Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells

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The growth and repair of skeletal muscle after birth depends on satellite cells that are characterized by the expression of Pax7. We show that Pax3, the parologue of Pax7, is also present in both quiescent and activated satellite cells in many skeletal muscles. Dominant-negative forms of both Pax3 and -7 repress MyoD, but do not interfere with the expression of the other myogenic determination factor, Myf5, which, together with Pax3/7, regulates the myogenic differentiation of these cells. In Pax7 mutants, satellite cells are progressively lost in both Pax3-expressing and -nonexpressing muscles. We show that this is caused by satellite cell death, with effects on the cell cycle. Manipulation of the dominant-negative forms of these factors in satellite cell cultures demonstrates that Pax3 cannot replace the antiapoptotic function of Pax7. These findings underline the importance of cell survival in controlling the stem cell populations of adult tissues and demonstrate a role for upstream factors in this context.

Introduction

Pax genes play key roles during development. Members of this family of paired box/homeodomain transcription factors regulate the contribution of progenitor cells to different tissue types (Tremblay and Gruss, 1994). Pax3 and its parologue Pax7 have been implicated in the specification of cells that will enter the myogenic program. In the absence of both Pax3 and -7, there is a major deficit in skeletal muscle, with arrest of myogenesis occurring during later embryonic and fetal development (Relaix et al., 2005). Cells in which the genes are activated become incorporated into other tissues or die in the double mutants. Normally, Pax3/7-positive skeletal muscle progenitor cells, which are derived from the central dermomyotome region of the somites (Ben-Yair and Kalcheim, 2005; Gros et al., 2005), activate the myogenic regulatory genes and differentiate into skeletal muscle fibers or remain as a proliferating reserve cell population within the muscle mass (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). In late-stage fetal muscle, these cells begin to adopt a satellite cell position (Kassar-Duchossoy et al., 2005; Relaix et al., 2005), suggesting that this somite-derived population also provides the progenitor cells of postnatal skeletal muscle (Gros et al., 2005). In these cells, the expression of Myf5 and MyoD results in muscle cell determination. During the formation of early embryonic skeletal muscle in the somite, Myf5 and Mrf4 play a critical role in myogenic progenitors, which at this stage are derived from the edges of the dermomyotome (Braun et al., 1992; Tajbakhsh et al., 1996; Kassar-Duchossoy et al., 2004). Pax7 is not expressed in these cells in the mouse, where Pax3 is present. Early myogenesis occurs in the Pax3 mutant; however, in a triple Pax3\Myf5\Mrf4 mutant no skeletal muscle forms and MyoD, which is required for skeletal muscle determination in the absence of Myf5 and Mrf4, is not expressed (Tajbakhsh et al., 1997). Therefore, Pax3, together with Myf5/Mrf4, regulates the activation of MyoD. Consistent with this conclusion, MyoD is up-regulated in embryos in which PAX3-FKHR, which acts as a strong transcriptional activator, has been targeted to an allele of Pax3 (Relaix et al., 2003). Pax3 is essential for the survival of cells at the edges of the dermomyotome, particularly to those located hypaxially, where it is also required for the delamination and migration of muscle progenitor cells to other sites where skeletal muscle will form, such as the limbs (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994). When the coding sequence is targeted to the Pax3 gene, Pax7 can substitute for Pax3 function in the trunk, but not in the...
Figure 1.  
**Pax3 expression in muscle satellite cells.** (A–C) Expression of Pax3 in different muscles from 3-wk-old Pax3<sup>Δβ-gal</sup>/<sup>−/−</sup> mice, revealed by X-Gal staining. (A) Diaphragm muscle; (B) hindlimb muscles; (C) trunk muscles, (d) serratus dorsalis caudalis. (D) Semiquantitative RT-PCR of Pax3 and -7 transcripts in adult tibialis anterior (TA) and diaphragm muscle. The number of cycles is indicated on the top of each lane. M, molecular weight markers (see Materials and methods). (D') Pax3 protein is detected by Western blot in protein extracts from different muscles (D, diaphragm; L, hindlimb; T, ventral trunk muscles) in 3-wk-old mice. Tubulin (Tub) expression is shown as a loading control. (E and F) Pax3<sup>Δβ-gal</sup>/<sup>−/−</sup> is expressed in a subset of diaphragm muscle nuclei (arrows) from 3-wk-old mice, as revealed by X-Gal and DAPI staining of a transverse section (E) or isolated fiber (F). (G and G') Detection of Pax3/7-dependent β-gal expression in transverse sections of adult diaphragm muscle from the transgenic line P34, which reports Pax3/7 transcriptional activity. (G) Immunodetection of laminin (red) and DAPI (blue) staining. (G') Coimmunodetection of laminin (red) and β-galactosidase (β-gal, green). As indicated in the figure, β-gal is shown in red in H, and in green in I and J. (H–I) Coimmunodetection of β-gal–positive cells in the diaphragm muscle of 3-wk-old Pax3<sup>Δβ-gal</sup>/<sup>−/−</sup> mice with laminin (H', green), CD34 (I', red), or M-cadherin (J', red). (K) Coimmunodetection of laminin (red) and DAPI (blue) staining. (L–N) Coimmunohistochemistry on diaphragm muscle from 3-wk-old Pax3<sup>Δβ-gal</sup>/<sup>−/−</sup> mice for Pax7 (red) or β-gal (green). (L–N') Coimmunodetection of Pax3 (red) and laminin (green, K'). Corresponding DAPI staining is shown, with the labeled nucleus indicated by arrows for each panel. Arrow indicates the labeled satellite cell nuclei. (K) Coimmunodetection of Pax3 (red) and laminin (green, K'). Corresponding DAPI staining is shown, with the labeled nucleus indicated by an arrow (K). (L–N') Coimmunohistochemistry on diaphragm muscle from 3-wk-old Pax3<sup>Δβ-gal</sup>/<sup>−/−</sup> mice for Pax7 (red) or β-gal (green). (L–N) Corresponding DAPI staining is presented with labeled nuclei indicated by arrows for each panel.

**Results**

**Pax3 expression in the satellite cells of adult skeletal muscle**

Because Pax3 plays a key role during the onset of skeletal myogenesis in the embryo, we investigated its status in adult muscle in relation to Pax7. Analysis of adult mice in which the Pax3 gene is targeted with nLacZ reporters (Relaix et al., 2003) revealed the presence of β-galactosidase (β-gal)–positive cells in adult skeletal muscle. The number of such cells varies between muscles. They are particularly abundant in the diaphragm.
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(Fig. 1 A), but are much less frequent in hindlimb muscles, with the exception of the gracilis muscle (Fig. 1 B). In contrast, ~50% of forelimb muscles express Pax3. As in the embryo (Relaix et al., 2004), expression of Pax3 is not detectable in head muscles. Most ventral trunk muscles are positive, with a striking juxtaposition in the rib area, where intercostal muscles are mainly negative, whereas body wall muscles such as the serratus caudalis dorsalis are positive (Fig. 1 C). This difference is confirmed by semiquantitative RT-PCR analysis of Pax3 versus -7 transcripts in the tibialis anterior hindlimb muscle compared with the diaphragm (Fig. 1 D). The Pax3 protein is also present, as shown by Western blot analysis of different muscles (Fig. 1 D'). The Pax3-lacZ-expressing cells are found to be associated with muscle fibers (Fig. 1 E and F). The Pax3/7 protein is transcriptionally active in adult muscle, as indicated by activation of the P34 reporter transgene in which Pax3/7 binding sites regulate nLacZ expression (Fig. 1, G and G'; Relaix et al., 2004). These Pax3-expressing nuclei are present in satellite cells, as shown by immunolocalization of β-gal with the satellite cell markers CD34 and M-cadherin and by the inclusion of β-gal–positive cells within the basal lamina of the muscle fiber (Fig. 1, H–K'). Because Pax3 is present in satellite cells (Seale et al., 2000), the question of Pax3 expression in relation to Pax7 was addressed. In the diaphragm, the majority of Pax7-positive satellite cells also coexpress Pax3. About 15% of the cells only label with Pax7, and, occasionally, cells that express only Pax3 are also detected (Fig. 1 L–N'). We therefore conclude that Pax3, like Pax7, is expressed in quiescent satellite cells and that the frequency of this event varies between muscles. There is no direct relation to fiber type (Kelly and...
Rubenstein, 1994) because the diaphragm muscle (type IIX, IIA, and I fibers) is positive, whereas in the hindlimb the soleus (type I and IIA) is negative. Similarly, in the hindlimb the gastrocnemius (mostly type IIB) is negative, whereas other fast muscles (type IIA and IIB) in the trunk and forelimb have Pax3-expressing satellite cells.

We next examined the expression of Pax3 and -7 in primary cultures of satellite cells prepared from different muscles (Fig. 2). Activated satellite cells, from the trunk muscle of $Pax^3_{\beta\text{-gal}}$ mice, which are Pax3 (β-gal)-positive, also express MyoD and down-regulate Pax3 (β-gal) in differentiated muscle fibers (Fig. 2, A–C). This is also seen in cultures from $Pax^{\text{GFP+/+}}$ mice (Relaix et al., 2005), where expression of myogenin, which marks the onset of differentiation, is associated with rapid reduction in Pax3 (GFP) expression (Fig. 2, G–L). MyoD-positive cells in which Pax3 (β-gal) is low (Fig. 2, B and C) have probably activated myogenin. MyoD (Fig. 2 C), or later myogenin (Fig. 2, I and L), is expressed in most cells sorted from the muscle of the lower hindlimb and cultured, no (Fig. 2 T). However, when Pax3-negative satellite cells are analyzed by flow cytometry, they remain Pax3 (GFP)-positive (Fig. 2). The distribution of these two types of colonies from different muscle sources is quantified in Fig. 2 S. To confirm that Pax3 is not activated in satellite cells that only express Pax7, we isolated these cells using flow cytometry. Based on the isolation of GFP-positive satellite cells from the diaphragm muscle, we had previously established the gating window that contains these cells (Montarras et al., 2005), which is shown as a boxed area (R2) in Fig. 2 (T and U). When satellite cells isolated on this basis from the diaphragm muscle are cultured and reanalyzed by flow cytometry, they remain Pax3 (GFP)-positive (Fig. 2 T). However, when Pax3-negative satellite cells are sorted from the muscle of the lower hindlimb and cultured, no GFP-positive cells were found in the R2 window after re-sorting by flow cytometry (Fig. 2 U). These results demonstrate that activated satellite cells from Pax3-negative muscles, do not activate this Pax gene in cell culture.

The myogenic function of Pax3 and -7 in satellite cells

In the genetic hierarchy that regulates the onset of myogenesis in the embryo, Pax3 activates MyoD (Tajbakhsh et al., 1997; Relaix et al., 2003), and Pax7 can replace Pax3 in this function (Relaix et al., 2004). Therefore, we investigated the myogenic activity of the two Pax proteins in adult muscle by infecting cultured satellite cells with adenoviral vectors expressing wild-type or dominant-negative forms of Pax3 and -7, together with a GFP reporter. The dominant-negative proteins contain the repression domain of the Drosophila melanogaster engrailed transcription factor (Han and Manley, 1993) fused to the NH2-terminal region of the Pax sequence, which retains its DNA-binding domain. An initial series of experiments was performed with satellite cells isolated from a Pax reporter line, $P34$, in which the transgenic mice express β-gal.
from an nLacZ reporter that is regulated by multimerized Pax3/7 binding sites (Relaix et al., 2004). Overexpression of Pax3 or -7 resulted in no obvious increase in reporter activity, which was already expressed at a high level in the Pax3/7-positive satellite cells. However, the dominant-negative versions of both of these factors (Pax3DN and -7DN) resulted in down-regulation of the reporter (Fig. 3 A). With decreasing levels of Pax3DN or -7DN, no significant difference in the repression exerted by either dominant-negative Pax was detectable (not depicted). We conclude that Pax3 and -7 bind to the consensus site with similar affinities and that their dominant-negative forms compete effectively with both endogenous proteins, which normally function as transcriptional activators in these adult muscle cells, as in the embryo (Relaix et al., 2003). When dominant-negative forms of Pax3 or -7 were expressed in satellite cell culture media, the level of the myogenic factor Myf5 was not markedly affected (Fig. 3 B), whereas MyoD was down-regulated in infected cells (Fig. 3 C). As for the Pax3/7 reporter transgene, similar dosage effects were seen for both Pax3DN and -7DN, with reduction, but not elimination, of MyoD in cells in which the adenoviral expression vector was expressed at a lower level (Fig. 3 C, arrowheads). These results are quantified in Fig. 3 D. In contrast to the striking down-regulation of MyoD seen with the dominant-negative constructs, overexpression of wild-type Pax3 or -7 is compatible with MyoD expression (Fig. 3 E). Because myogenesis still occurs in the absence of MyoD (Fig. 3 F and not depicted), we investigated whether this is regulated by Myf5, which is expressed independently of Pax3 or -7 in the satellite cell cultures. These cells were prepared from hindlimb muscles of Myf5GFP/+ and Myf5GFP/GFP mice and infected with Pax3DN and -7DN adenoviral vectors (Fig. 3 F). Unlike infected cells from heterozygous mice, Myf5 mutant cells do not express myogenin or differinative in the presence of the dominant-negative Pax vectors. This demonstrates that either Pax3/7, acting via MyoD, or Myf5 are required for the myogenic differentiation of adult satellite cells.

The presence of satellite cells and muscle differentiation in Pax3-expressing muscles of Pax7 mutant mice

Because Pax3-expressing satellite cells are found in adult muscles, their potential contribution to muscle growth and regeneration was investigated in the Pax7 mutant mouse. We first evaluated whether Pax3 continues to be expressed in diaphragm and trunk muscles. This is the case as shown in Fig. 4 at postnatal day 3 (P3). Western blots show that Pax3 is present, although at a reduced level (Fig. 4 A), and immunohistochemistry confirms that Pax3 is expressed in satellite cells (Fig. 4 B). Satellite cells are also revealed by β-gal labeling of Pax7lacZ/lacZ muscle at P2 (Fig. 4 C) and, indeed, even at P10 cultures from the diaphragm contain MyoD-positive cells that differentiate into myofibers expressing troponin T (Fig. 4 D), although such cells are much rarer (≤15% of wild type). Similarly, single fiber experiments with the extensor digitorum longus hindlimb muscle (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200508044/DC1) show that satellite cells are still present in the mutant, but that their number is reduced to ~10% of normal levels at P10. The number of myonuclei is also reduced by ~50% at this stage (Fig. S1 F) and muscle fibers are smaller (not depicted), consistent with the reduced size of mutant mice (Mansouri et al., 1996; Seale et al., 2000). The number of β-gal-positive satellite cells were counted on sections of Pax7lacZ/lacZ muscles and compared with heterozygotes at P2 and P11 (Fig. 4 E). At P2 there is only a small reduction...
Satellite cell proliferation in the Pax7 mutant. [A] Satellite cells from the diaphragm (dia) or hindlimb muscles (limb) of Pax7<sup>+/--</sup> and Pax7<sup>lacz/lacz</sup> (−/−) mice at P4 were plated at low density. After 3 d, when colonies had formed, cells were processed for immunocytochemistry using a MyoD antibody. The number of cells per myogenic colony was determined in three independent experiments for each type of muscle and after counting at least 10 colonies per experiment. Results are expressed as the percentage of cells per myogenic colony from the Pax7 mutant (−/−) with respect to Pax7 heterozygotes (+/−), taken as 100%. (B) Determination of the percentage of cycling cells in normal and mutant myogenic colonies cultured as in A. Cells were costained with antibodies against MyoD and Ki67, which marks all phases of the cell cycle. Examples shown are for myogenic colonies from hindlimb muscles. Quantitation is from two independent experiments for each type of muscle. Results expressed as percentage of Ki67-positive cells per myogenic colony indicate that both mutant and wild type cells proliferate equally well. (C) Determination of the percentage of cyclin A-positive cells per myogenic colony cultured as in A. Cells were costained with antibodies against MyoD and cyclin A, which marks the S and G2 phases of the cell cycle. Examples shown are from hindlimb muscles treated for immunocytochemistry as in B. Results are expressed as percentage of cyclin A-positive cells per myogenic colony.

Satellite cell proliferation in the Pax7 mutant

Because the numbers of satellite cells fall during postnatal development in Pax7 mutant mice, and muscle fiber size is reduced, it is possible that activated satellite cells, which contribute to muscle growth, do not proliferate normally. This was examined in primary cultures from the diaphragm and hindlimb muscles of Pax7<sup>+/+</sup> and Pax7<sup>lacz/lacz</sup> mice, plated at low density so that the number of cells per colony could be monitored. A reduction of 25–30% in the number of cells per myogenic colony was observed (Fig. 5 A), indicating that, in the absence of Pax7, proliferation is affected and that this is also the case for colonies from a muscle, such as the diaphragm, which expresses Pax3. To determine whether some cells have withdrawn from the cell cycle, a Ki67 antibody was used, which marks proliferating cells in all phases of the cell cycle (Scholzen and Gerdes, 2000). There was no difference between colonies from mutant or heterozygous mice, where the proportion of proliferating cells is concerned (Fig. 5 B). We next examined progression of cells through the cell cycle, using a cyclin A antibody that marks cells in the S and G2 phases (~18%) in the numbers of satellite cells in trunk, diaphragm, or hindlimb muscles. By P11, this reduction is striking (~83%) and is similar for all three muscle sources, whether Pax3 is expressed in most satellite cells (e.g., diaphragm) or not (hindlimb). This demonstrates that Pax3 cannot compensate for Pax7 function during the postnatal development of skeletal muscle. This is not because of a failure in myogenesis in the mutant satellite cells. MyoD is still expressed and, as in wild-type cultures (Fig. 3), its expression is inhibited by a dominant-negative form of Pax3 (Fig. 4, F and G).

Apoptosis of satellite cells in the absence of Pax7

To investigate the survival of satellite cells in postnatal muscle in vivo, in the absence of Pax7, we used an antibody to the activated form of caspase-3 to label cells undergoing apoptosis (Patel et al., 1996). Coimmunohistochemistry was performed with an antibody to desmin, which marks activated satellite cells as they assume a myoblast phenotype (Creuzet et al., 1998), as well as muscle fibers. In the postnatal trunk muscle of Pax7 mutant mice, activated caspase-3–labeled cells are observed, whereas in control mice labeled cells are not detected (Fig. 6, A–D). These results are quantitated in Fig. 6 E. The decrease observed from P0 to P6 reflects the decreasing numbers of satellite cells in the mutant. These cells also express desmin, suggesting that they correspond to activated satellite cells, probably contributing to the postnatal growth of muscle (Fig. 6, A and B, arrowheads). The identification of these cells was confirmed by labeling with a laminin (Fig. 6 C) or β-gal anti-
body (Fig. 6 D). The latter detects Pax7 transcription, which marks satellite cells. During postnatal development, apoptotic cells were detected in all trunk and limb muscles examined in the Pax7 mutant, whereas they were very rare in the muscles of normal mice (Fig. 6 E). Therefore, we conclude that Pax7 has an antiapoptotic function and that in its absence satellite cells die, despite the presence of Pax3.

To compare the roles of Pax3 and -7 in protecting against apoptosis, wild-type satellite cells were transfected with GFP-marked adenoviral vectors coexpressing a dominant-negative form of Pax3 or -7. These cells were analyzed by flow cytometry on the basis of GFP expression, and their viability was measured by propidium iodide (PI) staining, which detects dying cells (Matteucci et al., 1999). Such an experiment is shown in Fig. 7 A for cells from hindlimb muscle infected with a dominant-negative form of Pax3 or -7, which led to 71% of dying cells in the GFP-positive population when Pax7DN was expressed. The results of these experiments are summarized in Fig. 7 B. Whereas Pax7DN led to substantial cell death, the Pax3DN-expressing virus at similar or sixfold higher multiplicities of infection did not show any effect on these cells, relative to control values. Similar results were seen in primary cultures from young mice (P7; not depicted) to those shown here for 3–4-wk-old animals (Fig. 7). No such apoptotic effect was observed when muscle cultures were infected with adenoviral vectors expressing Pax7. The induction of apoptosis is not related to the presence of MyoD (Peschiaroli et al., 2002) because in satellite cell cultures from MyoD/+/H11002/mice infected with Pax7DN (Fig. 7 A for cells from hindlimb muscle infected with a dominant-negative form of Pax3 or -7, which led to 71% of dying cells in the GFP-positive population when Pax7DN was expressed. The results of these experiments are summarized in Fig. 7 B. Whereas Pax7DN led to substantial cell death, the Pax3DN-expressing virus at similar or sixfold higher multiplicities of infection did not show any effect on these cells, relative to control values. Similar results were seen in primary cultures from young mice (P7; not depicted) to those shown here for 3–4-wk-old animals (Fig. 7). 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Pax7DN a similar extent of cell death was observed (not depicted). Furthermore, Pax7DN (or Pax3DN) did not provoke cell death in nonmyogenic cells, such as the OP9 bone marrow stromal cell line (not depicted). When cells isolated from the diaphragm (Fig. 7 C) were similarly infected, cell death caused by Pax7DN was about half of that witnessed in cells from hindlimb muscle. Equivalent levels of Pax3DN showed no effect, but in contrast with the observations on the hindlimb (Fig. 7 B), when the concentration of Pax3DN was increased, some cell death was observed. These results suggest that Pax3 can have a limited antiapoptotic effect in the muscles in which it is expressed. In keeping with this conclusion, more myogenic colonies are present in cells isolated from the diaphragm of Pax7 mutant mice at P4, although in both diaphragm and hindlimb preparations, this number was strikingly lower than with the wild type (Fig. 7 D). At later stages no difference was detectable.

**Discussion**

Pax7 is a satellite cell marker; however, we now show that its parologue, Pax3, is coexpressed in these cells in many skeletal muscles. This raises the question of their respective roles and whether Pax3 can compensate for the Pax7 mutant phenotype. Both function similarly in regulating MyoD during the onset of myogenesis in satellite cell cultures, and our analysis of Myf5 mutant cells demonstrates that Myf5 and Pax3 or Pax7 control the entry of these adult muscle progenitor cells into the myogenic program. The progressive postnatal loss of satellite cells that we document for Pax7 mutant mice is seen in the presence of Pax3, and it is caused by a requirement for Pax7 in muscle satellite cell survival and cell cycle progression for which Pax3 cannot compensate.

**Muscle progenitor cell specification and Pax3 expression in a subset of skeletal muscles**

At birth the great majority of satellite cells are still present in the trunk and limb muscles of Pax7 mutant mice (Oustanina et al., 2004), showing that Pax7 is not required for satellite cell specification as previously suggested (Seale et al., 2000). During prenatal development, a Pax3/7 population of myogenic progenitor cells is present and these somite-derived cells take up a satellite cell position in late fetal muscle (Gros et al., 2005). It is only when both Pax3 and -7 are absent that these cells die or fail to enter the myogenic program, with a major deficit in skeletal muscle in the double mutant (Relaix et al., 2005). We would therefore propose that satellite cell progenitors are specified prenatally by the action of Pax3 in the Pax7 mutant.

It is not clear why Pax3 should be present in the quiescent, as well as in the activated satellite cells of some muscles and not others. Most hindlimb muscles, usually used as a source of satellite cells (Seale et al., 2000; Conboy and Rando, 2002), and some forelimb and trunk muscles are negative for Pax3. Even within a muscle that is positive, like the diaphragm, some satellite cells express only Pax7. These differences do not correlate with fiber type and, thus, they do not correlate with the type of innervation. A correlation with the embryological origin of the muscle is also not evident (Tajbakhsh and Buckingham, 2000).

Heterogeneity between muscles is a well known feature of myopathies in which the mutation of a gene expressed in all muscles may have a pathological effect on particular muscle groups (Hadhchouel et al., 2003). It is also evident from the study of regulatory genes in the embryo that different sites of myogenesis are coordinated by different regulatory strategies. This is illustrated by the number of distinct sequences that control the spatiotemporal activation of the Myf5 gene (Bucherger et al., 2003; Hadchouel et al., 2003) or by the effects of mutations in genes encoding homeobox proteins, such as Lbx1 (Schafer and Braun, 1999; Brohmann et al., 2000; Gross et al., 2000) or Meox2 (Mankoo et al., 1999), which lead to the loss of certain limb muscles and not others. Understanding the basis of such myogenic heterogeneity represents a challenge for the muscle field, which has tended not to think in these terms because of the apparently generalized effects of the MyoD family of myogenic regulatory factors in the embryo.

**The role of Pax3 and -7 in myogenesis**

Myogenesis in the embryo is initially orchestrated by the myogenic regulatory factors Myf5 and Mrf4 that, together with Pax3, lead to the subsequent activation of MyoD. Mrf4 is not detectable in adult satellite cells (unpublished data), although it is expressed when they differentiate (Cooper et al., 1999), and this gene does not play a role as a determination factor during late embryonic and fetal development (Kassar-Duchossoy et al., 2004, 2005). However, we show that the genetic hierarchy that controls the onset of myogenesis in the embryo is conserved in the adult and that Pax3/7 and MyoD control the entry of cells into the myogenic program, acting in parallel genetic pathways. In the absence of all three factors, skeletal muscle differentiation does not occur, whereas in satellite cells from the Myf5 mutant or from Pax3-negative hindlimb muscles of the Pax7 mutant, myogenin is activated and the cells differentiate. In keeping with previous observations on MyoD mutant embryos (Rudnicki et al., 1992), Myf5 can activate myogenin directly, whereas activation of myogenesis by Pax3/7 depends on MyoD, as shown in the experiments reported here (Tajbakhsh et al., 1997). Expression of Myf5 in satellite cells, which we show is not affected by dominant-negative forms of Pax3 or -7, suggests that these cells have progressed beyond the progenitor cell state, characterized by the presence of Pax3/7 and the absence of any myogenic regulatory factor (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). In prenatal progenitor cells, expression of both Myf5 and MyoD depends on the presence of Pax3/7 factors (Relaix et al., 2005).

Dominant-negative forms of Pax3 or -7 down-regulate MyoD expression in the presence of Myf5. Presumably, the engrailed repression domain present in these constructs overrides transcriptional activation by Myf5. The direct repression exerted by these constructs was demonstrated by their effect on the P34 reporter transgene, which is dependent on Pax3/7 binding sites. In these experiments, and in situations where
Pax3DN or -7DN are expressed at a lower level, as indicated by the GFP reporter, with only partial repression of MyoD, we have never detected any difference between the two Pax dominant-negative forms. Furthermore, satellite cells isolated from hindlimb muscles expressing only Pax7 down-regulate MyoD in the presence of Pax3DN. We therefore conclude that Pax3 and -7 play a similar role in the activation of MyoD and subsequent skeletal muscle differentiation. In experiments with adenoviral vectors in which Pax3 or -7 were overexpressed in satellite cells, we saw no down-regulation of MyoD. This is in contrast to the results of the study conducted by Olguin and Olwin (2004), although in the C2 myogenic cell line, we did see some effect on MyoD (unpublished data). This may be a question of the cell system and the extent of overexpression, but we conclude that Pax3 and -7 normally act as activators of the myogenic program. The overexpression of MyoD that we observe in embryos that express the constitutively active PAX3-FKHR protein is consistent with this role. We show that Pax3, like Pax7 (Olguin and Olwin, 2004; Zammit et al., 2004), is rapidly down-regulated as satellite cells begin to differentiate. We observed the presence of Pax3/7-positive, MyoD-negative cells in older cultures in which differentiated myotubes were present. These cells probably correspond to reserve cells that will reconstitute the satellite cell pool, taking up a satellite cell present. These cells probably correspond to reserve cells that will reconstitute the satellite cell pool, taking up a satellite cell present. These cells probably correspond to reserve cells that will reconstitute the satellite cell pool, taking up a satellite cell present. These cells probably correspond to reserve cells that will reconstitute the satellite cell pool, taking up a satellite cell present.

Satellite cell proliferation

In satellite cultures from hindlimb muscles, we frequently saw colonies that expressed only Pax7, with no detectable expression of the Pax3 reporter. In contrast, Pax7-positive satellite cells from the tibialis anterior muscle, also used in our experiments, have never been to systematically activate Pax3 in culture and it has been proposed that Pax3 is necessary for their proliferation (Conboy and Rando, 2002). This discrepancy may reflect problems with the Pax3 antibody used, which, in our experience, works poorly on cultured cells and may show cross-reactivity with Pax7. This is why we rely on different Pax3 reporter lines, which consistently give reproducible results (Relaix et al., 2003, 2004, 2005). When we isolate satellite cells from Pax3GFP/+/ mice by flow cytometry after injury of the tibialis anterior, these cells remain Pax3 (GFP)-negative (Montarras et al., 2005), consistent with our ex vivo observations. Grafting experiments (Montarras et al., 2005), in addition to cell culture observations, show that satellite cells retain their Pax3-positive or -negative status independent of their environment.

In the Pax7 mutant, satellite cells are present and can differentiate into skeletal muscle (Oustanina et al., 2004), but, as we show, there is a progressive loss of these cells during postnatal development. Their loss is equally evident in muscles where Pax3 is also expressed in satellite cells. This indicates that there must be a function of Pax7 for which Pax3 cannot compensate. It had been suggested that there may be a proliferative defect in satellite cells in the absence of Pax7 (Oustanina et al., 2004). We examined the proliferation of satellite cell cultures from Pax7 mutant mice and observed a 25–30% reduction compared with wild type. This was seen with Pax3-positive myogenic colonies from the diaphragm, as well as from Pax3-negative hindlimb muscles. All of the cells were still cycling, but the cell cycle was perturbed, with relatively more cells in S and G2 phases. We suggest that this effect may be attributable to the loss of cells in G1 caused by apoptosis (Abrams and White, 2004).

Pax7 and satellite cell survival

We show that apoptosis, marked by activated caspase-3, occurs in the skeletal muscle of the Pax7 mutant. This is detectable immediately after birth and appears to occur in activated satellite cells, marked by desmin expression that would normally contribute to muscle growth. This suggests that self-renewal of satellite cells takes place via an activated cell state, as previously proposed (Zammit et al., 2004). We do not detect cell death in cultured satellite cells from Pax7 mutant mice. This may be because the presence of serum in the medium provides some protection or because dying cells detach rapidly precluding detection with markers of apoptosis. Muscles, such as the diaphragm, in which Pax3 is expressed, have more satellite cells, giving rise to myogenic colonies when satellite cell loss in vivo is still relatively minor (15–20%). However, by 15 d after birth only 5% of satellite cells remain in Pax3-expressing as well as Pax3-negative muscles. The difference between Pax3 and -7 is demonstrated by the flow cytometry experiments on satellite cells expressing dominant-negative constructs. Pax3DN at high levels does have some effect on the viability of the satellite cells in which it is expressed, but Pax7DN is much more potent, suggesting that the antiapoptotic targets of these two Pax factors are different in postnatal muscle. In the embryo, muscle progenitor cells in the somite are lost in the absence of Pax3, which is necessary for the survival of the hyoplastic dermomyotome. Pax7 is not normally expressed in these cells, but can rescue this function (Relaix et al., 2004). The expression of a single Pax3/7 gene in the muscle progenitor cells present in the somites of the cephalochordate Amphioxus (Holland et al., 1999) also suggests that Pax3 and -7 have similar functions in this embryonic context and that the distinct antiapoptotic activity of Pax7 evolved during vertebrate radiation, perhaps in response to the requirements of postnatal muscle growth and regeneration.

Members of the Pax gene family, which play key roles in the formation of other tissues (Tremblay and Gruss, 1994; Chi and Epstein, 2002), have been implicated in several functions discussed here for Pax3 and -7 in the skeletal muscle context. In addition to affecting cell fate choices, roles in proliferation and cell survival have been described. Notably, in the latter context, cells that do not express Pax2 in the developing inner ear undergo apoptosis (Li et al., 2004) and apoptosis is seen in mesodermal cells, which would normally form the pronephros during kidney development, in the absence of Pax2 and -8, which are required for the emergence of the nephric cell lineages (Bouchard et al., 2002). Furthermore, Pax2 has been shown to prevent apoptosis in renal cell cultures (Torban et al., 2000; Cai et al., 2005) related to its function during later nephrogenesis (Porteous et al., 2000). In a screen of human cancer cell lines from a range of tissues, PAX gene expression was frequently observed, and repression of its expression led to ap-
optosis (Muratovska et al., 2003). The antiapoptotic function of Pax7, which we describe here in postnatal skeletal muscle, is therefore not unique to this Pax subgroup. A role for Pax proteins in assuring the survival of the progenitor cells in which they regulate cell fate determinants may be widespread. The fine-tuning of such an antiapoptotic function in progenitor cells in the adult may also be critical in regulating the maintenance of stem cell populations during tissue growth and regeneration.

Materials and methods

Mice

Pax3IRESnLacZ/ (Relaix et al., 2004) and Pax3340-405IRESnLacZ/ (Relaix et al., 2003) mice (Mansouri et al., 1996) were genotyped by PCR, on the basis of the “splotch” phenotype (Auerbach, 1954). Pax3340-405IRESnLacZ/ mice (Mansouri et al., 1996) were genotyped by PCR, using the following primers: D Pax3Ex2A: CTGGGCAAGGGCGAGTCAAT-CAGCTTGGTggg; R LacZ3: AAATTCGACAGCAACAAGCCTGCTTTGGC; and R Pax3Ex3C: gaTggACCAGCTCTGTATATCGgCACAg. The wild-type band was amplified using DPax3Ex2A/RPax3Ex3C (800 bp), whereas the mutated allele was amplified using DPax3Ex2A/R LacZ3 (500 bp).

Cell culture

Cells were prepared from muscle tissue of mice at different time points after birth by enzymatic dissociation, as previously described (Pinset and Montarras, 1998; Montarras et al., 2000). Cells were plated on gelatin-coated dishes in a 1:1 mixture (vol/vol) of Ham’s F12 and DME (GIBCO BRL) containing 20% (vol/vol) fetal calf serum (AB Cys) and 2% (vol/vol) ultraser (Biosepra). This medium, which supports both the proliferation and differentiation of muscle cells (Montarras et al., 1998; Montarras et al., 2000), was used in all experiments. To allow the formation of colonies of muscle cells, primary cultures were plated at a density of 100 and 200 cells/cm2. When plated under these conditions cultures were highly enriched in myogenic colonies. The number of mononucleated cells to be plated was determined by counting after labeling an aliquot with 5 µg/ml of the DNA dye bis-benzimide (Hoechst).

Single fiber preparation and culture were performed according to Beauchamp et al. (2000).

Immunocytochemical analysis

Cells were treated as previously described (Montarras et al., 2000). In brief, after fixation with 4% (wt/vol) paraformaldehyde and permeabilization with 0.2% (wt/vol) Triton X-100, cells were incubated with antibodies diluted in PBS containing 0.2% (wt/vol) gelatin. All incubations were at room temperature. For immunofluorescence, cells were mounted in mowiol (Calbiochem) after the staining of DNA with 5 µg/ml bis-benzimide (Hoechst).

Antibodies used were as follows: MyS5, rabbit polyclonal (Lindon et al., 2000), at a 1:1,000 dilution; MyoD, either a rabbit polyclonal (Santa Cruz Biotecnology, Inc.), at a 1:200 dilution, or a mouse monoclonal (clone 5 8A; DAKO), at a 1:200 dilution; troponin T, mouse monoclonal (clone J T12, Sigma-Aldrich), at a 1:200 dilution; desmin, mouse monoclonal (clone D03; DakoCytomation), at a 1:200 dilution; laminin, mouse monoclonal (clone 12G4; Nanotools GmbH), at a 1:200 dilution; antiactive caspase-3, rabbit polyclonal (BD Biosciences), at a 1:250 dilution; cyclin A, a rabbit polyclonal (gift from A. Fernandez and N. Lamb, Institut de Génétique Humaine, Montpellier, France), at a 1:200 dilution; Ki67, a mouse monoclonal (BD Biosciences), at a 1:100 dilution; Pax7, mouse monoclonal (Developmental Studies Hybridoma Bank), at a 1:100 dilution; Pax3, mouse monoclonal (provided by M. Bronner-Fraser, California Institute of Technology, Pasadena, CA), at a 1:100 dilution; β-gal, either rabbit polyclonal (Invitrogen), at a 1:4,000 dilution in cell culture experiments, or another rabbit polyclonal used on sections (provided by J-F. Nicolas, Institut Pasteur, Paris, France), at a 1:500 dilution, or mouse monoclonal (clone GolI3; DakoCytomation), at a 1:100 dilution. Secondary antibodies were coupled to a fluorochrome, either Alexa 488 or 594 (Invitrogen), at a 1:250 dilution.

For X-Gal (Roche) staining, single fibers were fixed for 30 min with 4% paraformaldehyde in PBS, on ice. Fibers were rinsed twice with PBS, and then stained with X-Gal, using 0.4 mg/ml X-Gal in 2 mM MgCl2, 0.02% NP-40, 0.1 M PBS, pH 7.5, 20 mM K3Fe(CN)6, and 20 mM KFe(CN)6 for 4–16 h at 37°C, with shaking. Fibers were rinsed in PBS, postfixed overnight in 4% paraformaldehyde, and mounted after washing in PBS-buffered mowiol with DAPI. Similar conditions were used for X-Gal staining of sections. X-Gal staining of cells was performed after a 5-min fixation in 4% paraformaldehyde in the same solution used for fibers, but without NP-40.

Images of cultured cells and sections were acquired using an Axiophot or an Axiophan equipped with an Axioimaging camera and Axioskop software (Carl Zeiss Microlmaging, Inc.). Neofluor lenses, 40×, NA 0.75, and 20×, NA 0.50, were used. Images were optimized globally for contrast and brightness and assembled using Photoshop CS software (Adobe).

Analysis of extracts by semiquantitative RT-PCR

RNA extracts were prepared from tibialis anterior and diaphragm muscles of 8-wk-old mice, using TRIzol (Invitrogen). Reverse transcripts were generated using Power Script reverse transcriptase (BD Biosciences). The primers for Pax3 were DPax3-740 [GTCCCTCTGTAAGTCTACAO and RPax3-1100 [GCTAACCAAGACCTGCACCTCGGCC], which generate a 360-bp PCR fragment. The primers for Pax7 were D Pax7-140 (TGG-AAGTGTCCACCCCTCTTGGC) and RPax7-650 (ATCCAGACGGTC- CCTTTGTCGCC), which generate a 510-bp PCR fragment. Three other primer pairs were used and gave similar results. PCR products were separated on 1.5% agarose gels, using standard techniques, and revealed by UV light (Image Master CV; GE Healthcare).

Analysis of extracts by Western blotting

Protein extracts were prepared and analyzed by Western blotting, as previously described (Lindon et al., 2000). The antibuin antibody (clone 5H); BD Biosciences) was used at a 1:2,000 dilution and the Pax3 antibody at a 1:400 dilution.

Preparation of adenoviral vectors and cell infection

Adenoviral vectors were generated using standard molecular biology techniques. In brief, dominant-negative Pax3 and -7 constructs were made by fusing in-frame sequences encoding the D. melanogaster engrailed repression domain (298 amino acids; Han and Manley, 1993) to the first 340 amino acids of murine Pax7 (to generate the Pax7DN construct) or the first 374 amino acids of Pax3 (to generate the Pax3DN construct). Dominant-negative activity of Pax7DN and -3DN was verified by cotransfection in 293 cells with a plasmid containing polymerized Pax3/7 binding sites (Epstein et al., 1996) in front of a thymidine kinase (Herpes virus) minimal promoter followed by a LacZ reporter gene. Before their introduction into the adenovector, Pax7DN and -3DN were cloned into the Adtrack-CMV shuttle vector and recombinant adenoviruses with a GFP reporter sequence were generated as described previously (He et al., 1996). High-titer viral stocks were prepared by repeated infection into the packaging cell line 293T. Viruses were purified by CsCl banding followed by passage through a 2.5-mL Sephadex G25 column (GE Healthcare) for desalting and stored in aliquots at −80°C. The titer of each preparation was determined after infection of 293 cells by limiting dilution of virus and detection of GFP expression.

Primary cultures of muscle cells were infected 3 d after plating on 35-mm dishes. The medium was removed, but a film of medium (0.3 ml) was left to prevent drying and virus was added to each dish for 30 min at 37°C in a multiplicity of infection of 5–30 (Results). 1.5 ml of medium was added to the cells that were analyzed 3 d later by immunofluorescence and flow cytometry.

Flow cytometry

Flow cytometry analysis and characterization of Pax3 GFP-positive cells present in skeletal muscles of adult Pax3340IRESnLacZ/ mice have permitted us to define parameters for isolating adult muscle progenitor cells both from Pax3-expressing muscles (e.g., diaphragm) and non-Pax3–expressing muscles (e.g., lower hindlimb muscles; Montarras et al., 2005). In the case of the latter, this was on the basis of the size and granularity of CD34+ cells. GFP-positive muscle progenitor cells from diaphragm muscle and GFP-negative muscle progenitor cells from lower hindlimb muscles, isolated by flow cytometry, were maintained in culture for 6 d as proliferating cells before a second flow cytometric analysis for detecting the presence of GFP-positive cells.

Flow cytometry analysis was performed with an LSR analyzer (Becton Dickinson) and cell sorting was performed with a MoFlo (Cytometry, Inc.). Antibody against CD34 was a mouse monoclonal clone (clone Ram [34]; Becton Dickinson), coupled to biotin, and detected with streptavidin coupled to phycoerythrin. Cell death was measured by PI staining of

K3Fe(CN)6 for 4–16 h at 37°C, with shaking. Fibers were rinsed in PBS, postfixed overnight in 4% paraformaldehyde, and mounted after washing in PBS-buffered mowiol with DAPI. Similar conditions were used for X-Gal staining of sections. X-Gal staining of cells was performed after a 5-min fixation in 4% paraformaldehyde in the same solution used for fibers, but without NP-40.

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adenovirus-infected cells, which were identified by GFP fluorescence. Cells were trypanized and pooled with the culture supernatants before addition of 1 μg/ml PI and flow cytometry. Each determination was from triplicate plates.

Online supplemental material
Fig. S1 presents an analysis of satellite cells present on single fibers isolated from the extensor digitorum longus hindlimb muscle of Pax7+/− and Pax7−/− mice at P10. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508044/DC1.

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In the original paper, an incorrect kiloDalton measurement appeared in the “Pax3” lane of Fig. 1 D’. The corrected image is shown below.

Figure 1. Pax3 expression in muscle satellite cells. (A–C) Expression of Pax3 in different muscles from 3-wk-old Pax3IRESnLacZ/+ mice, revealed by X-Gal staining. (A) Diaphragm muscle; (B) hindlimb muscles; (C) trunk muscles, [sdc] serratus dorsalis caudalis. (D) Semiquantitative RT-PCR of Pax3 and -7 transcripts in adult tibialis anterior (TA) and diaphragm muscle. The number of cycles is indicated on the top of each lane. M, molecular weight markers (see Materials and methods). (D’) Pax3 protein is detected by Western blot in protein extracts from different muscles (D, diaphragm; L, hindlimb; T, ventral trunk muscles) in 3-wk-old mice. Tubulin (Tub) expression is shown as a loading control. (E and F) Pax3IRESnLacZ/+ is expressed in a subset of diaphragm muscle nuclei (arrows) from 3-wk-old mice, as revealed by X-Gal and DAPI staining of a transverse section (E) or isolated fiber (F). (G and G’) Detection of Pax3/7-dependent β-gal expression in transverse sections of adult diaphragm muscle from the transgenic line P34, which reports Pax3/7 transcriptional activity. (G) Immunodetection of laminin (red) and DAPI (blue) staining. (G’) Coimmunodetection of laminin (red) and β-gal (green). (H–J’) Coimmunodetection of β-gal-positive cells in the diaphragm muscle of 3-wk-old Pax3IRESnLacZ/+ mice with laminin (H’, green), CD34 (I’, red), or M-cadherin (J’, red). As indicated in the figure, β-gal is shown in red in H’ and in green in I’ and J’. Corresponding DAPI staining is shown [H, I, and J] for each panel. Arrows indicate the labeled satellite cell nuclei. (K) Coimmunodetection of Pax3 (red) and laminin (green, K’). Corresponding DAPI staining is shown, with the labeled nucleus indicated by an arrow (K). (L–N’) Coimmunohistochemistry on diaphragm muscle from 3-wk-old Pax3IRESnLacZ/+ mice for Pax7 (red) or β-gal (green). [L–N] Corresponding DAPI staining is presented with labeled nuclei indicated by arrows for each panel.