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Pax3 induces differentiation of juvenile skeletal muscle stem cells without transcriptional upregulation of canonical myogenic regulatory factors

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Summary

Pax3 is an essential myogenic regulator of fetal and embryonic development, but its role in postnatal myogenesis remains a topic of debate. We show that constitutive expression of Pax3 in postnatal, juvenile mouse skeletal muscle stem cells, a subset of the heterogeneous satellite cell pool highly enriched for myogenic activity, potently induces differentiation. This differentiation-promoting activity stands in contrast to the differentiation-inhibiting effects of Pax3 in the commonly used mouse myoblast cell line C2C12. *Pax3* mRNA levels in distinct muscles correlate with the rate of myogenic differentiation of their muscle stem cells. Although Pax3 controls embryonic myogenesis through regulation of the canonical myogenic regulatory factors (MRFs) Myf-5, MyoD, myogenin and Mrf4, we find that in postnatal muscle stem cells, ectopic Pax3 expression fails to induce expression of any of these factors. Unexpectedly, overexpression of neither Myf-5 nor myogenin is sufficient to induce differentiation of juvenile stem cells; and knockdown of Myf-5, rather than inhibiting differentiation, promotes it. Taken together, our results suggest that there are distinct myogenic regulatory pathways that control the embryonic development, juvenile myogenesis and adult regeneration of skeletal myofibers.

Key words: Pax3, Pax7, Muscle stem cells, Satellite cells, C2C12, Myogenic differentiation, Myogenic regulatory factors

Introduction

The PAX family consists of nine transcription factors, which have common paired, homeodomain and octapeptide domains. Each member of this family has a crucial role in organogenesis during embryonic and fetal development (Tremblay and Gruss, 1992). PAX proteins regulate a wide variety of cellular processes, including cell proliferation, self-renewal, apoptosis, migration and differentiation (Tremblay and Gruss, 1992). They further are divided into four subfamilies based on their structural and expression domains (Gruss and Walther, 1992). The Pax3/7 subfamily regulates both myogenesis (Buckingham and Relaix, 2007) and neurogenesis in the neural crest (Koblar et al., 1999).

Pax3 is first expressed in the presomitic mesoderm (Williams and Ordahl, 1994) and is required for survival of the ventro-lateral dermomyotome, which gives rise to the hypaxial and limb musculature (Buckingham et al., 2002). Pax7 is expressed later, in the central dermomyotome (Jostes et al., 1990), and is thought to be essential only during postnatal myogenesis (Seale et al., 2000). A cell population has been identified that expresses both Pax3 and Pax7, but no additional markers of skeletal muscle such as MyoD or desmin (Kassar-Duchossoy et al., 2004; Relaix et al., 2005); these cells proliferate in embryonic and fetal muscles of the trunk and limbs throughout development (Relaix et al., 2005). In the absence of both Pax3 and Pax7, muscle development is arrested and precursor cells do not leave the myotome (Relaix et al., 2005). However, Pax3 and Pax7 exhibit divergent functions in development: Pax7 can substitute for Pax3 in dorsal neural tube, neural crest cell, somite and muscle formation in the trunk, but not in muscle formation in the limbs (Relaix et al., 2004). At the same time, Pax3 cannot substitute for Pax7 in postnatal skeletal muscle (Lagha et al., 2008). Deletion of Pax3 leads to death of progenitor cells in the hypaxial somite (Buckingham et al., 2002), and Pax3 also is required for the delamination and migration of muscle progenitor cells to sites where skeletal muscle will form, such as the limbs (Franz et al., 1993). Ectopic Pax3 can drive myogenesis of embryonic carcinoma cells (Ridgeway and Skerjanc, 2001) and embryonic stem cells (Darabi et al., 2008). In the adult, Pax3 is expressed in a variety of muscles, but to differing degrees in different muscles (Buckingham et al., 2002). These observations collectively raise important questions about the potential developmental and anatomic diversity of the functions of Pax3.

Similarly to Pax proteins, the four canonical myogenic regulatory factors (MRFs) Myf-5, MyoD, myogenin and Mrf4 have also been intensively studied in early development. All these MRFs were initially characterized by virtue of their ability to convert certain non-muscle cell lines, such as fibroblasts, into myoblasts or myotubes (Weintraub et al., 1991). Studies in targeted mice revealed that the MRFs are essential, to differing extents, for prenatal skeletal muscle development (Weintraub et al., 1991). Pax3 can directly transactivate Myf-5 and myogenin expression during embryonic myogenesis (Bajard et al., 2006), and Pax3 also appears to activate MyoD in some circumstances (Maroto et al., 1997; Tajbakhsh et al., 1997), although this regulation is probably indirect (Buckingham and Relaix, 2007). In contrast to developing muscle, Pax3 does not transactivate Myf-5 in the adult (Relaix et al., 2006). Here, using a previously described population that is highly purified for postnatal skeletal muscle stem cells, we evaluate the impact of increasing levels of Pax3 in this specific cellular compartment.

Pax3 inhibits differentiation of immortalized mouse myoblasts

To facilitate ectopic expression in a variety of poorly transfectable cells, we generated hemagglutinin epitope (HA)-tagged mouse Pax3 and Pax7 cDNAs, and cloned these into the MSCV retroviral vector. This vector also contained an IRES-GFP cassette, such that GFP is translated from the same transcript as the transgene, which enables visualization of virally infected cells. Western blot using an antibody against the HA tag confirmed the presence of HA-tagged Pax3 and Pax7 proteins in whole-cell extracts of C2C12 cells transduced with these viruses (Fig. 1A).

C2C12 is an immortalized mouse myoblast cell line commonly used for studies of myogenic processes. C2C12 cells in growth phase exhibit myoblast morphology, and when confluent can be induced to differentiate into multinucleated myotubes by switching to low-mitogen medium. C2C12 cells can also be induced to differentiate in vitro into adipocyte-like and osteocyte-like cells (Fux et al., 1994; Mancini et al., 2007), unlike primary skeletal muscle precursor cells, which maintain myogenic commitment in vivo and in vitro (Sherwood et al., 2004; Cerletti et al., 2008).

In our experiments, C2C12 cells transduced with control retroviral vector (containing only the IRES-GFP) began to adopt an elongated morphology approximately 2 days after switching to low-mitogen medium, and the percentage of cells exhibiting this morphology increased at 4 and 8 days after transduction (Fig. 1B, top panels). By contrast, and consistent with a previous report (Epstein et al., 1995), ectopic expression of Pax3 by retroviral

transduction strongly inhibited differentiation as assessed by the percentage of elongated myotubes (Fig. 1B,C) ($0\pm0\%$ elongated cells in Pax3-transduced cultures vs $43\pm5\%$ elongated cells in control-transduced cultures, Fig. 1C) and MyHC-positive cells (Fig. 1B, right panels). Ectopic expression of Pax3 did not affect the proliferation rate of C2C12 cells, as assessed by cell counting (Fig. 1D). And, despite the visual appearance, Pax3 expression did not induce cell death, but actually had a modest protective effect (Fig. 1E).

Pax3 induces differentiation of primary skeletal muscle stem cells

We recently described a population of muscle-fiber-associated cells that are highly enriched for muscle stem cell activity (Sherwood et al., 2004; Cerletti et al., 2008). These skeletal muscle precursor (SMP) cells are harvested from freshly dissected mouse muscle tissue, and isolated by fluorescence-activated cell sorting of the Sca1⁻ CD45⁻ Mac1⁻ β 1-integrin⁻ CXCR4⁺ subset of myofiber-associated cells. SMPs make up ~3-10% of the myofiber-associated live cell population (Sherwood et al., 2004; Cerletti et al., 2008), and exhibit robust in vitro myogenic activity at the single cell level and efficient engraftment into injured muscle in vivo (Cerletti et al., 2008).

To examine the effect of ectopic Pax3 expression on SMP myogenic activity, we used the retroviral vectors to transduce SMPs from juvenile mice (3-6 weeks of age) 16 hours after sorting (Fig. 2A). Control experiments, using the vector encoding green fluorescence protein (GFP) alone, demonstrated a typical



Fig. 1. Pax3 expression in C2C12 cells inhibits myogenic differentiation. (A) Western blot of C2C12 cells infected with the indicated retroviruses. (B) Brightfield photomicrographs of C2C12 cells infected with the indicated retroviruses, grown for 5 days, and then switched to differentiation medium for the indicated times. MyHC staining of C2C12 cells 2 days after switch to differentiation medium is shown on the right. Scale bars: $150 \,\mu\text{m}$. (C) Quantification of results shown in B (4 days; *n*=3 replicates/experiments). (D) Proliferation curve of C2C12 cells infected with the indicated retroviruses, assessed by Trypan Blue staining. **P*<0.01. Results are means ± s.e.m.



Fig. 2. Pax3 expression in skeletal muscle stem cells induces myogenic differentiation. (A) Photomicrographs of SMPs transduced with an IRES-GFPexpressing retrovirus, 3 days after transduction (4 days after sorting) (left and middle panels) and 6 days after transduction (right panels). Scale bars: 200 μ m. (B) Percentage of elongated cells observed out of total cells infected with the indicated retroviruses (*n*=10). (C) Percentage of cells positive for myosin heavy chain, as assessed by immunofluorescence (*n*=3). (D) Percentage of multinucleated cells as assessed by DAPI staining (*n*=3). (E) Fluorescence micrographs of control SMPs or SMPs infected with retrovirus encoding *Pax3* and plated on Matrigel, and stained with anti-myosin heavy chain, DAPI or for GFP visualization. Scale bars: 150 μ m. (F) Percentage of BrdU-positive, GFP-positive cells infected with the indicated retroviruses after a 1 hour pulse as assessed by flow cytometry. Means ± s.e.m. **P*<0.01; ***P*<0.05. B, C, D and F were taken 3 days after transduction (4 days after sorting).

transduction efficiency in excess of 90% (Fig. 2A and data not shown). GFP was expressed at higher levels in vector-transduced SMPs than in Pax3-transduced SMPs (Fig. 2A), but nonetheless, was easily detected in both experimental conditions. This decreased GFP fluorescence was probably due to a decrease in GFP expression, with a transgene inserted on the transcript, rather than to a difference in viral titer, because we used a fluorescencebased retroviral titer kit to normalize before infection (data not shown).

In contrast to results obtained following transduction of C2C12 cells, ectopic expression of Pax3 in sorted juvenile SMPs promoted differentiation. Primary myoblasts elongate as they begin to differentiate (Springer et al., 2002), and we saw a significant increase in the frequency of elongated cells [whereas undifferentiated SMPs are round (Sherwood et al., 2004)] in Pax3-trandsuced cells compared with the control, GFP-only-transduced cells (Fig. 2A,B). These cells were not contaminating nonmyogenic, fibroblast-like Sca-1⁺ colony-forming cells (Sherwood et al., 2004), because reanalysis by FACS 7 days after culturing showed 0.0% Sca-1⁺ cells (data not shown). Additionally, an increase in the frequency of myosin heavy chain (MyHC, a marker of myogenic differentiation) positivity (Fig. 2C,E) and an increase in the frequency of multinucleated cells (Fig. 2D,E) in Pax3-transduced compared with GFP-only-transduced SMPs established that we were assessing true

myogenic differentiation. Induction of SMP differentiation was visible within 3 days of retroviral transduction (4 days after cell isolation), and by 7 days after isolation, dense colonies of elongated cells were observed in Pax3-transduced cultures (Fig. 2A). (Visual quantification of elongated cell morphology was not possible at this later time-point, because cells were too numerous to count.) Among Pax3-transduced SMPs, a fraction of both round cells and elongated cells were MyHC⁺ (Fig. 2E). Costaining with MyHC antibody and DAPI revealed both myoblasts and myotubes with multiple nuclei (Fig. 2E).

Since myogenic differentiation of SMPs can depend, to a certain extent, on the density of cells in culture (our unpublished observations), we asked whether ectopic Pax3 affected SMP proliferation. BrdU labeling followed by flow-cytometry analysis showed that proliferation was unaffected by Pax3 transduction (Fig. 2F).

Endogenous levels of Pax3 in mouse muscle

Differing estimates of the endogenous levels of Pax3 in adult satellite cells have been presented: Kuang and colleagues (Kuang et al., 2006) reported that sublaminar satellite cells in hindlimb muscle do not express *Pax3* mRNA, whereas others (Relaix et al., 2006; Sacco et al., 2008) reported that a subset of satellite cells in the hindlimbs do express *Pax3*. We were able to detect endogenous



Fig. 3. Endogenous expression of Pax3 in SMPs. (A) RT-PCR (non-quantitative) for Pax3 in freshly isolated hindlimb SMPs and SMPs transduced with Pax3 for 3 days. (B) Relative endogenous Pax3 mRNA levels in freshly sorted hindlimb SMPs (day 0), followed by in vitro culture for the indicated times. (C) Comparison of Pax3 mRNA levels by real-time RT-PCR (quantitative) in freshly isolated hindlimb, triceps and diaphragm SMPs. **P=0.07; *P<0.01. (D) Relative endogenous Pax3 mRNA levels in freshly sorted triceps SMPs (day 0), followed by in vitro culture for the indicated times. (E) Spontaneous differentiation of hindlimb vs triceps SMPs, as assessed by the percentage of elongated cells, 3 days (left) and 6 days (right) after isolation (n=3). *P<0.01; **P<0.05.

Pax3 mRNA in freshly sorted hindlimb juvenile SMPs by RT-PCR (Fig. 3A).

Pax3 mRNA levels were dramatically decreased in SMP daughter cells once they were cultured (Fig. 3b). Consistent with previous reports (Relaix et al., 2006), we detected higher levels of endogenous Pax3 mRNA in SMPs from triceps and diaphragm, compared with hindlimbs (Fig. 3C). Pax3 expression in triceps SMPs decreased even more rapidly than did Pax3 expression in hindlimb SMPs upon culturing (Fig. 3D). It has been reported that expression of Pax3 and Pax7 decreases as cells undergo differentiation (Brzoska et al., 2009); however, we found that Pax3 (Fig. 3B,D) and Pax7 (Fig. 4D) mRNA decreased sharply well before any of the morphological signs of differentiation discussed above could be detected. This decline in Pax3 expression appears to coincide with the entrance of normally quiescent SMPs into the cell cycle (Cerletti et al., 2008). Indeed, hindlimb SMPs contained nearly undetectable levels of Pax3 mRNA after 2 days of culture (Fig. 3B), whereas triceps SMPs had nearly undetectable levels after only 1 day of culture (Fig. 3D).

Because we observed higher levels of *Pax3* mRNA in SMPs isolated from triceps than those from hindlimb muscles, we asked whether these differences in *Pax3* expression might predict differences in the differentiation kinetics of muscle precursor cells isolated from these different muscle beds. Indeed, at both early (3 days) and late (6 days) time points, cultures initiated from triceps SMPs appeared to be more differentiated than equivalent cultures initiated with SMPs from hindlimb muscles (Fig. 3E).

Pax7 does not induce SMP differentiation

Similarly to Pax3, ectopic expression of Pax7 in C2C12 cells completely inhibited their myogenic differentiation (Fig. 4A,B). In SMPs, retroviral transduction of Pax7 had a similar, though more modest effect, inhibiting SMP differentiation as assessed by cell morphology (Fig. 4D), but not by MyHC staining (Fig. 4E). This was consistent with previous reports (Olguin and Olwin, 2004; Zammit et al., 2006; Olguin et al., 2007). The majority of SMPs express Pax7 protein in vivo and in vitro just after isolation (Cerletti et al., 2008), but *Pax7* mRNA expression is reduced substantially soon after placing SMPs in culture (Kumar et al., 2009) (Fig. 4F). This decrease parallels the decrease in *Pax3* expression that also occurs upon in vitro culture of SMPs (compare Fig. 3B,D with Fig. 4F).

Pax3 regulates differentiation without transcriptional upregulation of canonical myogenic regulatory factors

Pax3 and Pax7 have previously been reported to induce transcription of Myf5, a myogenic regulatory factor in the MyoD family, in embryonic development (Maroto et al., 1997; Bajard et al., 2006) and postnatal myogenesis (McKinnell et al., 2007), respectively. Most reports indicate that Pax3 lies upstream of MyoD1 (Maroto et al., 1997; Borycki et al., 1999; Relaix et al., 2006; Hu et al., 2008), but there is some evidence suggesting that this regulation is indirect (Buckingham and Relaix, 2007). In our experiments, ectopic expression of Pax3 in SMPs did not induce significant increases in the expression of any of the four canonical myogenic regulatory factors: Myf-5, MyoD, myogenin or Myf-6/Mrf4, either at 3 days or at 2 days after Pax3 transduction (Fig. 5A and data not shown). Myf-5, MyoD and myogenin were detectable in both control and Pax3-transduced SMP cultures, but were not significantly changed, whereas Mrf4 was undetectable in either condition, despite using three independent primer pairs. Met is another direct target of Pax3, and it has a functional role in development of limb musculature (Epstein et al., 1996); however, Met was also not upregulated in SMPs transduced to express Pax3 (Fig. 5A). QPCR for Pax3 itself demonstrated elevated levels in Pax3-transduced SMPs (Fig. 5B).



Fig. 4. Pax7 inhibits differentiation in C2C12 cells and has little effect in SMPs. (A) Bright-field photomicrographs of C2C12 cells infected with the indicated retroviruses, grown for 5 days, and then switched to differentiation medium for 4 days. Scale bars: $150 \,\mu\text{m}$. (B) Quantification of results in A (*n*=3). (C) Immunofluorescence for Pax7 in SMPs infected with the indicated retroviruses. Scale bars: $200 \,\mu\text{m}$. (D) Percentage of elongated SMPs infected with the indicated retroviruses (*n*=4). (E) Percentage of SMPs positive for myosin heavy chain, as assessed by immunofluorescence (*n*=2). (F) Relative endogenous *Pax7* mRNA levels in freshly sorted SMPs (day 0), followed by in vitro culture for the indicated times. **P*<0.01.

Our results with Myf-5 are consistent with an earlier report that direct control of the Myf5 promoter by Pax3 occurs in prenatal but not postnatal myogenic precursors (Bajard et al., 2006; Relaix et al., 2006). Suprisingly, however, enforced expression of Myf-5 or myogenin in SMPs did not accelerate the differentiation of juvenile SMPs, as assessed by morphology (Fig. 5C) or MyHC positivity (Fig. 5D). No increase in differentiation was observed at 2, 3, 4 and 5 days after transduction with MRFs (Fig. 5C and data not shown). Real-time RT-PCR confirmed that Myf-5 was expressed in Myf-5-transduced SMPs at significantly higher levels compared with control-vector-transduced SMPs (Fig. 5E). We were able to also detect an increase in myogenin in myogenin-transduced SMPs, although it was relatively weak (1.6-fold, P<0.01; Fig. 5F); and for unknown reasons, we were unable to detect ectopic expression of MyoD (data not shown). Visualization of GFP confirmed that transduction efficiency was similar to that obtained with the Pax3 or Pax7 retroviruses (Fig. 5G). To ensure that our retroviruses expressed functional Myf-5 or myogenin, C3H10t1/2 mouse fibroblasts were transduced with the same retrovirus preparations. Myf-5-transduced C3H10t1/2 cells formed myotubes that were long and thick, even before switching to differentiation medium; myogenin induced a similar effect (supplementary material Fig. S1), albeit with delayed kinetics (data not shown).

Knockdown of Myf-5 by lentiviral shRNA transduction showed that Myf-5 was not essential for spontaneous differentiation. Surprisingly, *Myf5* shRNA markedly induced differentiation (Fig. 5H,I). QPCR confirmed knockdown of endogenous Myf-5 (Fig. 5J).

Discussion

C2C12 myoblasts are one of the most commonly used models of in vitro myogenesis. Consistent with a previous report (Epstein et al., 1995), we found that ectopic expression of Pax3 inhibits myogenic differentiation of C2C12 cells; however, in contrast to the effects of Pax3 in C2C12 cells, ectopic expression of Pax3 augmented differentiation of primary mouse SMPs that were freshly isolated from skeletal muscle. These opposite effects on myogenic differentiation following Pax3 transduction might reflect differences in the expression of Pax3 cofactors in C2C12 versus SMP cells, which could result in induction by Pax3 of a different set of target genes in these two cellular contexts. Alternatively, Pax3 might induce the same target genes in both cells, but the downstream pathways that respond to these target genes might be distinct. It must be noted that C2C12 cells and SMPs were cultured under different conditions, so one must be circumspect about drawing a definitive comparison between the two; nevertheless, Pax3 blocked differentiation of C2C12 cells when grown in pro-differentiation conditions, and Pax3 induced differentiation of SMPs when grown in pro-proliferation conditions. Our data therefore indicate that caution must be taken in interpreting results from experiments using C2C12 cells as a model of normal satellite cells or myogenic precursor cells.

Although Pax3 significantly increased the frequency of cells that acquired an elongated morphology, MyHC positivity and multiple nuclei, the overall rate, at least as presented in our data, was still quite low. In comparison, it has been claimed that the majority of myogenic precursors contribute to myofibers when transplanted into injured muscle. Several factors might contribute to our relatively lower rate: (1) For most of our assays, cells were cultured in proliferation-enhancing conditions [high serum (20%) medium, daily addition of FGF, plating on collagen-laminin]. When plated on Matrigel, the percentage of Pax3-transduced SMPs which became elongated was ~20% (data not shown). (2) It is probable that cells in culture do not have the full complement of signals that would come from a niche in vivo, which would support myogenic differentiation. (3) Even in highly efficient engraftment in vivo, approximately 30 SMPs give rise to each single myofiber (Cerletti et al., 2008), a rate that is not markedly different than what we see in vitro. (4) At later time-points (6 days after sorting), a much higher proportion of SMPs showed differentiation, with Pax3 much



Fig. 5. Pax3 and myogenic regulatory factors. (A) Relative mRNA levels of the indicated genes in SMPs 3 days after transduction with Pax3 compared with vector (n=4). (B) Relative Pax3 mRNA levels 3 days after transduction with Pax3 compared with vector. (C) Percentage of elongated SMPs infected with the indicated retroviruses, 3 days after transduction (n=3). (D) Percentage of myosin heavy chain positive cells of total cells infected with the indicated retroviruses, as assessed by immunofluorescence, 3 days after transduction. (E) Relative My/5 mRNA levels of SMPs infected with the indicated retroviruses. (F) Relative Myog mRNA levels of SMPs infected with the indicated retroviruses. (G) GFP photomicrographs of SMPs transduced with the indicated retroviruses, 3 days after transduction. (H) Percentage elongated cells infected with the indicated lentiviruses (luc, non-targeting control shRNA) (two independent shRNA sequences; only one is shown). (I) Percentage myosin heavy chain positivity of total cells infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated set with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (J) Relative My/5 mRNA levels in SMPs infected with the indicated lentiviruses. (L) Relative My/5 mRNA levels in SMPs infected wit

greater than the control (Fig. 2A, right panels); but it was not possible to accurately quantify this because cells were too dense to count. Finally, (5) both retroviral and lentiviral transduction had an inhibitory effect on cell proliferation and differentiation, when vector-transduced cells were compared with untransduced cells. The appropriate comparison was therefore between vectortransduced and *Pax3*-transduced samples.

Our results in primary muscle stem cells suggest that Pax3 has a continuing role in promoting the differentiation of precursor cells in postnatal skeletal muscle. Consistent with other groups (Relaix et al., 2006), we found that triceps SMPs expressed higher levels of *Pax3* mRNA than hindlimb SMPs, and triceps SMPs exhibited a correspondingly higher predisposition to spontaneously differentiate. Although correlative, these data suggest that differences in the endogenous expression levels of *Pax3* might help to determine the relative rate of myogenic differentiation in postnatal muscles.

As mentioned above, Pax3 has been extensively characterized in embryonic and fetal developing muscle, whereas its role in postnatal muscle is less clear. Several groups have been able to detect Pax3 in adult satellite cells: (1) in a nuclear *lacZ* reporter knock-in model (Relaix et al., 2006); (2) with RT-PCR at a singlecell level, indicating that a minority of cells in the tibialis anterior muscle express appreciable levels of Pax3 (Sacco et al., 2008); and (3) with myofibers isolated from hindlimb muscles and cultured to release myogenic progenitor cells (Conboy and Rando, 2002). With these observations in mind, it was deemed worthwhile to assess the impact of Pax3 expression on postnatal muscle precursor cells. In a provocative recent paper, Pax3 was shown to be dispensable for adult muscle regeneration (Lepper et al., 2009). However, the study did find that Pax7 was essential up to the juvenile period, and nonessential thereafter. Thus, we restricted our studies of Pax3 to juvenile stem cells. Our experiments sought to uncover the role of Pax3 by using a relatively new method for obtaining a highly enriched population of skeletal muscle stem cells. Data from these analyses suggest that cultured SMPs from the juvenile period can indeed respond to induced expression of Pax3.

We observed a rapid and dramatic decrease in endogenous Pax3 expression upon placing SMPs in culture. This observation raises a perplexing question: how can Pax3 regulate myogenic differentiation in a positive fashion when it is itself downregulated as cells differentiate? We speculate that there exists a negative feedback loop whereby Pax3 sends an initial signal to facilitate the commitment to differentiation, and then later effectors in the process signal back to downregulate expression of Pax3. However, it has been reported that Notch-1 activation, which is associated with satellite cell activation, induces an upregulation of Pax3 expression (Conboy and Rando, 2002). These data seem difficult to reconcile; but satellite cell activation induced by Notch-1 might not be identical to spontaneous activation. It is possible that downregulation of Pax3 relates more to stem cell activation than to differentiation, and because induced Pax3 expression predisposes to differentiation, an initial reduction of Pax3 levels could be required for effective proliferation before differentiation.

Expression of dominant-negative Pax3 or Pax7 in cultured satellite cells results in the downregulation of MyoD, but myogenesis still takes place (Relaix et al., 2006). However, the effect of Pax3 fused to the engrailed repressor, which probably represents a more 'active' repressor, might not precisely mimic loss of Pax3 function. Also, the authors did find that in satellite cells lacking Myf-5, dominant-negative Pax3 prevents differentiation (Relaix et al., 2006). In contrast to our results, Boutet and co-workers (Boutet et al., 2007) found that levels of Pax3 mRNA and Pax3 protein increase with satellite cell activation and that enforced expression of stable Pax3 mutants in primary myoblasts inhibits differentiation. The disparity between these results and ours might reflect different cell populations, because our experiments used sorted cells from the myofiber-associated compartment freshly isolated from mouse muscle tissue (only 3-10% of this compartment). With regard to Pax7, our finding that overexpression of this transcription factor has a modest negative effect on differentiation of SMPs, whereas it completely abolishes differentiation of immortalized myoblasts, is consistent with earlier reports that Pax7 prevents MyoD-induced conversion of C3H10T1/2 cells (Olguin and Olwin, 2004), prevents myogenic differentiation of C2C12 cells (Zammit et al., 2006) and inhibits myogenesis of adult primary myoblasts independently of its transcriptional activity (Olguin et al., 2007).

Forced expression of any of the bHLH group of myogenic transcriptional regulators - Myf-5, MyoD, myogenin or Mrf4 converts a variety of cell types (e.g. fibroblasts, chondrocytes, neurons, amniocytes) to myoblasts (Berkes and Tapscott, 2005). Myf5 mRNA is first detected in the 8 day somite, and then is markedly reduced after day 14 (Ott et al., 1991). MyoD is expressed only in skeletal muscle and its precursors, and is repressed in nonmuscle lineages (Weintraub et al., 1991). Mice that are null for either Myf5 or Myod have apparently normal skeletal muscle, but closer analysis reveals mild defects in trunk skeletal muscle in Myf5-null mice (Braun et al., 1992), and a delay in early limb and branchial muscle development in Myod-null mice (Rudnicki et al., 1992). Mice that lack both Myf-5 and MyoD are born alive but are immobile and die soon after birth, and have no skeletal muscle. Immunohistochemical analysis showed an absence of desminexpressing myoblast-like cells (Rudnicki et al., 1993), suggesting that these factors are required for determination and/or propagation of skeletal myoblasts during embryonic development. Despite the severe phenotype of the Myf5 Myod double mutant, however, another group found that skeletal muscle was in fact present but only when Mrf4 expression was not compromised, and their conclusion was that Mrf4 is an additional myogenic determination gene that directs embryonic, but not fetal, development (Kassar-Duchossoy et al., 2004). Mice lacking myogenin have very poorly developed skeletal muscle, although myoblasts are present (Hasty et al., 1993; Nabeshima et al., 1993).

In our model of juvenile skeletal muscle stem cells, differentiation was induced by Pax3 without the transcriptional upregulation of any of the MRFs, because increases in MRF mRNA were not detectable despite >80% of SMPs transduced with high levels of Pax3. We cannot rule out the possibility that only a small minority of cells respond to Pax3 expression by upregulating the MRFs, and go on to differentiate. However, neither Myf-5 nor myogenin was capable of inducing differentiation of juvenile SMPs, despite their ability to convert C3H10t1/2 fibroblasts into myotubes; and surprisingly, knockdown of Myf-5 appeared to augment differentiation rather than inhibit it. These observations raise interesting questions about the transferability of conclusions drawn from prenatal muscle development to postnatal muscle development, especially juvenile muscle stem cell biology, and suggest that the latter might be driven by a distinct set of regulators. Identification of such novel regulators of postnatal myogenesis will probably contribute new approaches for enhancing muscle regeneration in the context of acute injury or aging.

Materials and Methods

Vectors

Mouse open reading frames for *Pax3*, *Pax7d*, *Myf5* and *Myog* were PCR amplified and cloned into the MSCV IRES-GFP retroviral vector. cDNAs encoding these four genes were kindly provided by A. Lassar, Harvard Medical School, Boston, MA. The hemagglutinin epitope tag was placed at the N-terminus of all the constructs.

Cells

SMPs were isolated as described (Cerletti et al., 2008). After myofiber-associated cell isolation, cells were separated by fluorescence-activated cell sorting based on the immunophenotype: Scal⁻ CD45⁻ Macl⁻ β 1 integrin⁺ CXCR4⁺. Growth media for these cells was F10, 20% horse serum, penicillin-streptomycin and non-essential amino acids. Cells were plated onto 96-well plates pre-coated with collagen (1 µg/ml) and laminin (10 µg/ml). For retroviral infections, 6000-7500 cells per well were plated. For the time-course experiments (e.g. Fig. 4B), 5000 cells per well were plated. Basic fibroblast growth factor (FGF) was added to 5 ng/ml daily without removing medium. For experiments where photomicrographs of myofibers were taken, cells were plated on 2% Matrigel. C2C12 cells were grown in DMEM + 10% fetal bovine serum. To differentiate, cells were grown to complete confluence, and then medium was switched to DMEM + 2% horse serum. C3H 10t1/2 cells were grown in DMEM + 10% fetal bovine serum, and were differentiated in the same way as C2C12 cells. HEK293T cells for retroviral production were grown in DMEM + 10% fetal bovine serum.

Viral transduction

HEK293T cells were transfected in 60 mm dishes with pcLEco (the ecotropic helper retroviral vector) and the above retroviral vectors in a 1:1 ratio, using Lipofectamine 2000 (Invitrogen). Supernatant was collected four times every 8-16 hours (2 ml per collection) and stored at 4°C until the final harvest. SMPs were infected 18 hours after FACS isolation, C2C12 cells 24 hours after plating and C3H10t1/2 cells 24 hours after plating. For SMPs, virus was incubated with 8 µg/ml polybrene for 1 hour. Medium was removed from the wells down to 50 µl and 100 µl virus was added with 10 µl horse serum and 3 µl FGF, and 3 hours later, 100 µl virus, 10 µl horse serum and 2 µl FGF were added. 26 hours later, medium (including virus) was removed down to 25 µl, and replaced with fresh growth medium with FGF. For lentiviral infections (shRNAs), glycerol stocks of plasmid were obtained from Sigma. Virus was produced in HEK293T cells with VSV-g and pHR8.10R in a 2:1:1 ratio. Supernatant was collected as with retrovirus, and virus was concentrated by ultracentrifugation for 3 hours at 20,000 r.p.m. and titered with a p24 ELISA kit (Cell Biolabs). In experiments where cell phenotype was quantitatively determined, two wells per sample were analyzed, and for elongated cell quantification (which could be done on live cells), at least three different timepoints were assessed and the entire well counted each time (2000-4000 cells).

Immunofluorescence

MyHC staining was carried out using a combination of anti-myosin Fast (My-32, Sigma M4276, 1:100) and anti-myosin Slow (NOQ7.5.4.D, Sigma M8421, 1:200). Before staining, cells were either transferred to LabTek tissue culture slides and allowed to grow for a further 2 days, or harvested and spot-dried on frosted slides.

QPCR

RNA was prepared with the RNeasy mini kit (Qiagen) and reverse transcribed with SuperScript II (Invitrogen). QPCR was performed using SYBR green cocktail (Qiagen) on a Stratagene Mx3005P. Primer sequences are as follows: *Pax3* F,

5'-ACTACCCA-GACATTTACACCAGG-3'; R, 5'-AATGAGATGGTTGAAAG-CCATCAG-3'; *Pax7* F, 5'-GCAGCTGGAGGAGGCTAGAGAAG-3'; R, 5'-GTCTCCTGGCTTGATGG-AGTCG; *Myf5* F, 5'-ACSAGAGCTGCTCTG-AGCCCACC-3'; R, 5'-TACATCA-GGACAGTAGATGCTGTC-3'; *Myod1* F, 5'-AGAATGGCTACGACACCGCCTAC-3'; R, 5'-TGGTGCATCTGCCAAAA-GCAGC-3'; *Myog* F, 5'-TGTTTGTAAAGC-TGCCGTCTGAC-3'; R, 5'-AAAAATTGGCAAAACCACAACAATGC-3'; *Met* F, 5'-GTCAATGACTTC-TTCAACAAGATTG-3'; R, 5'-ACAGCCGGAAGAGGTTTCT-CAG-3'. Data was normalized to 18S RNA.

Statistical analysis

Error bars in all figures depict s.e.m. For significance analysis, we used a Student's *t*-test.

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Supplementary material available online at

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