Pax7 and myogenic progression in skeletal muscle satellite cells

Peter S. Zammit[1,2,*], Frederic Relaix[3], Yosuke Nagata[1,‡], Ana Pérez Ruiz[1], Charlotte A. Collins[1,§], Terence A. Partridge[1,¶] and Jonathan R. Beauchamp[1]

[1]Muscle Cell Biology Group, Medical Research Council Clinical Sciences Centre, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN, UK
[2]Randall Division of Cell and Molecular Biophysics, King's College London, New Hunt's House, Guy's Campus, London, SE1 1UL, UK
[3]CNRS URA 2578, Département de Biologie Du Développement, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris CEDEX 15, France

*Author for correspondence (e-mail: peter.zammit@kcl.ac.uk)
‡Present address: Department of Life Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
§Present address: The Dubowitz Neuromuscular Unit, Department of Paediatrics, Imperial College London, Hammersmith Campus, Du Cane Road, London, W12 0NN, UK
¶Present address: Children's National Medical Center, 111 Michigan Ave NW, Washington, DC 20010, USA

Accepted 23 January 2006
Journal of Cell Science 119, 1824-1832 Published by The Company of Biologists 2006
doi:10.1242/jcs.02908

Summary

Skeletal muscle growth and regeneration are attributed to satellite cells – muscle stem cells resident beneath the basal lamina that surrounds each myofibre. Quiescent satellite cells express the transcription factor Pax7 and when activated, coexpress Pax7 with MyoD. Most then proliferate, downregulate Pax7 and differentiate. By contrast, others maintain Pax7 but lose MyoD and return to a state resembling quiescence. Here we show that Pax7 is able to drive transcription in quiescent and activated satellite cells, and continues to do so in those cells that subsequently cease proliferation and withdraw from immediate differentiation. We found that constitutive expression of Pax7 in satellite-cell-derived myoblasts did not affect MyoD expression or proliferation. Although maintained expression of Pax7 delayed the onset of myogenin expression it did not prevent, and was compatible with, myogenic differentiation. Constitutive Pax7 expression in a Pax7-null C2C12 subclone increased the proportion of cells expressing MyoD, showing that Pax7 can act genetically upstream of MyoD. However these Pax7-null cells were unable to differentiate into normal myotubes in the presence of Pax7. Therefore Pax7 may be involved in maintaining proliferation and preventing precocious differentiation, but does not promote quiescence.

Key words: Pax7, MyoD, Satellite cell, Skeletal muscle, Regeneration, Myogenic differentiation, Self-renewal

Introduction

The cellular unit of adult skeletal muscle is the myofibre, a highly specialised syncytium evolved to generate force by contraction. Myofibres are sustained by hundreds of post mitotic myonuclei and are efficiently repaired and regenerated by satellite cells (Mauro, 1961), a pool of stem cells located beneath the basal lamina that surrounds each fibre (reviewed by Zammit and Beauchamp, 2001). Satellite cells are the main source of new myonuclei for growing myofibres (Moss and Leblond, 1971), before they become quiescent in normal mature muscle (Schultz et al., 1978). However, satellite cells retain the ability to proliferate and differentiate in response to the routine needs of myonuclear turnover, or the more sporadic demands of myofibre hypertrophy and muscle regeneration (Bischoff, 1986; Schmalbruch and Lewis, 2000; Snow, 1977; Snow, 1978).

This effective restoration of structure and function in the face of repeated injury (Luz et al., 2002; Sadeh et al., 1985) demonstrates that mechanisms are in place to maintain the satellite cell compartment. It has been proposed that satellite cells may be part of a hierarchical system and merely represent a committed myogenic precursor that is restricted to providing myonuclei, where replacement of satellite cells occurs from a stem cell located within the muscle interstitum and/or outside muscle tissue (Gussoni et al., 1999; Asakura and Rudnicki, 2002; Fukada et al., 2002; LaBarge and Blau, 2002; Polesskaya et al., 2003; Kuang et al., 2006). Evidence though, is lacking for this mechanism accounting for more than a very minor contribution (Sherwood et al., 2004). It is more likely that the predominant mode of satellite cell replenishment is direct self-renewal (reviewed by Dhawan and Rando, 2005; Zammit and Beauchamp, 2001). Indeed, we have recently shown that when transplanted in association with a myofibre, satellite cells proliferate extensively to give rise to many new viable satellite cells in the host muscle (Collins et al., 2005). In culture, satellite cell progeny adopt divergent fates. Quiescent satellite cells express the paired box transcription factor family member Pax7 (Seale et al., 2000). When activated, they coexpress Pax7 with MyoD (Grounds et al., 1992; Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2002), a key transcription factor for myogenic differentiation and a member of the myogenic regulatory factor (MRFs) family, comprising MyoD, Myf5, myogenin and Mrf4 (reviewed by Tajbakhsh and Buckingham, 2000). Most activated satellite cells then proliferate, downregulate Pax7 and differentiate. By contrast, other proliferating cells maintain Pax7 but lose MyoD and withdraw from both cell cycle and immediate myogenic

differentiation, returning to a state resembling quiescence (Zammit et al., 2004; Nagata et al., 2006). A similar mechanism has also been described during muscle growth in chicken (Halevy et al., 2004).

Interplay between *Pax* and *MRF* genes is likely, therefore, to be important for self-renewal of satellite cells. Pax3 and Myf5 act genetically upstream of MyoD during embryogenesis (Tajbakhsh et al., 1997) where Pax3 and Pax7 identify myogenic stem cells in mouse somite (Relaix et al., 2005; Kassar-Duchossoy et al., 2005). Later in development however, both Myf5 and MyoD lie genetically downstream of Pax3 and Pax7 (Relaix et al., 2005). In adult muscle, Pax7 is expressed by the majority of quiescent satellite cells (Seale et al., 2000), whereas Pax3 is only present in satellite cells in particular muscles (Relaix et al., 2006), although it has been reported that Pax3 is transiently expressed in satellite cells during activation (Conboy and Rando, 2002). Although deletion of Pax3 results in the absence of diaphragm and limb muscles (Bober et al., 1994; Goulding et al., 1994), loss of Pax7 does not affect muscle formation, but mice do exhibit reduced postnatal muscle growth (Seale et al., 2000). Pax7-null mice have significant numbers of satellite cells at birth, but the population is progressively depleted as a result of cell-cycle defects and increased apoptosis (Oustanina et al., 2004; Relaix et al., 2006; Kuang et al., 2006). In the rare mice that survive to adulthood, the regenerative capacity of muscle is compromised (Oustanina et al., 2004; Kuang et al., 2006). Thus it appears that Pax7 is required for maintenance and survival of satellite cells. A recent suggestion that Pax7 has a role in self-renewal by suppressing MyoD and causing cells to exit the cell cycle (Olguin and Olwin, 2004), is inconsistent with a series of observations that *Pax* genes have a positive role in proliferation in many tissues (Mansouri et al., 1999). Indeed, when *PAX3* or *PAX7* DNA binding domain is translocated with the *FOXO1a* (formerly *FKHR*) transcriptional activation domain, the resulting fusion protein is thought to contribute to the development of alveolar rhabdomyosarcoma (reviewed by Barr, 2001; Mansouri, 1998). Furthermore, mice transgenic for *PAX3-FOXO1a* have been shown to overexpress MyoD (Relaix et al., 2003).

Here we sought to determine the effects of Pax7 on the choice between satellite cell differentiation and self-renewal. Using a transgenic mouse that reports Pax3/Pax7 transcriptional activity (Relaix et al., 2004), we found that Pax7 was able to drive transcription in quiescent satellite cells. However, following activation of satellite cells, Pax7 then progressively became transcriptionally inactive in most satellite cell progeny as they committed to differentiation. Significantly, in those satellite cells that adopted an alternative fate and returned to a state resembling quiescence, Pax7 remained able to activate transcription. We next sought to directly determine the effects of Pax7 on satellite-cell proliferation, differentiation and self-renewal. Satellite-cell progeny continued to divide and express MyoD following retroviral infection with Pax7-expressing vectors. Although the onset of myogenin expression was delayed in some infected cells, Pax7 expression was still compatible with myogenic differentiation; indeed Pax7 protein persisted in the nuclei of the resultant myotubes. To explore the role of Pax7 in a Pax7-null myogenic background, we used a clone of C2C12 that lacks Pax7. We found that in these cells, Pax7 upregulates MyoD, but also disrupts differentiation into normal myotubes.

Results

Pax7 is able to activate transcription in quiescent satellite cells

Pax7 is known to be a poor transcriptional activator, and in some cases represses transcription from target genes. To determine whether Pax7 is transcriptionally active in quiescent satellite cells, we used a transgenic mouse line (P34) in which *Tk-nlacZ* is controlled by concatermerised Pax3/Pax7 binding sites. In this mouse, the presence of β-galactosidase (β-gal) shows that Pax3 and/or Pax7 is able to bind its cognate site and activate transcription (Relaix et al., 2004). In muscles from the lower hindlimb, such as EDL, satellite cells very rarely express Pax3 (Relaix et al., 2006) and so the presence of β-gal is almost exclusively a result of Pax7 transcriptional activity.

Entire EDL, tibialis anterior, soleus, diaphragm and intercostal muscles from the Pax3/Pax7 reporter mice were rapidly dissected, immediately fixed and incubated in Xgal. These muscles contained many cells with β-gal activity (data not shown). Isolated myofibres had limited numbers of cells with strong β-gal activity, identified as satellite cells by co-immunostaining for β-gal and Pax7 (Fig. 1a,b). Indeed, on freshly isolated myofibres, the vast majority of cells identified by Pax7 expression were also β-gal$^+$ (97.0±1.4%; n=49 myofibres from three animals), showing that Pax7 is able to activate transcription in quiescent satellite cells.

Pax7 transcriptional activity is maintained in satellite cells that escape immediate differentiation

When myofibres are incubated in mitogen-rich medium, the associated satellite cells are activated and proliferate, modelling early events in muscle regeneration. After 24 hours of culture, immunostaining showed that most satellite cells on myofibres isolated from P34 mice remained β-gal$^+$ but had also begun to express MyoD (88.9%, n=171 cells on 23 myofibres). In this preparation, most satellite cells undergo their first division at around 36-40 hours (Zammit et al., 2004). After 48 hours, 88.5% (n=156 cells on 13 myofibres) remained both β-gal$^+$ and MyoD$^+$, although levels of β-gal were variable, even within the same cluster (Fig. 1c,d). Later, clusters of satellite cell progeny were observed to contain cells both with and without β-gal activity (Fig. 1e). Satellite cell progeny with Pax7 contained β-gal protein, showing that Pax7 remained able to activate transcription in some cells (Fig. 1f-h). Around this 3-day time point, some Pax7$^+$/MyoD$^-$ cells in clusters withdraw from immediate differentiation (Zammit et al., 2004). Pax7 transcriptional activity may contribute to this decision, since at this time only 39.3% (n=61 cells on 14 myofibres) of β-gal$^+$ satellite cell progeny in clusters still expressed MyoD (Fig. 1i,j). In general, cells containing myogenin did not express *nlacZ* (Fig. 1k,l), indicating that the P34 transgene was no longer active in these cells undergoing differentiation, consistent with the downregulation of *Pax* genes upon terminal differentiation (Zammit et al., 2004; Relaix et al., 2005).

Although culturing satellite cells attached to a myofibre provides an excellent model to study the initial events of regeneration, it is less suited for examination of later events

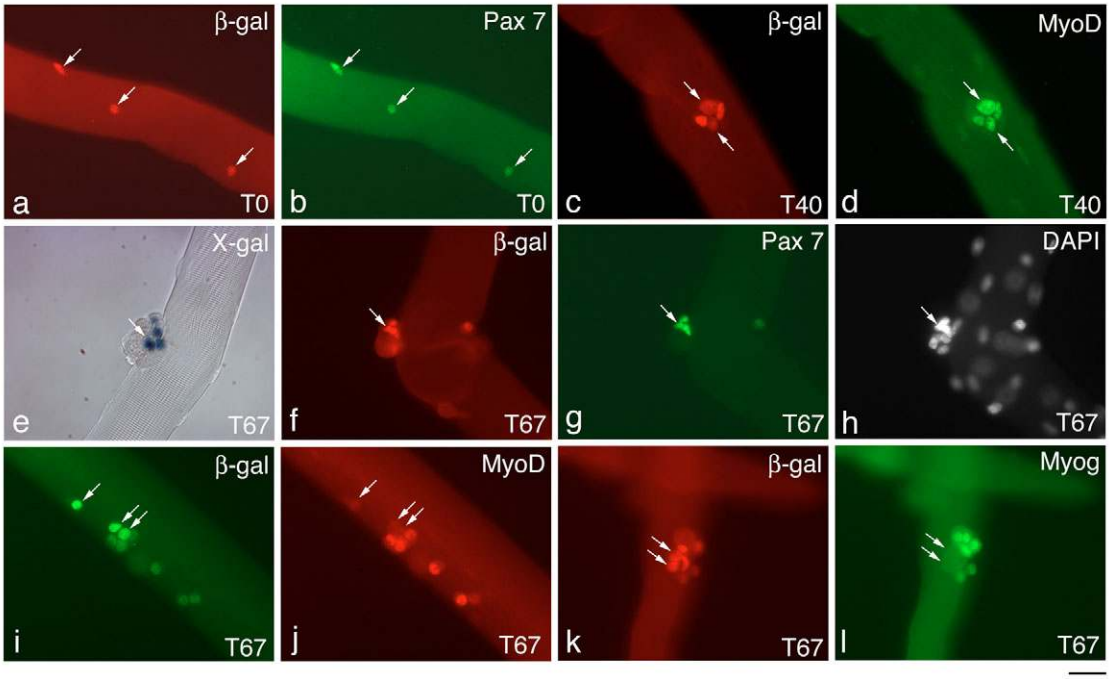


Fig. 1. Pax7 remains able to drive transcription in some satellite cell progeny. Co-immunostaining of freshly isolated (T0) EDL myofibres derived from P34 mice, that report Pax3/Pax7 transcriptional activity, demonstrate that β-gal protein (a, arrows) is present in quiescent satellite cells, as shown by the presence of Pax7 protein (b, arrows). After 40 hours (T40) in culture, β-gal levels are variable in proliferating MyoD$^+$ satellite cells, even within the same cluster (c and d, arrows). Clear divergence in the fate of satellite-cell progeny is evident by ~3 days (T67) in culture, with most cells downregulating Pax7 and committing to differentiation whereas others maintain Pax7 and lose MyoD. At this time, there are few satellite cell progeny with β-gal activity after incubation in X-gal (e) and immunostaining shows these cells contain Pax7 (f-h, arrows). β-gal levels are low/absent in cells committed to differentiation (i-l) as shown by the presence of MyoD (j, arrows) and myogenin (l, arrows). Counterstaining with DAPI was used to identify all nuclei present. Bar, 30 μm.

such as fusion. Plating myofibres on Matrigel however, allows satellite cells to migrate from the myofibre onto the tissue-culture substrate and proliferate, before differentiating and fusing into large multi-nucleated myotubes. Satellite-cell-derived myoblasts from the P34 mouse initially contained β-gal (data not shown). Cultures immunostained a week after the myofibres were plated however, showed that *nlacZ* expression was restricted to single cells, often closely associated with large multinucleated myotubes, whereas the myonuclei themselves were β-gal$^-$ (Fig. 2a,b). These findings therefore, are consistent with observations obtained from satellite cells cultured while remaining associated with a myofibre, where Pax7 is downregulated and no longer transcriptionally active as cells differentiate.

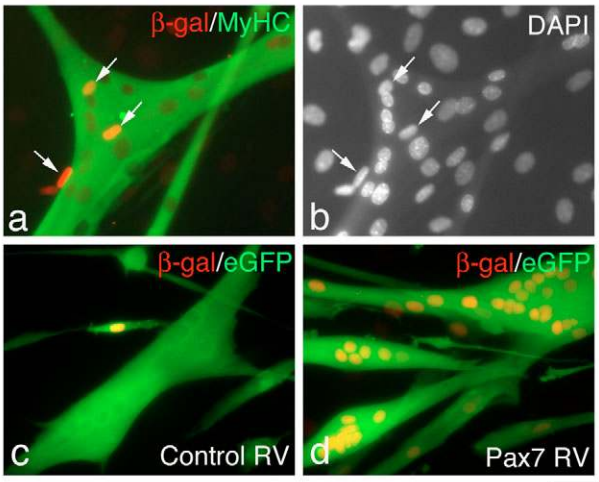


Fig. 2. Although endogenous Pax7 is not present in myotubes, ectopically expressed Pax7 can still activate its transcriptional targets in myonuclei. In order to investigate Pax7 transcriptional activity as satellite cell-derived myoblasts differentiate, myofibres were plated on matrigel. This preparation allows satellite cells to emigrate from the myofibre and proliferate extensively before fusing into multi-nucleated myotubes. Such plated myofibres from the Pax3/Pax7 transcriptional activity indicator mouse line P34, gave rise to large myosin heavy chain (MyHC$^+$) multinucleated myotubes, the nuclei of which were β-gal$^-$ (a,b). Some single cells however, often located close to myotubes, contained β-gal protein, indicating that Pax7 remained transcriptionally active in these cells (a and b, arrows). To test the retroviral vectors (RV), P34-derived myofibres were plated and the satellite-cell-derived myoblasts infected with either control pMSCV-IRES-eGFP (c) or pMSCV-Pax7-IRES-eGFP (d) and allowed to fuse into large multinucleated myotubes. Immunostaining for β-gal and eGFP revealed that pMSCV-IRES-eGFP-infected cultures only gave rise to eGFP$^+$/β-gal$^-$ myotubes (c), indistinguishable from uninfected myotubes (a). However, those infected with pMSCV-Pax7-IRES-eGFP had eGFP$^+$ myotubes with β-gal$^+$ myonuclei (d), showing that the introduced Pax7 protein was able to activate is transcriptional targets in myonuclei. Counterstaining with DAPI was used to identify all nuclei present. Bar, 30 μm.

Retrovirally delivered Pax7 is able to activate the Pax3/Pax7 reporter transgene in myonuclei

Having shown that Pax7 remains able to drive its transcriptional targets in some activated satellite cells that escape immediate differentiation, we next wished to explore the effects of constitutively expressed Pax7 on regenerative myogenesis. To do this we used a retrovirus to express Pax7 (pMSCV-Pax7-IRES-eGFP). To test the effectiveness of this construct, we again used the P34 Pax3/Pax7 reporter mouse. Infecting proliferating satellite cells with control pMSCV-IRES-eGFP retrovirus and then allowing them to differentiate and fuse resulted in eGFP$^+$ myotubes that did not contain nuclei with Pax7 transcriptional activity, as shown by a lack of *nlacZ* expression from the P34 transgene (Fig. 2c). However, when infected with pMSCV-Pax7-IRES-eGFP, high levels of β-gal protein were detected in the nuclei of large eGFP$^+$ myotubes (Fig. 2d), showing that Pax7 protein from the introduced retroviral construct was functional and was able to bind its cognate sequence and activate transcription.

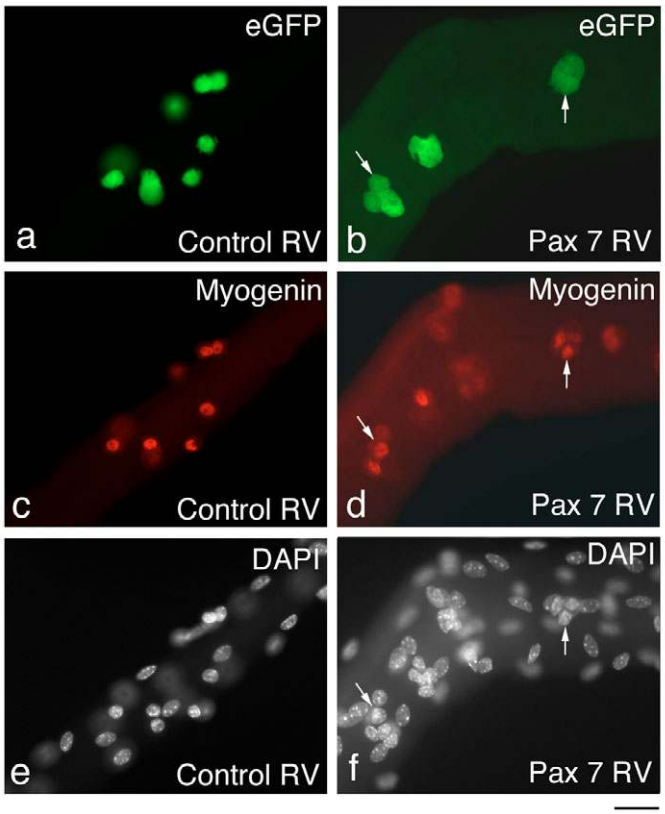
Maintaining Pax7 expression delays the appearance of myogenin in satellite cells

pMSCV-Pax7-IRES-eGFP was used to infect satellite cells retained beneath the basal lamina of their associated myofibre. Wild-type myofibres were isolated and cultured in plating medium with retrovirus and then fixed between 72 and 96 hours later and immunostained. Myofibre-associated satellite cell progeny infected with pMSCV-IRES-eGFP or pMSCV-Pax7-IRES-eGFP were identified by immunostaining for eGFP (Fig. 3). To determine if differentiation was compromised in the presence of Pax7, cultures were co-immunostainined for eGFP and myogenin. Infected cells entering terminal differentiation were readily detectable (Fig. 3a-f). There were however, significantly less (P<0.05) eGFP$^+$ satellite cell progeny coexpressing myogenin in cultures of pMSCV-Pax7-IRES-eGFP infected cells (38.8±4.6%, n=542 cells on 24 myofibres) compared with parallel cultures infected with control virus (70.4±2.6%, n=619 cells on 26 myofibres) (Fig. 3g).

Pax7 does not prevent fusion of satellite-cell-derived myoblasts

To study the later stages of differentiation, we again analysed satellite-cell-derived myoblasts following adherent myofibre culture. To determine whether the delay in myogenic differentiation was caused by the presence of Pax7 perturbing the cell cycle, we infected wild type satellite cell progeny with either pMSCV-IRES-eGFP or pMSCV-Pax7-IRES-eGFP and then after 72 hours, pulsed them with BrdU. Co-immunostaining for eGFP and BrdU (Fig. 4a,b) showed that infected cells continued to proliferate with the level of BrdU incorporation not significantly different between eGFP$^+$ satellite cell progeny infected with pMSCV-Pax7-IRES-eGFP (52.5±0.9%, n=206) or pMSCV-IRES-eGFP (58.0±11.3%, n=318). Co-immunostaining for eGFP and MyoD also showed that Pax7-infected satellite cell progeny maintained MyoD expression (Fig. 4c-f).

After 1 week of culture, many multi-nucleated satellite-cell-derived myotubes could be observed. Immunostaining of cultures infected with control pMSCV-IRES-eGFP showed that Pax7 protein was not present in eGFP$^+$ myotubes, but was only maintained in some single cells (Fig. 4g). By contrast,



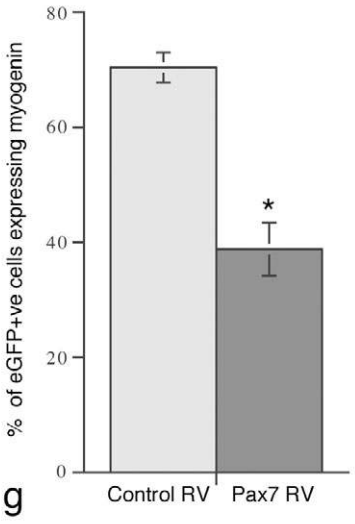


Fig. 3. Maintained Pax7 expression delays the onset of myogenin expression. Myofibres were cultured in suspension, exposed to retrovirus and then immunostained between 72 and 96 hours later. Infected satellite cell progeny were readily identified by the presence of eGFP. Most cells infected with pMSCV-IRES-eGFP coexpressed eGFP myogenin (a,c,e). Cells infected with pMSCV-Pax7-IRES-eGFP also coexpressed eGFP (Pax7) and myogenin (arrows in b,d,f), showing that the presence of Pax7 did not prevent differentiation. Importantly though, fewer cells coexpressed eGFP and myogenin after pMSCV-Pax7-IRES-eGFP infection compared with control cultures (g). Values are population means ± s.e.m. from 542 (Pax7 RV) and 619 (control RV) satellite cells from two mice and *P<0.05 denotes significant difference from levels in the control using Student's t-test. Counterstaining with DAPI was used to identify all nuclei. Bar, 30 μm.

Pax7 protein was present in the nuclei of eGFP$^+$ myotubes in pMSCV-Pax7-IRES-eGFP infected cultures (Fig. 4h). As in myotubes from non-infected and control pMSCV-IRES-eGFP infected cultures, eGFP$^+$ myotubes from cultures infected with pMSCV-Pax7-IRES-eGFP contained MyoD and myogenin (Fig. 4i-l). As expected, only a few single cells in differentiated cultures infected with Pax7 incorporated BrdU after a 60-minute pulse immediately prior to fixation (data not shown). Therefore, maintained expression of Pax7 in satellite-cell-derived myoblasts is compatible with differentiation and fusion into multinucleated myotubes.

Pax7 can act genetically upstream of MyoD in myogenic cells

The models used thus far involve determining the effects of Pax7 in cells with functional endogenous Pax7 protein. In order to explore the effects on a myogenic background lacking Pax7, we isolated a clone of C2C12, termed YMCA. These cells did not contain detectable levels of Pax7 protein (Fig. 5a), yet readily differentiated into large myotubes upon serum withdrawal (Fig. 6a).

Proliferating YMCA were infected with either pMSCV-Pax7-IRES-eGFP or the retroviral backbone pMSCV-IRES eGFP as a control, and the cells fixed 48 hours later. Immunostaining showed that the pMSCV-Pax7-IRES-eGFP produced nuclear localised Pax7 protein in eGFP$^+$ cells (Fig. 5b), whereas control pMSCV-IRES eGFP did not (Fig. 5a). The presence of Pax7 did not prevent the cells from cycling as shown by the incorporation of BrdU (Fig. 5c,d), with 51.3±1.9% (n=342 cells) of eGFP$^+$ YMCA cells being BrdU$^+$ following infection with pMSCV-Pax7-IRES-eGFP, not significantly different from either control pMSCV-IRES-eGFP infected or non-infected cultures (Fig. 5g). Not only was Pax7 expression compatible with MyoD in YMCA cells (Fig. 5e,f), there were significantly more eGFP$^+$ YMCA cells with robust MyoD expression following infection with pMSCV-Pax7-IRES-eGFP (62.3±2.9%, n=407 cells) than in cells infected with control pMSCV-IRES-eGFP (28.1±4.0%, n=284 cells) or non-infected cells (27.3±2.7%, n=233) (Fig. 5h). In cycling Pax7-null myogenic cells therefore, introduction of Pax7 results in a greater number of cells containing MyoD.

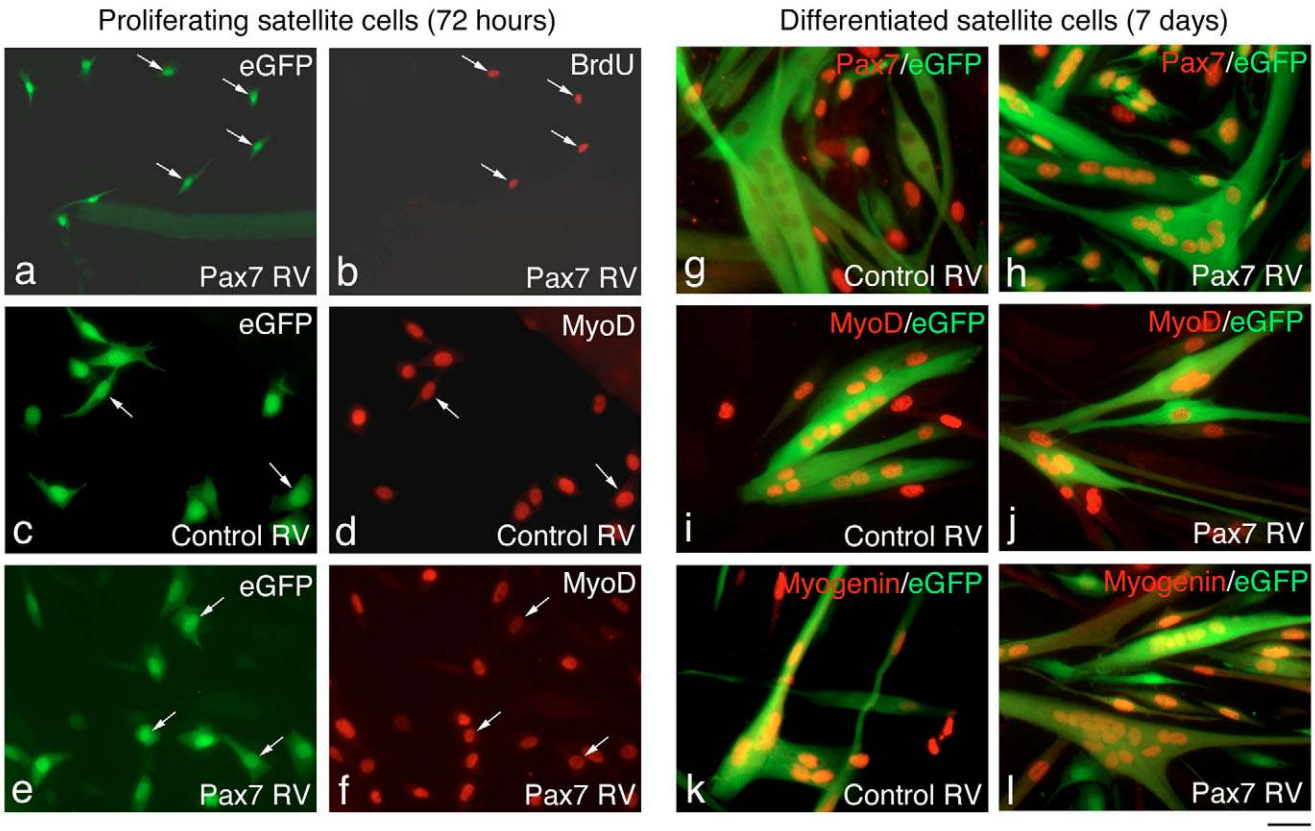


Fig. 4. Maintained Pax7 expression does not prevent the myogenic differentiation of satellite cells. To determine the effects of Pax7 expression on satellite-cell-derived myoblasts, plated myofibres were infected with retrovirus. After a further 72 hours of culture, immunostaining showed that the presence of Pax7, as shown by eGFP expression, did not affect proliferation of satellite-cell-derived progeny as shown by BrdU incorporation (a and b arrows). Similarly, cultures infected with pMSCV-IRES-eGFP (c,d) or pMSCV-Pax7-IRES-eGFP (e,f) were indistinguishable, showing that Pax7 did not alter MyoD expression (examples indicated with arrows). After 1 week of culture, many multinucleated satellite-cell-derived myotubes could be observed. Cultures infected with control pMSCV-IRES-eGFP showed that Pax7 protein was not present in eGFP$^+$ myotubes, but was only maintained in single cells (g). By contrast, Pax7 protein was present in the nuclei of eGFP$^+$ myotubes in pMSCV-Pax7-IRES-eGFP-infected cultures (h). As in myotubes from non-infected and control pMSCV-IRES-eGFP infected cultures, eGFP$^+$ myotubes from cultures infected with pMSCV-Pax7-IRES-eGFP contained MyoD and myogenin (i-l). Bar, 60 μm (a,b); 30 μm (c-l).

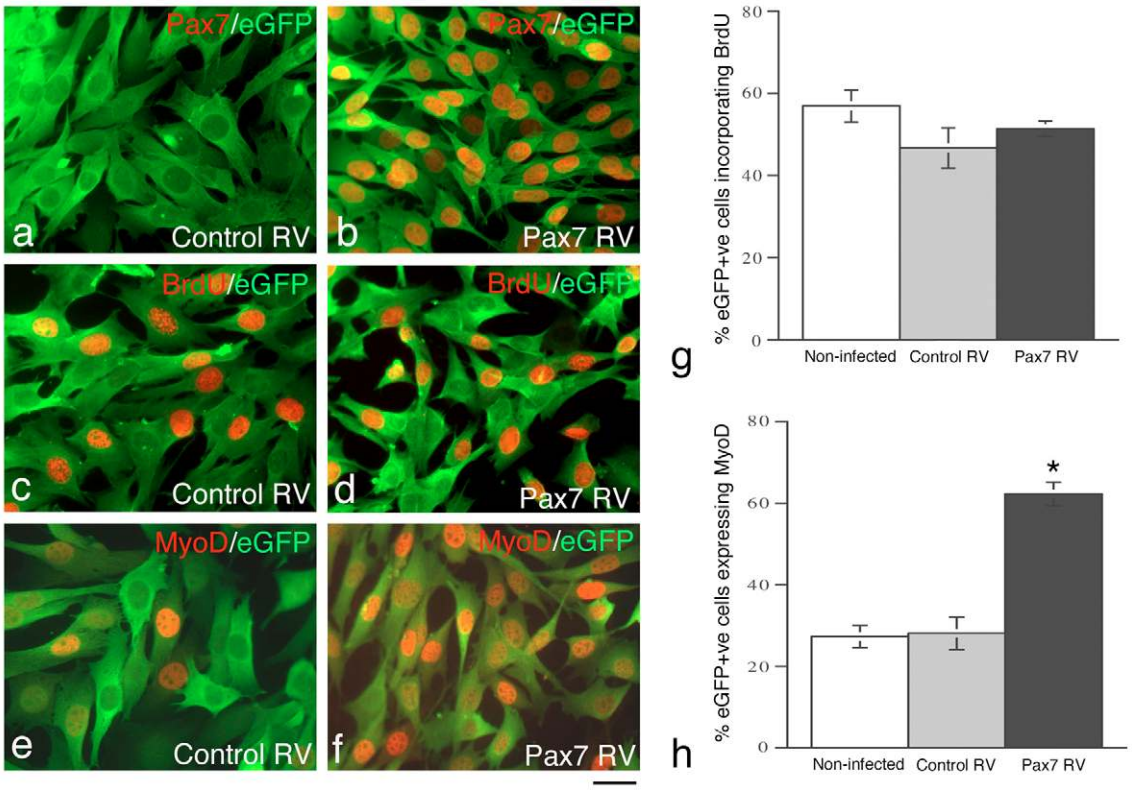
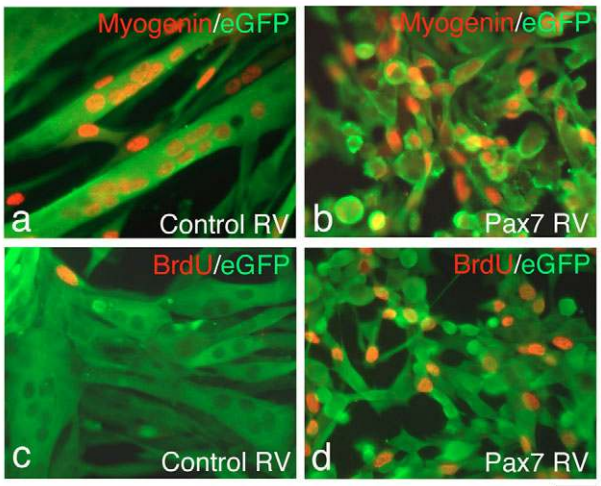


Fig. 5. Ectopic Pax7 increases MyoD expression in Pax7-null myogenic cells. YMCA cells, a subclone of C2C12, did not contain detectable levels of Pax7 (a). Cycling YMCA cells were infected with pMSCV-IRES-eGFP or pMSCV-Pax7-IRES-eGFP, left for 48 hours, fixed and immunostained. Infection with pMSCV-Pax7-IRES-eGFP resulted in Pax7 protein in nuclei of YMCA cells (b). Infected cultures were also co-immunostained for eGFP and BrdU (c,d) or eGFP and MyoD (e,f). Random fields were then selected and the total number of cells containing eGFP and either BrdU (g) or MyoD determined (h). The presence of Pax7 from pMSCV-Pax7-IRES-eGFP did not significantly affect the number of cells able to incorporate BrdU compared with pMSCV-IRES-eGFP-infected or control cells (g). However, the presence of Pax7 significantly increased the number of YMCA cells expressing MyoD when compared with cells infected with control vector or non-infected cells (h). Values are from at least eight random fields and are expressed as population mean ± s.e.m., where **P*<0.05 denotes significant difference from non-infected cells using Student's *t*-test. Bar, 30 μm.

Pax7 perturbs myogenic differentiation of Pax7-null myogenic cells

YMCA cells infected with control pMSCV-IRES-eGFP retrovirus fused into large multinucleated myotubes (Fig. 6a).



By contrast, whereas some cells infected with pMSCV-Pax7-IRES-eGFP expressed myogenin, the majority did not fuse (Fig. 6b); a similar observation was previously reported for C2 cells containing exogenous Pax3 (Epstein et al., 1995). Other pMSCV-Pax7-IRES-eGFP infected cells were myogenin$^-$ and more rounded in appearance (Fig. 6b). Few differentiated YMCA cells that had been infected with control pMSCV-IRES-eGFP incorporated BrdU, as expected from a differentiated culture, and those that did were generally single cells (Fig. 6c). Interestingly, many eGFP$^+$ (Pax7$^+$) YMCA cells were also BrdU$^+$, even when the pulse was administered after several days in differentiation medium (Fig. 6d).

Fig. 6. Ectopic Pax7 perturbs differentiation in Pax7-null myogenic cells. YMCA cells infected with pMSCV-IRES-eGFP while proliferating and then stimulated to differentiate, readily fused into large multi-nucleated myotubes (a). When stimulated to differentiate however, pMSCV-Pax7-IRES-eGFP infected cells either remained single or fused into aberrantly shaped myotubes (b). As expected of a differentiated myogenic culture, most cells were refractory to BrdU incorporation when infected with control pMSCV-IRES-eGFP (c) whereas many cells infected with pMSCV-Pax7-IRES-eGFP continued to divide (d). Bar, 30 μm.

Discussion

Compared with the basic helix-loop-helix family of MRFs, the role of Pax7 in adult regenerative myogenesis remains poorly defined. Pax7 protein is known to be expressed in quiescent satellite cells, is maintained during the progression from quiescence to activation and proliferation and is then lost during differentiation. Recently however, we showed that some activated satellite cells maintain Pax7, downregulate MyoD and adopt an alternative fate, returning to a state that resembles quiescence (Zammit et al., 2004; Nagata et al., 2006). Previous studies have implicated Pax7 in the specification of myogenic identity (Seale et al., 2000; Seale et al., 2004), satellite cell survival (Oustanina et al., 2004; Relaix et al., 2006; Kuang et al., 2006) and maintenance of quiescence (Olguin and Olwin, 2004): each or all of which could be consistent with the above observations. We sought to investigate this further by using retroviral expression of Pax7 in several in vitro models of regenerative myogenesis.

Pax7 protein is present in quiescent satellite cells (Seale et al., 2000; Zammit and Beauchamp, 2001). The mere presence of a transcription factor however, does not necessarily mean that it is able to activate transcription, as for example co-factors or phosphorylation of specific amino acid residues may also be required. Therefore, we first set out to determine whether the presence of Pax7 protein correlated with active transcription from its target genes. To do this we used a mouse line transgenic for a construct containing concatermerised Pax3/Pax7 binding sites controlling *TK-nlacZ* (Relaix et al., 2004). The presence of β-gal in virtually all quiescent satellite cells of EDL muscle, where Pax3 expression is extremely rare (Relaix et al., 2006), shows that Pax7 is able to drive transcription. Furthermore, the loss of Pax7 protein in most proliferating satellite-cell progeny is mirrored by a progressive loss of reporter gene expression, until undetectable in differentiated cells. Conversely, in satellite-cell progeny where Pax7 protein is maintained, the activity of the P34 transgene indicates that Pax7 remains able to bind its cognate DNA sequence and activate transcription. Together, these observations show that Pax7 is transcriptionally active in quiescent satellite cells and remains so throughout activation and proliferation in cells that subsequently return to a state resembling quiescence.

We further explored the effects of Pax7 on regenerative myogenesis by infecting satellite cells with a retrovirus encoding Pax7. Coexpression of endogenous Pax7 and MyoD in proliferating satellite cells shows that Pax7 does not prevent cell cycle progression (Halevy et al., 2004; Zammit et al., 2004). Indeed, other studies have shown that *Pax* genes strongly stimulate cell proliferation in various tissues and are also implicated in several cancers (Mansouri, 1998). Constitutive Pax7 did not suppress MyoD, in contrast to a previous report (Olguin and Olwin, 2004) (see below). Furthermore, although Pax7 is normally downregulated during myogenic differentiation, maintained expression did not prevent satellite cell progeny from expressing myogenin and fusing into large multinucleated myotubes. Therefore, the presence of Pax7 is compatible with myogenic differentiation and downregulation of this protein is not prerequisite for this process.

The phenotype of Pax7-null mice shows that Pax7 is not required for embryonic, and to a certain extent, foetal myogenesis to proceed. $Pax7^{-/-}$ mice are born alive but are smaller in size and the majority fail to thrive, dying at approximately 2 weeks of age (Seale et al., 2000). Importantly, satellite-cell-mediated postnatal muscle growth still occurs, although the number of satellite cells declines sharply in the first weeks of life (Oustanina et al., 2004; Relaix et al., 2006; Kuang et al., 2006). A small proportion of Pax7-null mice survive to adulthood, indicating that sufficient satellite-cell function remains to continue muscle growth in the absence of Pax7 (Oustanina et al., 2004; Kuang et al., 2006). However, there is debate about the stability of muscle in adult Pax7-null mice and the extent of its regenerative capacity (Oustanina et al., 2004; Kuang et al., 2006). When cultured, myoblasts from Pax7-null mice can still activate MyoD, proliferate and differentiate (Oustanina et al., 2004; Relaix et al., 2006; Kuang et al., 2006). Importantly, infection of wild-type myoblasts with a dominant-negative form of Pax7 leads to many cells dying (71%), showing that Pax7 has anti-apoptotic functions (Relaix et al., 2006). Therefore, Pax7 would not appear to be required for the specification of satellite cells but to have a role in their maintenance and survival.

The phenotype of YMCA cells, our $Pax7^{-}$ C2 clone, is consistent with the phenotype of the $Pax7^{-/-}$ mouse, in that these cells can activate MyoD, proliferate and differentiate into multi-nucleated myotubes. Since wild-type myoblasts can still express myogenin in the presence of a Pax7 dominant-negative protein, which suppresses MyoD but not Myf5, it seems that Myf5 can regulate myogenesis independently of *Pax* genes in adult muscle (Relaix et al., 2006). When our YMCA C2 clone was manipulated to express Pax7, cells continued to divide and the percentage containing MyoD actually increased. This suggests that Pax7 can act genetically upstream of MyoD in myogenic cells and may be involved in the rapid upregulation of MyoD during satellite-cell activation. Such a role is consistent with the induction of MyoD during development, where a lack of Pax3 and Myf5 results in a failure of MyoD activation in the epaxial somite (Tajbakhsh et al., 1997) and where a constitutively active *PAX3-FOXO1A* transgene leads to overexpression of MyoD (Relaix et al., 2003). Furthermore, in adult muscle it has been shown that Pax7 is sufficient to activate the myogenic program in CD45/Sca1-expressing muscle-derived cells (Seale et al., 2004).

In a recent study, it was found that overexpression of Pax7 suppressed MyoD, stopped cells from cycling and prevented differentiation (Olguin and Olwin, 2004), results contradictory to our own. The most likely explanation for this discrepancy lies in the different methods used to express *Pax7*. In the Olguin and Olwin study, cells were transfected with an expression vector that used the *CMV* promoter to drive *Pax7*. This method results in many copies of the plasmid entering a low proportion of cells and producing high levels of Pax7 protein. These transfected cells were then identified by brief exposure to a Pax7 antibody, an immunostaining protocol designed to exclude near-normal endogenous levels of Pax7. In our hands, transfection with pMSCV-Pax7-IRES-eGFP plasmid resulted in a decrease in the number of satellite-cell-derived myoblasts expressing MyoD compared with transfection with control plasmid (52.3% versus 75%). Parallel infections of the same primary myoblast population with packaged retrovirus to generate Pax7, actually caused a slight increase in MyoD expressing cells (83.3% versus 72.1%). Our

results indicate that the lower levels obtained by retroviral infection are compatible with MyoD expression. Again, this is consistent with the situation in proliferating myogenic cells where Pax7 and MyoD are coexpressed (Zammit et al., 2004; Halevy et al., 2004).

Interestingly, although primary cultures were able to differentiate efficiently in the presence of Pax7, our Pax7-null YMCA clone was not, consistent with the effects of forced expression of Pax3 in C2 cells (Epstein et al., 1995). Instead, YMCA cells infected with Pax7 continued to proliferate and formed only a limited number of small, abnormal myotubes. It is therefore probable that Pax7 does not function alone and that other regulatory mechanisms are involved. We would predict that our YMCA cells, which do not require Pax7 for differentiation, may also lack these mechanisms, such that ectopic expression of Pax7 is not appropriately regulated. This phenotype also has parallels with alveolar rhabdomyosarcoma, which is often characterized by the presence of *PAX3* or *PAX7* translocations with *FOXO1A*, such that the *PAX* DNA binding region is fused to this powerful transcriptional activator (Mansouri, 1998; Barr, 2001).

In summary, we show that Pax7 protein is able to drive transcription in quiescent, activated and proliferating satellite cells. Expression is then downregulated in cells that initiate terminal differentiation, but is maintained (and transcriptionally active) in those that opt out of immediate differentiation. Pax7, either endogenous or when ectopically expressed following retroviral infection, does not prevent satellite cells from dividing and expressing MyoD. Significantly, persistent ectopic expression of Pax7 is compatible with the differentiated state, although it delays the onset of myogenin expression. Therefore Pax7 may be involved in maintaining proliferation and preventing precocious differentiation, but does not promote quiescence.

Materials and Methods

Animal models

The P34 construct has five copies of the Pax3/Pax7 binding site ccctCGTCACGCTTgaatgt from the Trp-1 gene, highly similar to the consensus site as determined by CASTing (Epstein et al., 1996), cloned upstream of a thymidine kinase *(Tk)* minimal promoter controlling *nlacZpA*. This construct was used to make a transgenic mouse line in which the presence of β-gal reports transcriptional activity of Pax3 and Pax7 (Relaix et al., 2003; Relaix et al., 2004). Adult (>6 weeks of age) mice were used in this study.

Tissue preparation and single myofibre isolation

Mice were killed by cervical dislocation and the muscles were carefully removed and immediately fixed in 4% paraformaldehyde. Myofibres were isolated from the extensor digitorum longus (EDL) muscle as described in detail elsewhere (Rosenblatt et al., 1995).

Myofibre culture

For suspension culture, myofibres were incubated in plating medium [DMEM with 10% (v/v) horse serum (PAA Laboratories) and 0.5% (v/v) chick embryo extract (ICN Flow)] at 37°C in 5% CO_2. For adherent cultures, isolated myofibres were placed in LAB-TEK® eight-well chamber slides (Nunc) coated with 1 mg/ml Matrigel (Collaborative Research). Plating medium was added and the cultures maintained at 37°C in 5% CO_2. Where used, BrdU was added to the medium at a final concentration of 10 μM. Myofibres and cells were then fixed in 4% paraformaldehyde/PBS for 5-20 minutes.

Immunostaining

Fixed myofibres were permeabilised with 0.5% (v/v) Triton X-100 in PBS and blocked using 20% (v/v) goat serum in PBS, as described previously (Beauchamp et al., 2000). Primary antibodies used were monoclonal rat anti-BrdU (clone BU1/75: Abcam), monoclonal mouse anti-myogenin (clone F5D: DakoCytomation or DSHB), anti-MyoD1 (clone 5.8a: DakoCytomation), anti-Pax7 (DSHB), anti-β-galactosidase (clone 40-1a: DSHB), anti-myosin heavy chain (MF20), rabbit polyclonal anti-MyoD (Santa Cruz), anti-myogenin (Santa Cruz), anti-β-galactosidase (Molecular Probes) and anti-GFP (Molecular Probes). Primary antibodies were visualised with fluorochrome-conjugated secondary antibodies (Molecular Probes) before mounting in DakoCytomation Faramount fluorescent mounting medium containing 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI).

Histology

To visualise β-gal activity, whole muscles or isolated myofibres were incubated in X-gal solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, 400 μg/ml X-gal and 0.02% NP40 in PBS) at room temperature. Myofibres were then rinsed several times in PBS and mounted in Faramount aqueous mounting medium containing DAPI.

Retroviral expression vectors

The retroviral backbone pMSCV-puro (Clontech) was modified to replace the puromycin selection gene with eGFP, to create pMSCV-IRES-eGFP, which served as the control vector. Murine Pax7 cDNA (Relaix et al., 2006) was then cloned in pMSCV-IRES-eGFP to produce pMSCV-Pax7-IRES-eGFP, producing Pax7 as a bisitronic message with eGFP. Retroviruses were then packaged in 293T cells using standard methods.

Retroviral infection

A total of 2×10^4 cells were plated in each well of LAB-TEK® eight-well chamber slides (Nunc). The following day the media was replaced with undiluted 293T supernatant with 4 μg/ml polybrene and left at 37°C for 3 hours before the cells were rinsed and placed in fresh medium. To infect satellite cells associated with myofibres, 1:10 dilution of the supernatant was used.

Image capture

Immunostained myofibres and plated cells were viewed on a Zeiss Axiophot epi-fluorescence microscope using a 40×/0.75 Ph2-Neofluar lens. Digital images were acquired with a charge-coupled device (RTE/CCD-1300-Y; Princeton Instruments) at –10°C using Metamorph software version 4.5r5 (Universal Imaging). Images were optimized globally and assembled into figures using Adobe Photoshop 6.0.1.

We would like to thank the colleagues who made their antibodies available through the Developmental Studies Hybridoma Bank and Professor Margaret Buckingham for the P34 Pax3/Pax7 reporter mice and useful discussions. This work was supported by The Medical Research Council. We acknowledge the support of the MYORES Network of Excellence, contract 511978, from the European Commission 6th Framework Programme and EC Biotechnology Grant BIO4 CT 95-0228, EC Framework 5 QLRT-99-00020, EC Framework 5QLK6-1999-02034. P.S.Z. was funded by the Medical Research Council and The Muscular Dystrophy Association. The Muscle Cell Biology Group also received support from The Leopold Muller Foundation. F.R. is an INSERM fellow, Y.N. was funded by the Japan scholarship foundation, A.P.R. received funding from Fundación Ramón Areces post-doctoral fellowship and C.C. was supported by the Muscular Dystrophy Campaign (RA1/685 and RA3/711). We would also like to acknowledge Bradley Cobb for the pMSCV-IRES-eGFP retroviral backbone.

References

Asakura, A. and Rudnicki, M. A. (2002). Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. *Exp. Hematol.* **30**, 1339-1345.

Barr, F. G. (2001). Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma. *Oncogene* **20**, 5736-5746.

Beauchamp, J. R., Heslop, L., Yu, D. S., Tajbakhsh, S., Kelly, R. G., Wernig, A., Buckingham, M. E., Partridge, T. A. and Zammit, P. S. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J. Cell Biol.* **151**, 1221-1234.

Bischoff, R. (1986). Proliferation of muscle satellite cells on intact myofibers in culture. *Dev. Biol.* **115**, 129-139.

Bober, E., Franz, T., Arnold, H. H., Gruss, P. and Tremblay, P. (1994). Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* **120**, 603-612.

Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A. and Morgan, J. E. (2005). Stem cell function, self-renewal and behavioural heterogeneity of cells from the adult muscle satellite cell niche. *Cell* **122**, 289-301.

Conboy, I. M. and Rando, T. A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev. Cell* **3**, 397-409.

Dhawan, J. and Rando, T. A. (2005). Stem cells in postnatal myogenesis: molecular

mechanisms of satellite cell quiescence, activation and replenishment. *Trends Cell Biol.* **15**, 666-673.

Epstein, J. A., Lam, P., Jepeal, L., Maas, R. L. and Shapiro, D. N. (1995). Pax3 inhibits myogenic differentiation of cultured myoblast cells. *J. Biol. Chem.* **270**, 11719-11722.

Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. Y. and Maas, R. L. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. *Proc. Natl. Acad. Sci. USA* **93**, 4213-4218.

Fukada, S., Miyagoe-Suzuki, Y., Tsukihara, H., Yuasa, K., Higuchi, S., Ono, S., Tsujikawa, K., Takeda, S. and Yamamoto, H. (2002). Muscle regeneration by reconstitution with bone marrow or fetal liver cells from green fluorescent protein-gene transgenic mice. *J. Cell Sci.* **115**, 1285-1293.

Goulding, M., Lumsden, A. and Paquette, A. J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* **120**, 957-971.

Grounds, M. D., Garrett, K. L. and Beilharz, M. W. (1992). The transcription of MyoD1 and myogenin genes in thymic cells in vivo. *Exp. Cell Res.* **198**, 357-361.

Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M. and Mulligan, R. C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401**, 390-394.

Halevy, O., Piestun, Y., Allouh, M. Z., Rosser, B. W., Rinkevich, Y., Reshef, R., Rozenboim, I., Wleklinski-Lee, M. and Yablonka-Reuveni, Z. (2004). Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. *Dev. Dyn.* **231**, 489-502.

Kassar-Duchossoy, L., Giacone, E., Gayraud-Morel, B., Jory, A., Gomes, D. and Tajbakhsh, S. (2005). Pax3/Pax7 mark a novel population of primitive myogenic cells during development. *Genes Dev.* **19**, 1426-1431.

Kuang, S., Charge, S. B., Seale, P., Huh, M. and Rudnicki, M. A. (2006). Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. *J. Cell Biol.* **172**, 103-113.

LaBarge, M. A. and Blau, H. M. (2002). Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* **111**, 589-601.

Luz, M. A., Marques, M. J. and Santo Neto, H. (2002). Impaired regeneration of dystrophin-deficient muscle fibers is caused by exhaustion of myogenic cells. *Braz. J. Med. Biol. Res.* **35**, 691-695.

Mansouri, A. (1998). The role of Pax3 and Pax7 in development and cancer. *Crit. Rev. Oncog.* **9**, 141-149.

Mansouri, A., Goudreau, G. and Gruss, P. (1999). Pax genes and their role in organogenesis. *Cancer Res.* **59**, 1707s-1710s.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9**, 493-495.

Moss, F. P. and Leblond, C. P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anat. Rec.* **170**, 421-435.

Nagata, Y., Kobayashi, H., Umeda, M., Ohta, N., Kawashima, S., Zammit, P. S. and Matsuda, R. (2006). Sphingomyelin levels in the plasma membrane correlate with the activation state of muscle satellite cells. *J. Histochem. Cytochem.* doi:10.1369/jhc.5A6675.2006.

Olguin, H. C. and Olwin, B. B. (2004). Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Dev. Biol.* **275**, 375-388.

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

Polesskaya, A., Seale, P. and Rudnicki, M. A. (2003). Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell* **113**, 841-852.

Relaix, F., Polimeni, M., Rocancourt, D., Ponzetto, C., Schafer, B. W. and Buckingham, M. (2003). The transcriptional activator PAX3-FKHR rescues the defects of Pax3 mutant mice but induces a myogenic gain-of-function phenotype with ligand-independent activation of Met signaling in vivo. *Genes Dev.* **17**, 2950-2965.

Relaix, F., Rocancourt, D., Mansouri, A. and Buckingham, M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev.* **18**, 1088-1105.

Relaix, F., Rocancourt, D., Mansouri, A. and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* **435**, 948-953.

Relaix, F., Montarras, D., Zaffran, S., Gayraud-Morel, B., Rocancourt, D., Tajbakhsh, S., Mansouri, A., Cumano, A. and Buckingham, M. (2006). Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J. Cell Biol.* **172**, 91-102.

Rosenblatt, J. D., Lunt, A. I., Parry, D. J. and Partridge, T. A. (1995). Culturing satellite cells from living single muscle fiber explants. *In Vitro Cell. Dev. Biol. Anim.* **31**, 773-779.

Sadeh, M., Czyewski, K. and Stern, L. Z. (1985). Chronic myopathy induced by repeated bupivacaine injections. *J. Neurol. Sci.* **67**, 229-238.

Schmalbruch, H. and Lewis, D. M. (2000). Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. *Muscle Nerve* **23**, 617-626.

Schultz, E., Gibson, M. C. and Champion, T. (1978). Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. *J. Exp. Zool.* **206**, 451-456.

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

Seale, P., Ishibashi, J., Scime, A. and Rudnicki, M. A. (2004). Pax7 is necessary and sufficient for the myogenic specification of CD45+:Sca1+ stem cells from injured muscle. *PLoS Biol.* **2**, E130.

Sherwood, R. I., Christensen, J. L., Conboy, I. M., Conboy, M. J., Rando, T. A., Weissman, I. L. and Wagers, A. J. (2004). Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. *Cell* **119**, 543-554.

Snow, M. H. (1977). Myogenic cell formation in regenerating rat skeletal muscle injured by mincing. II. An autoradiographic study. *Anat. Rec.* **188**, 201-217.

Snow, M. H. (1978). An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats. *Cell Tissue Res.* **186**, 535-540.

Tajbakhsh, S. and Buckingham, M. (2000). The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. *Curr. Top. Dev. Biol.* **48**, 225-268.

Tajbakhsh, S., Rocancourt, D., Cossu, G. and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell* **89**, 127-138.

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

Zammit, P. and Beauchamp, J. (2001). The skeletal muscle satellite cell: stem cell or son of stem cell? *Differentiation* **68**, 193-204.

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A. and Beauchamp, J. R. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J. Cell Biol.* **166**, 347-357.

Zammit, P. S., Heslop, L., Hudon, V., Rosenblatt, J. D., Tajbakhsh, S., Buckingham, M. E., Beauchamp, J. R. and Partridge, T. A. (2002). Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. *Exp. Cell Res.* **281**, 39-49.