred as in the control experiments in the absence of mitomycin C (Figure 6, lanes 1–3). In some experiments, a very low activation of myogenic differentiation markers was observed, correlating with the onset of mitomycin C treatment (Figure 6, lanes 4–6 and data not shown). This might be due to the fact that it was necessary to perform mitomycin C treatment 1–2 days after infection since efficient expression by RCAS-based retroviruses requires cell proliferation. To further confirm that *Lbx1*- and *Pax3*-induced cell proliferation is critical for activation of myogenic differentiation, tissue culture explants were irradiated with 50 Gy (5000 rads) 2 days after viral infection. This dose was sufficient to abrogate any cell divisions in explant cultures since no bromodeoxyuridine (BrdU)-incorporating or PCNA-positive cell was detectable after irradiation (data not shown). Similar to mitomycin C treatment, irradiation prevented the activation of myogenic markers by *Lbx1* and *Pax3* (Figure 6, lanes 7 and 8), but did not prohibit the mutual up-regulation of *Lbx1* and *Pax3* and the stimulation of *Dach2* expression. It is feasible that treatment of explants with high doses of mitomycin C and radiation beyond the block of cell proliferation compromise cellular responses to an extent that activation of myogenic markers cannot occur anymore. Although this appears unlikely since the mutual up-regulation of *Lbx1* and *Pax3* and the stimulation of *Dach2* expression occurred readily in treated and untreated cultures, we incubated explants with 0.25 mg/ml *N*-ethyl-*N*-nitrosourea (ENU) for 2 h. ENU is a potent mutagen, which has been demonstrated to generate high mutation rates in various types of cells (Chen *et al*., 2000). As shown in Figure 6, lanes 10 and 11, activation of myogenic markers in response to *Lbx1* and *Pax3* and in the presence of ENU occurred similarly to experiments without the drug, thus excluding the possibility that any cellular stress would block activation of myogenic markers in this type of experiment.

The requirement of cell proliferation for Lbx1- and Pax3-induced myogenesis cannot be overcome by tissues inducing myogenesis

We reasoned that the failure of somitic explants to initiate myogenesis in the absence of further cell proliferation might be overcome by signals from neighboring tissues normally inducing myogenesis. Although it has been shown previously that a main input from adjacent tissues is the activation and/or maintenance of *Pax3* expression (Maroto *et al*., 1997), it could not be excluded that the activation of other pathways contributes to the activation of myogenesis.

In order to include possible effects of signals from NT and the notochord in our analysis, we explanted somites I–IV from HH10 embryos together with these tissues and treated the explants with mitomycin C as before. As shown in Figure 7, myogenesis was not rescued in the presence of notochord and NT. None of the myogenic markers *MyoD*, *myogenin* and *myosin heavy chain* (Figure 7, lanes 3 and 4) was induced by RCAS(A)–Lbx1 and RCAS(A)–Pax3 despite a considerable up-regulation of *Lbx1* and *Pax3*. Owing to the expression of *Pax3*, *Lbx1* and *Dach2* in NT, the basal levels of these markers were higher in the explants. It appears likely that this fact obscured the detection of a possible up-regulation of *Dach2* in somites.

To rule out the possibility that the treatment of NT with mitomycin C affected its ability to induce myogenesis in the paraxial mesoderm, a number of control experiments were performed. PSM was dissected from HH10 chicken embryos and cultured alone or in combination with the NT–notochord complex (NT–NC). The tissues were harvested after 3 days and subjected to RT–PCR analysis. As described previously, myogenic marker molecules were induced only in the presence of NT–NC (Figure 7, lanes 5 and 6; Münsterberg *et al*., 1995; Maroto *et al*., 1997). When the PSM was combined with NT–NC that had been previously treated with mitomycin C or irradiated at 50 Gy (5000 rads), essentially the same results were obtained (Figure 7, lanes 7 and 8), demonstrating that cell cycle arrest did not affect the power of NT–NC to induce myogenesis in the PSM.

In conclusion, our experiments demonstrate that activation of the *Pax3–Lbx1–Dach2* regulatory loop is not sufficient to initiate myogenesis, but requires an enlarge-

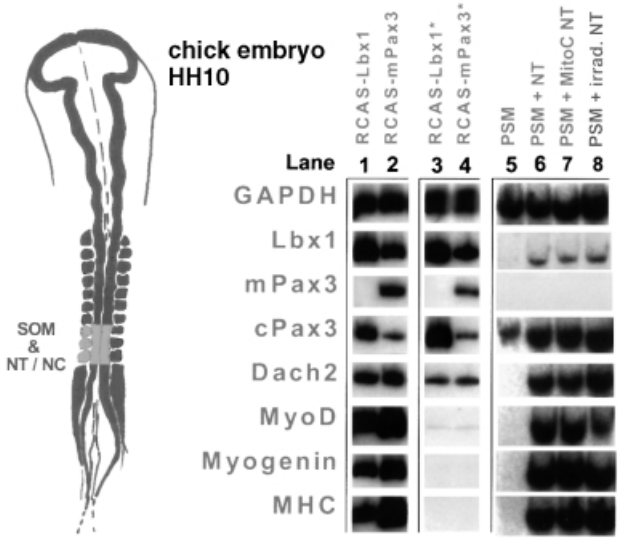

Fig. 7. The requirement of cell proliferation for *Lbx1–Pax3*-induced myogenesis cannot be overcome by tissues inducing myogenesis. Somites were dissected from HH10 embryos together with a segment of the adjacent NT and the notochord, and cultured in collagen gels. Tissues were infected with RCAS(A)–Lbx1 (lanes 1 and 3) and RCAS(A)–Pax3 (lanes 2 and 4) and cultured for 2 days. One set of tissues was left without additives (lanes 1 and 2), while the other set was treated with mitomycin C to inhibit cell proliferation (lanes 3 and 4). Four days after treatment, tissue explants were analyzed by RT–PCR. Similar to the experiment without muscle-inducing tissues, expression of *Lbx1* (lane 3) and *Pax3* (lane 4) did not result in an up-regulation of *MyoD*, *myogenin* and *MyHC*. To ensure that treatment of the NT–NC with mitomycin C or irradiation did not affect its ability to induce myogenesis in the PSM, the PSM was either cultured alone (lane 5) or with NT–NC that was not treated (lane 6), treated with mitomycin C (lane 7) or subjected to irradiation (lane 8). Myogenic marker molecules were induced in the PSM by the NT–NC (lanes 6–8), irrespective of a cell proliferation block in the NT–NC.

ment of the cell pool fated to become muscle. The requirement for this enlargement cannot be substituted for by signals from other tissues or by a combination of the *Pax3–Lbx1–Dach2* regulatory loop and signals released by the notochord and NT.

Discussion

In this work, we used a gain-of-function approach in chicken embryos to assess the role of the homeobox transcription factor *Lbx1* in initiation of myogenesis.

Lbx1 genetically acts upstream of myogenic factors and activates myogenesis in a cell type-dependent manner

The ability of muscle precursor cells to respond to environmental cues and to express mitogenic regulatory factors (MRFs) depends on numerous preceding decisions and the condition of a cell. *Pax3* has been demonstrated to positively influence the capability of mesodermal cells for myogenic differentiation, although its mode of action is enigmatic (Maroto *et al*., 1997; Tajbakhsh *et al*., 1997). In this study, we have shown that *Lbx1* has the same or even better potential than *Pax3* to activate myogenesis. From our data, we conclude that *Lbx1* is an important factor that might prepare and program muscle precursor cells to initiate myogenesis. In this regard, *Lbx1* might substitute for *Pax3* on a number of occasions. The ability of *Lbx1* to induce myogenesis is cell type dependent, another property shared by *Pax3*. Expression of *Lbx1* in 10T1/2 fibroblasts failed to induce myogenesis, while *Lbx1* activated the myogenic program in primary chicken dermal fibroblasts as well as in NT explants and tissue fragments derived from the mesoderm. *In vivo*, the capacity of *Lbx1* to activate the myogenic program is restricted, which is an interesting difference to the widespread activation of myogenesis *in vitro*.

Since forced expression of *Lbx1* always induced *Pax3*, and vice versa, it cannot be excluded that both genes are necessary for the activation of myogenesis or that their respective targets mediate the effect of *Lbx1* or *Pax3*. However, several arguments make the latter assumption more likely: (i) the activation of myogenic markers occurs more or less at the same time as the activation of *Pax3* or *Lbx1*; (ii) in *Lbx1* and in *Pax3* mutant mice, the ability of muscle precursor cells to respond to *Pax3* and *Lbx1* is not lost; (iii) *Lbx1* is expressed in *Pax3*-negative *Mox2* mutant limb muscle precursor cells and apparently fulfills its role in hypaxial muscle formation independent of *Pax3*; and (iv) *Lbx1* but not *Pax3* can stimulate myogenesis in somites of intact embryos. Therefore, it appears more likely that both genes funnel into a common pathway, which seems to include *Dach2*, *Eya2* and *Six1* (Heanue *et al*., 1999).

It is tempting to speculate that primary chicken cells contain co-factors not present in 10T1/2 cells that are necessary for *Lbx1*-dependent activation of myogenesis but dispensable for the myogenic conversion of 10T1/2 cells by MRFs. It will be interesting to analyze whether the *Dach2–Eya2–Six1* pathway, which might be instrumental for the effects of *Lbx1* and *Pax3*, also operates in 10T1/2 cells.

Genetically, *Pax3* is upstream of *Lbx1* since *Pax3* is expressed well before *Lbx1* expression starts and the expression of *Lbx1* is lost in somites of *Pax3* mutant mice (Mennerich *et al*., 1998). In addition, in *Lbx1* mutant mice, Pax3 can still be found in the remaining limb muscle precursor cell population. At first glance, it is therefore surprising that *Lbx1* induces *Pax3* even in the absence of cell proliferation. However, as outlined above, *Pax3* seems to be necessary only for the initiation of *Lbx1* expression but not for its maintenance. *Lbx1*, on the other hand, might be one of the factors by which *Pax3* maintains its own expression via a positive regulatory feedback loop.

The regulation of cell proliferation by Lbx1 and Pax3 might be a key step in the control of myogenesis

The mechanism by which *Lbx1* and *Pax3* induce myogenesis in some cell types is still poorly understood. Considering the role of *Pax3*, different models have been discussed that are based on either the absence or presence of positive or negative regulatory co-factors, which may determine whether a particular tissue is prone to muscle cell formation or not. Lassar and colleagues suggested the existence of negative regulatory co-factors to explain the capability of *Pax3* to activate myogenic factors in primary chicken cells but not in murine cells that are otherwise rather permissive for myogenesis (Maroto *et al*., 1997).

The molecular control of cell proliferation has been largely neglected in this context, although it is well known that the control of the proliferation rate might decisively contribute to the control of myogenesis. The rate of cell proliferation decides whether a given cell population reaches a critical size that is capable of creating its own micro-environment, which, in turn, may support further proliferation and finally differentiation. In addition, continuous cell divisions may dilute negative co-regulators, thus enabling cells to differentiate. Under certain conditions, proliferation appears to be linked to a lasting suppression of differentiation, while other circumstances that promote cell division simultaneously keep, or probably even increase, the propensity of a cell to undergo myogenic specification. *Lbx1* and *Pax3* seem to fall in the second category.

We have shown that forced expression of *Lbx1* and *Pax3* stimulates proliferation of recipient cells, and that cell proliferation is a prerequisite for myogenesis, but not for the activation of the *Pax3–Lbx1–Dach2* pathway. The time course of the appearance of proliferating cells in explants infected with RCAS–Lbx1 suggests that *Lbx1* induces proliferation of quiescent cells. It cannot be ruled out, however, that *Lbx1* maintains proliferation of cycling cells, but the initial decrease of proliferation followed by a sharp increase suggests that *Lbx1* is able to recruit resting cells into mitosis.

We postulate that enhanced proliferation enlarges the muscle precursor cell pool and thereby augments its tendency for myogenic differentiation. Enlarging cell populations might become increasingly independent from repressing signals, such as BMP4, which originate from the ectoderm and keep hypaxial muscle precursor cells in an undifferentiated state (Pourquie *et al*., 1996; Amthor *et al*., 1998).

Our findings that *Lbx1* and *Pax3* stimulate cell proliferation in chicken embryo explants are in line with previous reports that murine *Pax* genes can promote oncogenesis in tissue culture cells and in mice (Maulbecker and Gruss, 1993). In addition, Sauvageau *et al*. (1995) have shown that overexpression of another homeobox-containing gene, *HOXB4*, in hematopoietic cells causes the selective expansion of more primitive populations *in vitro* and *in vivo*. In a different approach, it has been demonstrated that loss of *Pax3* is accompanied by a marked increase in programmed cell death (Borycki *et al*., 1999). Likewise, simultaneous inactivation of *Pax3* and *Pax7* leads to a strong reduction in the number of cells in somites and to virtually complete loss of myogenic differentiation in the myotome. Since apoptosis is a characteristic response of cells deprived of their appropriate stimuli, it is conceivable that the absence of the activation of cell proliferation leads to increased or even excessive programmed cell death, as in the case of *Pax3* or *Pax3–Pax7* double mutants. Hence, the lack of growth stimulation might be the cause for the increase in apoptosis.

A modified view on the community effect

The community effect has been described by Gurdon in *Xenopus* embryos (Gurdon, 1988). Single cells derived from a region of the gastrula fated to give rise to muscle and placed in an ectopic location fail to differentiate into muscle, whereas a group of cells will readily do so. Similarly, paraxial mesoderm cells isolated from the limb and somites of developing mouse embryos require a minimum number of cells to differentiate into muscle (Cossu *et al*., 1995). It has been speculated that the community effect might be based on autocrine or paracrine effects mediated by unstable or slowly diffusing substances or by cell–cell contacts. The concentration at which such molecules reach a certain threshold might be much higher in solid aggregates or clusters of cells than in single or dispersed cells.

Cell autonomous factors that act along this line must therefore meet two criteria: (i) able to stimulate cell proliferation to increase the size of the cell cluster; and (ii) able to support and maintain the myogenic potential of these cells. *Lbx1* and *Pax3* appear to fulfill both requirements, and might therefore induce myogenesis by creating a muscle precursor cell pool that is sufficient for initiation of the community effect.

This concept adapts previous findings that dissociated cells derived from the mesoderm or the chicken epiblast have an intrinsic tendency to form muscle (George-Weinstein *et al*., 1996). Dissociated mesodermal cells kept in culture will proliferate and form clusters of identical or related cells that differentiate spontaneously, following a similar fate as enforced by exogenous expression of Lbx1 and Pax3.

It is evident that such a mechanism is dependent on a strong intrinsic myogenic potential of mesoderm. Several observations, particularly in lower vertebrates, have suggested that myogenesis might be a default program of the mesoderm. For example, in *Xenopus*, *MyoD* transcripts are found in the oocyte and zygotic expression starts ubiquitously at mid-blastula transition (Harvey, 1990; Rupp and Weintraub, 1991). Later, during development, *MyoD* transcripts become restricted to muscle-forming regions, suggesting an onset of repression in prospective non-muscle cells and a lack of repression in future muscle cells. Finally, in the chordate amphioxus, the dermal segments, analogs of somites in vertebrates, lack the dermomyotome and the sclerotome, and completely differentiate into muscle. The dominance of the myogenic differentiation pathway is emphasized by the presence of sarcomeric proteins in mid-line cells of the notochord (Holland *et al*., 1995).

Refinement of the genetic hierachy that controls skeletal myogenesis

Numerous components that control myogenesis have been inactivated in the mouse by targeted mutation. How do our results fit into the genetic hierarchy defined by analysis of these loss-of-function mutants?

One of the principal defects of *Lbx1* mutants is the failure of muscle precursor cells to migrate into their corresponding muscle anlagen (Schäfer and Braun, 1999). Expression analysis demonstrated that dislocated β-Gal$^+$ cells initially expressed *Pax3* and *c-Met*, although this ectopic expression faded quickly (Schäfer and Braun, 1999). No significant expression of the myogenic markers *Myf5* and *MyoD* was found in ectopically located β-Gal$^+$ cells, despite the ability of the remaining muscle precursor cells of forelimbs to switch on expression of *Myf5* and *MyoD*. These findings are in line with a role of *Lbx1* in the

maintenance of *Pax3* expression and the activation of myogenic bHLH genes in a subset of muscle precursor cells.

Myf5–Pax3 double mutants lack most of the muscles of the trunk and do not activate *MyoD* in the body. This dramatic phenotype is not seen in individual mutants. Therefore, it has been postulated that the expression of *MyoD* is dependent on both *Myf5* and *Pax3*, and that these genes can mutually rescue each other (Tajbakhsh *et al*., 1997). Our results may provide an alternative explanation of how *Pax3* might control myogenesis. We have shown that the stimulation of cell proliferation by *Pax3*, which leads to the amplification of myogenic progenitor cells, is required for the activation of *MyoD* and for myogenesis. In the absence of *Pax3*, *Myf5* may exert a similar function, i.e. expand the population of muscle progenitor cells. In contrast to *MyoD*, which probably inhibits cell proliferation by activation of *p21*, *Myf5* does not appear to restrict cell proliferation. *MyoD*$^{-/-}$ myoblasts continue to proliferate even in the presence of a 4-fold higher expression of *Myf5* under conditions that normally induce differentiation (Sabourin *et al*., 1999). Hence, *MyoD*-expressing cells may be dependent on an additional factor that promotes cell proliferation. *Pax3* (and in limb muscle, *Lbx1*) seems to be a good candidate for supplying such a function. In the absence of both *Pax3* and *Myf5*, expansion of the myogenic lineage will not occur, resulting in a virtually complete absence of body muscles. This view is different from direct activation of the *MyoD* gene by *Pax3* and focuses on the amplification of cells that subsequently express *MyoD*. Our interpretation is supported by results obtained in *Pax3–Pax7* double-mutant mice, which show a strong reduction in the number of cells in the dermomyotome and the myotome, and a severe impairment of myogenic differentiation (A.Mansouri and P.Gruss, personal communication).

Materials and methods

Plasmids and retroviral vectors

Generation of viral constructs and production of high titer virus stocks were performed as described previously (Morgan and Fekete, 1996). Chick *Lbx1* cDNA encoding the entire open reading frame was cloned by PCR, introducing an optimized translational initiation sequence and *Cla*I restriction sites. The retroviral construct encoding mouse *Pax3* was a kind gift from A.Lassar (Maroto *et al*., 1997). RCAS(A)–hAP contained a cDNA of placental hAP. Retroviral titers ranged from 7×10^7 to 2×10^9 c.f.u./ml.

Virus injections and explant cultures

Injections of virus solutions *in ovo* were performed essentially as described by Morgan and Fekete (1996). Embryos were injected several times in separate somites, giving rise to distinct virus infection patterns. For explant cultures, embryonic mesoderm and neural tissues were isolated and cultured in collagen matrix as outlined by Münsterberg *et al*. (1995). Retroviral infections of explanted tissues were performed as described (Maroto *et al*., 1997). Cultures were incubated for 6 days and analysed by RT–PCR or immunohistochemistry.

In some experiments, explanted tissues were incubated for 6 h with 10 μg/ml mitomycin C 2 days after retroviral infection. Cultures treated this way were washed three times and incubated for another 4 days. For irradiation, explanted tissues were subjected to a dose of 50 Gy (5000 rads) from a cobalt source and cultured as above. Treatment with ENU was for 2 h in tissue culture medium supplemented with 0.25 mg/ml ENU, analogous to the mitomycin C incubation. Co-cultures of PSM with NT–NC were performed as described (Münsterberg *et al*., 1995) either with untreated NT–NC or with NT–NC that had previously been treated with mitomycin C or irradiated. To achieve BrdU incorporation, explants were incubated for several hours in 15 ng/ml BrDU in cell tissue culture medium 2 days after retroviral infection.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed according to the protocol of Wilkinson (1992), using appropriate cRNA probes. The retroviral Lbx1 transcripts were stained with an anti-fluorescein antibody coupled to peroxidase using Fast Red (Sigma) as a substrate. For simultaneous staining of MyoD or myogenin, the anti-fluorescein antibody was used together with an anti-digoxygenin antibody coupled to hAP and NBT–BCIP as a substrate, following the double-staining protocol developed by Hauptmann and Gerster (1994).

Analysis of cell proliferation

Proliferating cells in explanted tissues were determined by immunohistochemical stainings with antibodies against PCNA (Dakopads, Hamburg) or BrDU (Roche Biochemicals) as described (Krüger *et al*., 2001). To visualize bound antibodies, a biotinylated anti-mouse IgG secondary antibody was used with diaminobenzidine as substrate. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to reveal the absolute number of cells. To determine the number of proliferating cells, 48–72 viewing fields originating from 12 different explants were counted for each type of explanted tissue at each time point. Viewing fields were selected on a random basis. The total number of cells in each viewing field was usually between 90 and 120. The absolute number of proliferating cells per viewing field ranged between 2 and 95. Each viewing field was normalized for the total number of cells in the field. Induction of cellular proliferation is depicted as the ratio of proliferating cells between infected and uninfected control explants. The time course of proliferating cells in explanted tissues is shown as the ratio between proliferating cells and the total cell count.

RT–PCR

RT–PCR analysis was performed according to Krüger *et al*. (2001). Information about primers and conditions can be obtained from the authors.

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