Chemistry & Biology
Article

Inhibitors of Tyrosine Phosphatases and Apoptosis Reprogram Lineage-Marked Differentiated Muscle to Myogenic Progenitor Cells

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DOI 10.1016/j.chembiol.2011.07.012

SUMMARY

Muscle regeneration declines with aging and myopathies, and reprogramming of differentiated muscle cells to their progenitors can serve as a robust source of therapeutic cells. Here, we used the Cre-Lox method to specifically label postmitotic primary multinucleated myotubes and then utilized small molecule inhibitors of tyrosine phosphatases and apoptosis to dedifferentiate these myotubes into proliferating myogenic cells, without gene overexpression. The reprogrammed, fusion competent, muscle precursor cells contributed to muscle regeneration in vitro and in vivo and were unequivocally distinguished from reactivated reserve cells because of the lineage marking method. The small molecule inhibitors downregulated cell cycle inhibitors and chromatin remodeling factors known to promote and maintain the cell fate of myotubes, facilitating cell fate reversal. Our findings enhance understanding of cell-fate determination and create novel therapeutic approaches for improved muscle repair.

INTRODUCTION

Skeletal muscle represents a classic example of terminal differentiation wherein myogenic proliferating cells expressing Pax7 and MyoD permanently withdraw from the cell cycle upon serum deprivation and physiologically fuse into multinucleated myotubes expressing muscle differentiation markers myogenin and eMyHC (Okazaki and Holtzer, 1966; Olson, 1992; Rudnicki and Jaenisch, 1995). The regenerative capacity of muscle stem cells declines upon aging and in certain diseases, exemplified by Duchenne muscular dystrophy. Hence, studying reprogramming of terminally differentiated muscle cells to their proliferating progenitors holds not only theoretical value but is also therapeutically relevant. The reprogramming from myotubes to myogenic precursor cells is particularly challenging because myogenic proliferating cells not only undergo postmitotic arrest, but also physically fuse with each other to form multinucleated myotubes during their terminal differentiation. Once these cells terminally differentiate, they are incapable of reentering into mitosis even when switched to serum-rich medium (Endo and Nadal-Ginard, 1986, 1998; Stockdale and Holtzer, 1961). In contrast, reserve cells (myoblasts, which remain mononucleated upon serum withdrawal) can reenter cell cycle when switched back to the mitogen-high serum-rich growth medium (Carnac et al., 2000; Friday and Pavlath, 2001; Yoshida et al., 1998). Several advances have been previously made in the field of muscle dedifferentiation. Overexpression of cyclin D1 and CDK4/6 or knocking down cell-cycle inhibitors alone or in combination is insufficient for myotubes to enter mitosis (Latella et al., 2001; Tiainen et al., 1996). Studies in C2C12 cells have shown that a fraction of myotubes derived from this cell line can dedifferentiate in the presence of newt extract, myoseverin, or when msx1 or twist are overexpressed (Duckmanton et al., 2005; Hjiantoniou et al., 2008; McGann et al., 2001; Odelberg et al., 2000; Rosania et al., 2000). However, the rare dedifferentiated cells were not tested for their ability to contribute to muscle regeneration in vivo. Earlier work has also reported that C2C12 myotubes responsive to thrombin-activated serum response factor triggers expression of immediate early genes but is not sufficient for S phase entry (Lööf et al., 2007). Interestingly, the same group also demonstrated that H3K9 di-methylation remains unperturbed in C2C12 myotubes in the presence of serum, as opposed to salamander myotubes, which readily enter cell proliferation. A recent study has shown deletion in Ink4a locus in C2C12 immortalized cell lines, which provides an advantage to C2C12 cells to enter the cell cycle upon knockdown of Rb. Knockdown of pRb in conjunction with Arf can induce cell-cycle entry in primary myocytes but not in primary myotubes, where nuclei get arrested at the onset of mitosis (Pajcini et al., 2010). Nevertheless, the process of dedifferentiation of primary multinucleated myotubes is still not well understood, and most of the previous studies relied on the overexpression of exogenous genes. Some of the previous studies have employed single myocyte and myotube isolation, which can lead to preferential selection of those myotubes that survive such process and does not clear ambiguity of reserve cells that can come along with myotubes. Sparse plating of myoblasts was also tried, but that prevents formation of multinucleated myotubes and limits the study to myocytes. To address these challenges, we performed muscle reprogramming studies in differentiated lineage-marked primary myotubes generated by the physiological fusion of Rosa26-Lox-YFP myoblasts with Cre-expressing myoblasts, where the multinucleated myotube cell fate results in the recombination of YFP locus and expression of YFP. Our work critically examined and identified small molecule inhibitors that are necessary and sufficient for the dedifferentiation of myotubes to their progenitor cells without forced expression of specific genes. Briefly, these

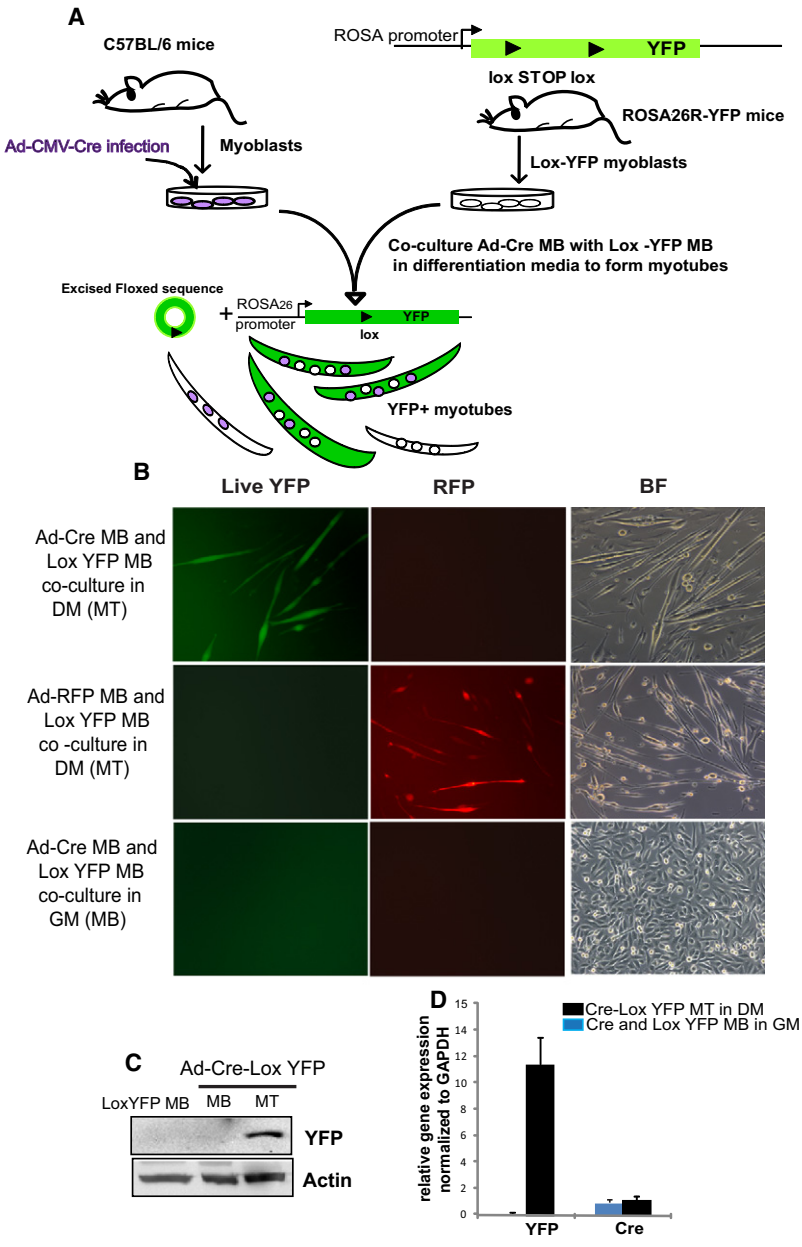

Figure 1. Lineage Marking of Primary Myotubes by Cre-Lox Method

(A) Schematic of the system. Wild-type myoblasts (MB) derived from C57BL/6 mice were infected with Ad-Cre and subsequently cocultured with Lox-YFP MB obtained from Rosa 26-YFP reporter mice in differentiation medium (DM) to form myotubes. The fusion of these two populations of MB led to the excision of stuffer sequence (green circle) by Cre recombinase activity to give rise to lineage marked YFP-expressing myotubes (green). Self fusion among the two populations of MB will give rise to YFP-negative myotubes (colorless). These lineage marked myotubes were then used in dedifferentiation studies.

(B) Fusion-dependent, Cre-Lox mediated labeling of myotubes upon coculture of Ad-Cre MB with Lox-YFP MB in DM. As described in (A), wild-type MB were cocultured with Lox-YFP MB (1:2 ratio) in DM to induce formation of myotubes. Endogenous YFP fluorescence in myotubes was observed by 72–96 hr, as shown by epifluorescent images. In control infection with control Ad-RFP virus, no YFP fluorescence was observed. No YFP expression was observed upon coculture of Ad-Cre MB and Lox YFP MB in GM where myoblasts did not undergo physiological fusion to form myotubes.

(C) Western blotting to determine YFP expression using lysates from Lox-YFP MB, Ad-Cre MB cocultured with Lox-YFP MB in GM and parallel in DM. YFP protein was observed in the myotubes, which arose from fusion of Ad-Cre and Lox-YFP MB in DM.

(D) qRT-PCR analysis for YFP gene expression. RNA was extracted from Ad-Cre, and Lox-YFP MB was cocultured in GM and in DM for 96 hr to detect the levels of YFP and Cre recombinase by qRT-PCR. Data were normalized to internal control GAPDH. Error bars indicate mean and standard deviation, n = 3. YFP mRNA levels was only observed in Ad-Cre-Lox-YFP$^+$ myotubes while Cre recombinase expressed in both the cocultures of Ad-Cre and Lox-YFP MB in GM and in DM. The fusion-dependent marking of myotubes was clearly and robustly mediated by this adaptation of the Cre-Lox method, and no mononucleated cells expressed YFP.

See also Figure S1.

studies demonstrate that, in the presence of tyrosine phosphatase (BpV) and apoptosis (Q-VD) inhibitors, Cre-Lox lineage-marked myotubes exhibited altered morphology, down-regulated terminal differentiation markers, up-regulated markers of myogenic progenitor cells, and attenuated the cell-cycle inhibitors p21, p15, and p16. According to BrdU incorporation, the dedifferentiation efficiency was ~12%. To further validate the labeling technique and dedifferentiation of labeled myotubes, the lineage-marked myotubes, followed by their dedifferentiation into mononucleated cells, were captured by time lapse microscopy. The dedifferentiated proliferating cells maintained their myogenic identity and were capable of expansion in culture, as well as redifferentiation into myotubes in vitro and in vivo. Furthermore, at the level of molecular mechanism, this work established that phosphatase and apoptosis inhibitors caused down-regulation of a number of chromatin remodeling factors and components that are necessary for the maintenance of terminal myogenic differentiation, thus predisposing myotubes for muscle precursor cell fate. The controlled reprogramming from postmitotic multinucleated terminally differentiated cell fate into a progenitor cell fate of the same lineage provides new insights into adult tissue formation and enables unique clinical strategies for enhancing muscle regeneration.

RESULTS

Fusion-Dependent Lineage Marking of Primary Myotubes

To overcome instances of mistaken cell identity during reprogramming studies, we first established a method to genetically and irreversibly label terminally differentiated myotubes using the Cre-Lox technique (Figure 1A). The Cre-Lox system has

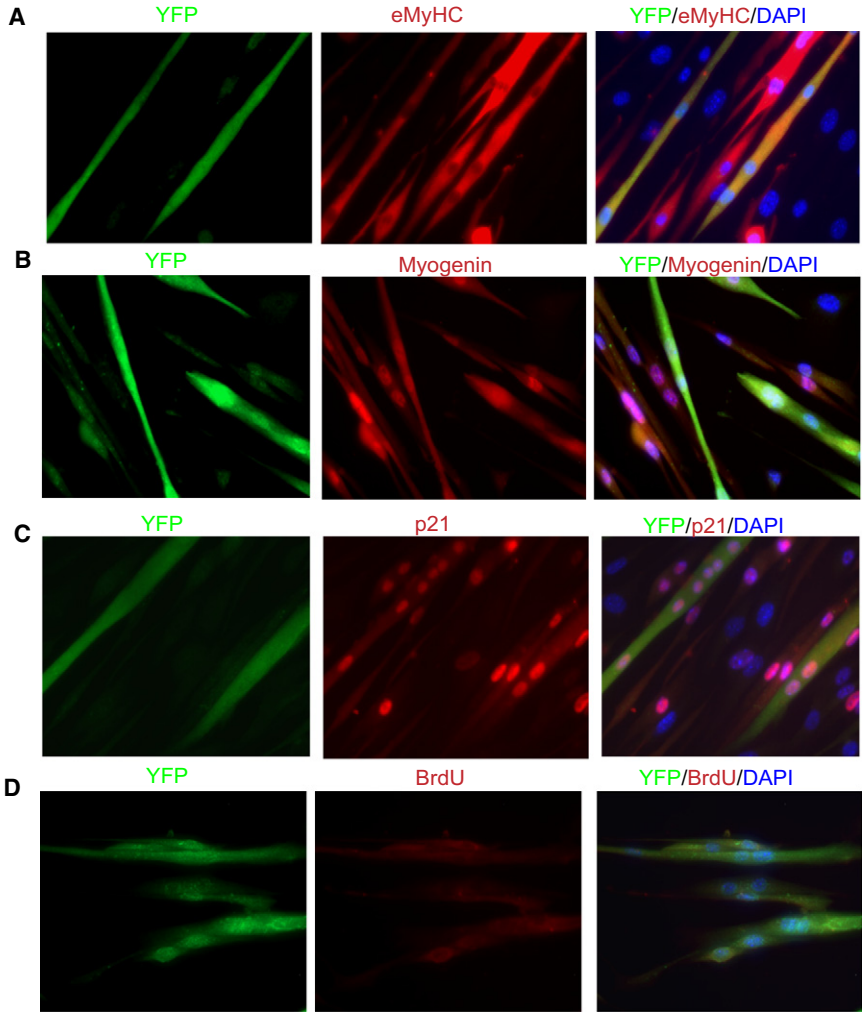

Figure 2. Immunodetection of YFP and Muscle Specific Markers

(A–D) YFP$^+$ myotubes obtained after Cre-Lox fusion express muscle differentiation marker and does not incorporate BrdU. Cre-Lox YFP$^+$ myotubes cultures were coimmunostained with muscle differentiation markers eMyHC (A), myogenin (B), p21 (C), and DNA synthesis label BrdU (D) along with anti-YFP antibody. Representative images are shown.

been widely used for tissue-specific disruption of genes, utilizing the P1 bacteriophage Cre recombinase, which specifically recognizes Lox sites and excises any DNA sequence flanked by these sites (Nagy, 2000). The schematic of the system is represented in Figure 1A. Cre recombinase was expressed exogenously by adenoviral mediated infection (Ad-Cre) in wild-type primary myoblasts (MB). Ad-Cre-infected myoblasts (Cre-MB) were cocultured with Lox-YFP myoblasts (Lox-YFP MB) derived from Rosa26 YFP reporter mice (Srinivas et al., 2001) in the ratio of 1:2, so that more numbers of Lox YFP myonuclei coexist with Ad-Cre myonuclei in single myotube and yield high expression of YFP. In standard low-mitogen differentiation-promoting medium (DMEM, 2% horse serum), these cocultured MB within 72–96 hr physiologically fused into YFP-expressing myotubes (henceforth, YFP$^+$ myotubes), where both Cre and Lox-YFP myonuclei coexisted (Figure 1B). No mononucleated cells expressing YFP were observed, thus confirming the validity of this lineage marking strategy. Adenoviral infection control was also performed by coculturing Ad-RFP infected MB with Lox-YFP MB in differentiation medium (DM) and this did not yield YFP$^+$ myotubes (Figure 1B).The YFP$^+$ myotubes accounted for around 70% of total myotubes formed within 96 hr. Of these, around 60% of YFP$^+$ myotubes had 2–4 myonuclei, whereas 30% had 5–7 myonuclei with an average number of 4.5 myonuclei per YFP$^+$ myotube. Non-YFP myotubes that arose from syngeneic fusion events of Cre-MB or Lox-YFP MB were also detected. This labeling strategy was captured by time lapse microscopy encompassing a total of 4 days from the coculture of Cre and Lox YFP myoblasts to their fusion into multinucleated myotubes (see Movie S1 available online). Representative images of time lapse microscopy are shown in Figure S1. To further rule out any possibility of YFP expression without physiological myoblast fusion, specific control experiments were conducted. The Cre–MB were cocultured with Lox-YFP MB in mitogenic growth medium (GM; Ham's F10, 20% BGS, 9 ng/ml bFGF-2), where cells remained mononucleated and did not fuse into myotubes (Figure 1B). After 96 hr of coculture in GM, cells were processed for Western blotting (Figure 1C), qRT-PCR analysis (Figure 1D), and immunostaining (Figure S1) for YFP expression. No YFP expression was observed by any of these techniques. A more stringent control experiment was performed to check the possibility of horizontal transfer of Cre recombinase, without complete fusion process. For this, Cre myoblasts were cultured in differentiation medium to form myotubes. Later, Lox YFP MB (2×10^5 cells) were added to 96-hr-old Cre-expressing myotubes, and cultures were switched to mitogenic growth medium for 72–96 hr. As seen in Figure S1E, both epifluorescent imaging and anti-YFP staining confirmed the absence of YFP-expressing mononucleated cells without physiological fusion of Cre-MB and Lox-YFP MB into myotubes. These results clearly show that indeed YFP$^+$ myotubes arose only from the fusion of Cre and Lox-YFP MB in DM. The results obtained and quantified with YFP live-direct fluorescence were completely consistent with the data produced using anti-YFP immunofluorescence, and both assays were routinely employed throughout these studies. The YFP$^+$ myotubes were positive for muscle differentiation markers myogenin and eMyHC and for CDK inhibitor p21 (which indicates the postmitotic state) (Figures 2A–2C). Furthermore, these YFP$^+$ myotubes did not incorporate BrdU, confirming that all YFP$^+$ marked cells produced by fusion

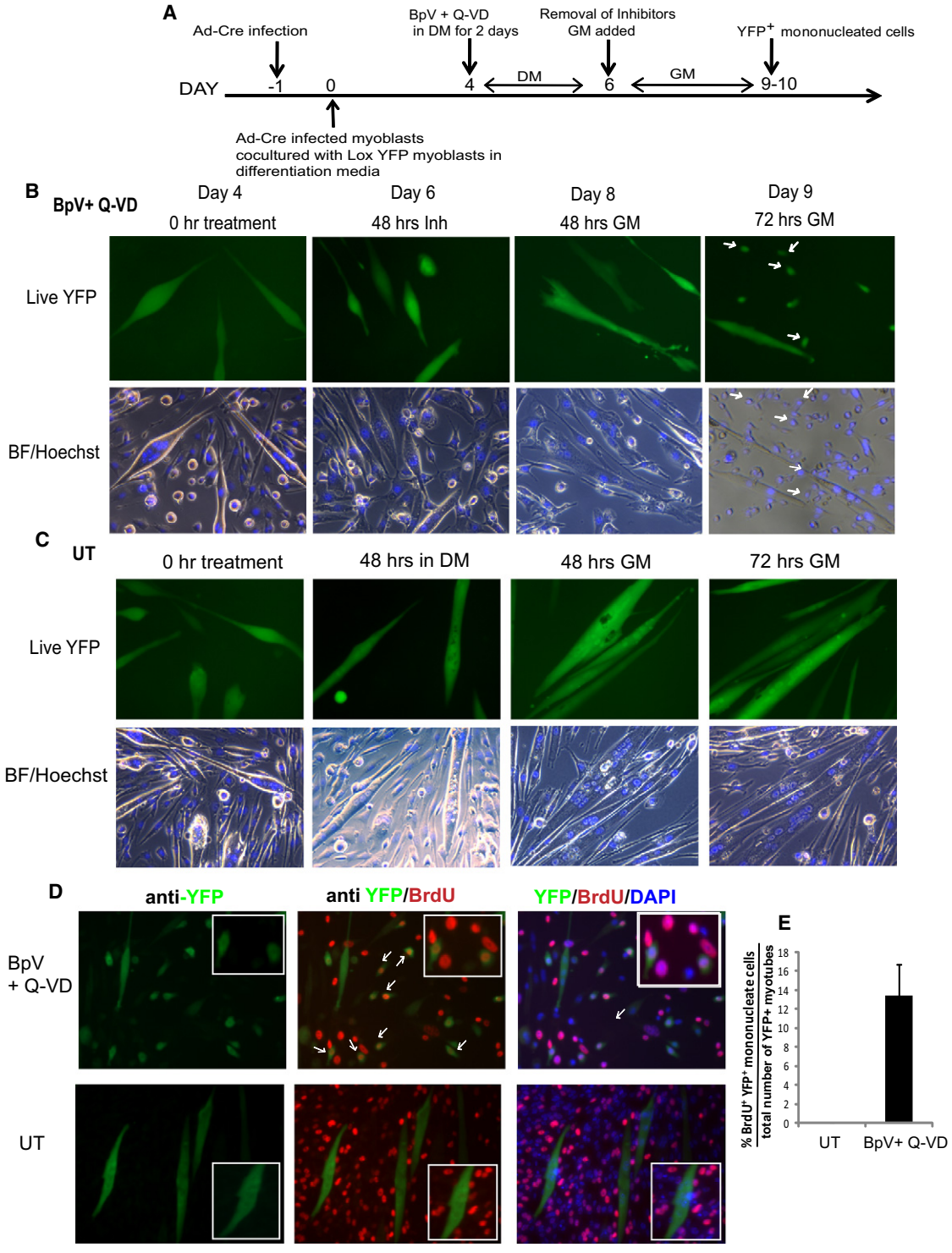

Figure 3. BpV with Q-VD Dedifferentiates the Irreversibly Labeled YFP$^+$ Myotubes to YFP$^+$ Proliferating Mononucleated Cells

(A) Myotube dedifferentiation strategy. MB infected with Ad-Cre were cocultured with Lox-YFP MB in DM for 4 days to give rise to YFP$^+$ myotubes. These were treated with 10 μM BpV plus 10 μM Q-VD in parallel with other experimental conditions for two days in DM. The treated myotubes were then switched to myoblast GM, which was replaced fresh every day. YFP$^+$ mononucleated cells were observed around day 10.

(B) Dedifferentiation of YFP$^+$ myotubes to YFP$^+$ proliferative cells. YFP$^+$ myotubes cultures were treated with the BpV plus Q-VD and were photographed every day. The addition of BpV plus Q-VD led to morphological changes and when switched to GM these cells expanded as YFP$^+$ mononucleated cells in 72 hr (white arrow shows YFP$^+$ mononucleated cells). Representative high magnification images of dedifferentiation experiment over the course of 10 days with live Hoechst is shown by epifluorescent microscopy.

of Cre and Lox-YFP MB have exited cell cycle and are in postmitotic arrest by 96 hr in DM (Figure 2D). These findings establish an unambiguous lineage marking of terminally differentiated myotubes that is dependent on physiological myoblast fusion.

Combination of a Tyrosine Phosphatase Inhibitor and Apoptosis Inhibitor Reprograms Lineage Marked Myotubes into Proliferating YFP$^+$ Mononucleated Cells

Myotube formation involves many events, such as changes in cytoskeletal assembly, sequential expression of differentiation-specific genes, modulation of signaling pathways, and up-regulation of tyrosine phosphatases (Bennett and Tonks, 1997; Delgado et al., 2003; Lassar et al., 1994; Weintraub, 1993). Hence, we reasoned that global transient inactivation of tyrosine phosphatases would reset signaling in myotubes, making them receptive to mitogens present in growth medium conditions and propelling them into cell cycle as well as toward a less-differentiated state. Earlier BpV (a tyrosine phosphatase inhibitor) was reported to delay differentiation of dividing C2C12 into myotubes (Castaldi et al., 2007), and other studies also indicated that a small percentage of myotubes that enter S-phase upon overexpression of genes fail to proliferate and succumb to apoptosis (Endo and Nadal-Ginard, 1998; Latella et al., 2000). Therefore, we reasoned that if BpV was able to trigger the process of myotube dedifferentiation and that the addition of an apoptosis inhibitor (Q-VD) in our studies may help in survival of those myotubes that might undergo massive restructuring of cell cytoskeleton simultaneously with the breakage of postmitotic arrest. Using our lineage marking technique for myotubes, we then explored whether BpV plus Q-VD (hence forth inhibitor mix) was capable to reprogram already differentiated primary myotubes to their muscle progenitor fate. For dedifferentiation assays, inhibitor mix (10 μM each) was added to YFP$^+$ myotube cultures daily for two days, after which cultures were switched to GM (Figure 3A). Remarkably, in the presence of inhibitor mix, a considerable number of YFP$^+$ myotubes showed altered morphology and cleaved into small cells, which were followed by the appearance of YFP$^+$ mononucleated progeny of the dedifferentiated myotubes (Figure 3B). When myotubes produced by the fusion of primary myoblasts were treated with BpV alone, cell death occurred as described in previous studies (Rumora et al., 2003). Interestingly, in the presence of BpV alone, myotubes did show apoptosis and few of them gave rise to YFP$^+$ mononucleated cells, albeit at very low frequency (~1.18%) in comparison to inhibitor mix treatment, which augmented the dedifferentiation frequency to~12%–13% (Figures S2A, S2C, and S2D) These results demonstrate that the inhibition of apoptosis is a critical requirement for the dedifferentiation of multinucleated primary myotubes. In control experiments, no YFP$^+$ mononucleated cells were observed in untreated YFP$^+$ myotubes that were switched to GM for 72 hr (Figure 3C), or in the presence of Q-VD alone (Figure S2B). Furthermore, the YFP$^+$ cells derived from the postmitotic multinucleated myotubes engaged in proliferation, where ~12%–13% of total lineage-marked population was found to incorporate BrdU during a 24-hr BrdU labeling interval (Figures 3D and 3E). The former myotube identity of these YFP$^+$ cells clearly discriminated them from the reactivation of reserve YFP$^-$ myoblasts, which also proliferated and expanded when myotube cultures were switched to the highly mitogenic GM (Figure 3D). To rule out any spurious YFP expression in the absence of Cre-expressing cells and in the presence of inhibitor mix, 4-day-old Lox YFP myotube cultures were treated with the inhibitor mix and then switched to growth medium. No YFP expression was ever observed in these cultures. This clearly establishes that Lox YFP cells do not spontaneously express YFP upon addition of inhibitor mix in the absence of Cre recombinase (Figure S4A). To capture reprogramming of lineage-marked myotubes, we also performed time lapse microscopy, where Ad-Cre-Lox YFP myotubes were labeled for 96 hr, followed by the addition of inhibitor mix for another 48 hr, and the cultures were then switched to GM. Live cell imaging was performed for the total period of 4 days, where inhibitor mix-treated YFP$^+$ multinucleated myotubes gave rise to YFP$^+$ mononucleated cells (Movie S2). Representative images of the time lapse imaging can be seen in Figure S3. A combination of a small molecule tyrosine phosphatase inhibitor, BpV, and an apoptosis inhibitor, Q-VD, was necessary and sufficient for such reprogramming of terminally differentiated muscle cells. Because inhibition of tyrosine phosphatases induces apoptosis, the possibility that some aspect of apoptosis may mediate the reprogramming of multinucleated myotubes to undergo dedifferentiation was also examined. Doxorubicin (0.2 μM), a classic inducer of apoptosis that has been studied in muscle (Latella et al., 2004), was added to Cre-Lox myotube cultures for 48 hr either alone or in combination with the apoptosis inhibitor. This was followed by removal of drugs and switching cultures to growth media conditions for 72–96 hr. We observed reduced apoptosis by doxorubicin in the presence of apoptosis inhibitor, but no YFP$^+$ mononucleated cells were observed in these cultures, in spite of altered morphology of myotubes (Figures S4B and S4C). This suggests that apoptosis does not mediate the dedifferentiation of myotubes.

In parallel to these experiments, we also addressed a possible role of Oct4 in myotube reprogramming, considering the pivotal reprogramming activity of Oct4 and the ability of this transcriptional factor alone to reprogram neural stem cells (Kim et al.,

(C) No-treatment (UT). Untreated YFP$^+$ myotubes were grown in similar conditions and did not show any dedifferentiation events. These data demonstrate that inhibitor mix is necessary and sufficient for dedifferentiation of genetically labeled myotubes into expanding mononucleated cells.
(D) Reprogrammed YFP$^+$ mononucleated cells rapidly divide. Cre-Lox-YFP$^+$ myotubes reprogrammed as depicted in Figures 3B and C, were pulsed with BrdU for 24 hr and costained with anti YFP and BrdU antibodies. Arrows indicate representative BrdU$^+$ YFP$^+$ cells in treated conditions. Untreated cultures of YFP$^+$ myotubes do not show any YFP$^+$ mononucleated cells, though BrdU incorporation is seen in non-YFP-cycling mononucleated cells. Inset shows magnified images.
(E) Quantification of percentage of BrdU$^+$ YFP$^+$ mononucleated cells out of total number of YFP$^+$ myotubes (shown are the mean and standard deviations, $n = 3$, $p < 0.05$). Note that many reserve myoblasts reentered cell cycle and incorporated BrdU in GM (both in the presence of BpV$^+$ Q-VD and in control untreated cultures); these cells, however, were reliably distinguished in our experiments by the absence of YFP.
See also Figures S2–S5.

2009). We used our published method (Conboy and Conboy, 2010) to activate endogenous muscle stem (satellite) cells by injury into hind limb muscle of Tet-Oct4 mice (Hochedlinger et al., 2005) and derived primary myoblasts that were kept in DM to form myotubes and then treated or untreated with doxycycline (dox) for 24 and 48 hr, to induce Oct4 protein and mRNA expression (Figure S5). These Tet-Oct4 myoblasts were infected with Ad-Cre and were cocultured with Lox-YFP MB in DM for 96 hr, where myotubes were readily formed (Figure S5). No significant changes in morphology of myotubes were observed for up to 48 hr of Oct4 induction, followed by growth media incubation for an additional 4 days (Figure S5D). In concert with earlier studies, where it has been reported that induction of Oct4 in the differentiated cells of the intestine and hair follicle has no effect on their cellular phenotype (Hochedlinger et al., 2005), our results showed that Oct4 induction up to 2 days was not sufficient to induce any morphological changes in myotubes or to promote their dedifferentiation in GM (Figure S5).

YFP$^+$ Mononucleated Cells from Dedifferentiated Myotubes Reexpress Markers of Myogenic Progenitors, Downregulate Markers of Myotubes, Attenuate p21, and Retain Their Myogenic Potential to Fuse into De Novo Myotubes

To further assess the properties of reprogrammed cells, mononucleated YFP$^+$ progeny of dedifferentiated myotubes was FACS-sorted and expanded in culture (Figure S6A). These YFP$^+$ mononucleated cells were immunostained with antibody against Ki67 (proliferation marker) and BrdU (S phase marker) along with YFP. As shown in Figure S6C, these FACS-sorted expanded YFP$^+$ cells positively immunostained for Ki67 and incorporated BrdU. Furthermore, cell-cycle analysis by propidium iodide DNA staining confirmed that these cells can proliferate and exist in different phases of cell cycle (Figure S6E). To confirm that the actively dividing reprogrammed YFP$^+$ cells are indeed myogenic, they were analyzed for the myogenic markers Pax7, MyoD1, and differentiation markers myogenin, eMyHC, and Cdk inhibitor, p21 (Figure 4A). On the basis of the quantification of the immunofluorescence, around 70% of YFP$^+$ mononucleated cells expressed high levels of Pax7, and $\geq$90% expressed MyoD1 (Figure 4B). The differentiation capability of the YFP$^+$ mononucleated cells was tested by switching the cultures to DM, where normal primary myoblasts exit cell cycle and fuse into multinucleated myotubes. The YFP$^+$ precursor cells were found to retain their myogenic potential as they underwent rapid physiological fusion de novo into myotubes that expressed typical muscle differentiation markers eMyHC, myogenin, and p21 (Figure 4C). Thus, the markers of terminal differentiation that were down-regulated upon myotube reprogramming with inhibitor mix treatment were up-regulated again when YFP$^+$ myogenic progenitor cells differentiated into de novo myotubes in the mitogen-low differentiation medium (Figure 4D). The changes in marker gene expression were also validated at the transcriptional level by qRT-PCR, which clearly showed the up-regulation of eMyHC, p21, and myogenin and down-regulation of Pax7 and MyoD mRNA levels upon differentiation of YFP$^+$ mononucleated cells (Figure 4E). Thus, according to the profile of myogenic markers and the functional properties, dedifferentiated genetically labeled progeny of primary myotubes acquired the fate of muscle precursor cells or myoblasts.

Reprogrammed YFP$^+$ Cells Can Contribute to In Vivo Muscle Regeneration

The ultimate test was to make sure that these reprogrammed cells could contribute to in vivo muscle regeneration under physiological conditions. The dividing dedifferentiated YFP$^+$ cells were expanded in GM for ~1.5–2 weeks and were injected into cardiotoxin-injured tibialis anterior (TA) of immunodeficient NOD-SCID mice. Injections of unrecombined Lox-YFP myoblasts and buffer medium served as negative controls. Two weeks after injection, muscles were dissected out, sectioned, and immunostained for YFP and laminin. YFP$^+$ reprogrammed cells readily fused with regenerating myofibers and contributed to muscle repair in vivo (Figure 5). These results establish that postmitotic myotubes can dedifferentiate into functional, proliferating myogenic precursor cells that regenerate muscle tissue after an injury. Importantly, cultured muscle precursor cells that eagerly regenerate muscle in vivo were produced from terminally differentiated primary myotubes without an exogenous gene expression, making this method therapeutically feasible.

Inhibitor Mix Treatment Modulates Gene Expression in Myotubes

To address the mechanism by which inhibitor mix facilitated dedifferentiation of YFP$^+$ myotubes, we analyzed early changes in expression of eMyHC (a terminal muscle differentiation marker), myogenin (a muscle marker expressed on onset of differentiation), and p21, p15, and p16 (CDK inhibitors) in Ad-Cre-Lox YFP$^+$ myotubes treated with inhibitor mix for 48 hr (Figures 6A and 6B). By immunofluorescence, over 60% of the myonuclei in YFP$^+$ myotubes down-regulated myogenin as compared to untreated myotubes, whereas the levels of p21, a negative regulator of mitosis that plays an important role in cell-cycle arrest (Bunz et al., 1998; Cayrol et al., 1998), were found to be attenuated in approximately 25% of the myonuclei present in YFP$^+$ myotubes (Figures 6C and 6D). The down-regulation of eMyHC, p21, p15, p16, and myogenin in Ad-Cre-Lox-YFP$^+$ myotubes treated with inhibitor mix was also confirmed by Western blotting experiments and qRT-PCR analysis (Figures 6E and 6F). These results demonstrate that the myogenic cell fate is reversed at the genetic level in multinucleated myotubes, before they split into single dividing cells. We also observed that inhibitor treatment increased the expression of YFP in Cre-Lox myotubes both at protein and RNA level. As shown earlier in Figure S4A, Lox YFP myotubes do not express YFP spontaneously upon inhibitor mix treatment and in the absence of Cre recombinase. However, when YFP locus is recombined, the broad-range phosphatase inhibitor mix, which is known to modulate numerous signaling pathways by acting at the transcriptional as well as posttranscriptional levels, might activate ROSA locus at the transcriptional level, thereby increasing YFP expression.

Studies have also shown that, upon myotube differentiation, widespread chromatin remodeling occurs, and genes necessary for differentiation are activated while those for proliferation are repressed (Forcales and Puri, 2005; Guasconi and Puri, 2009;

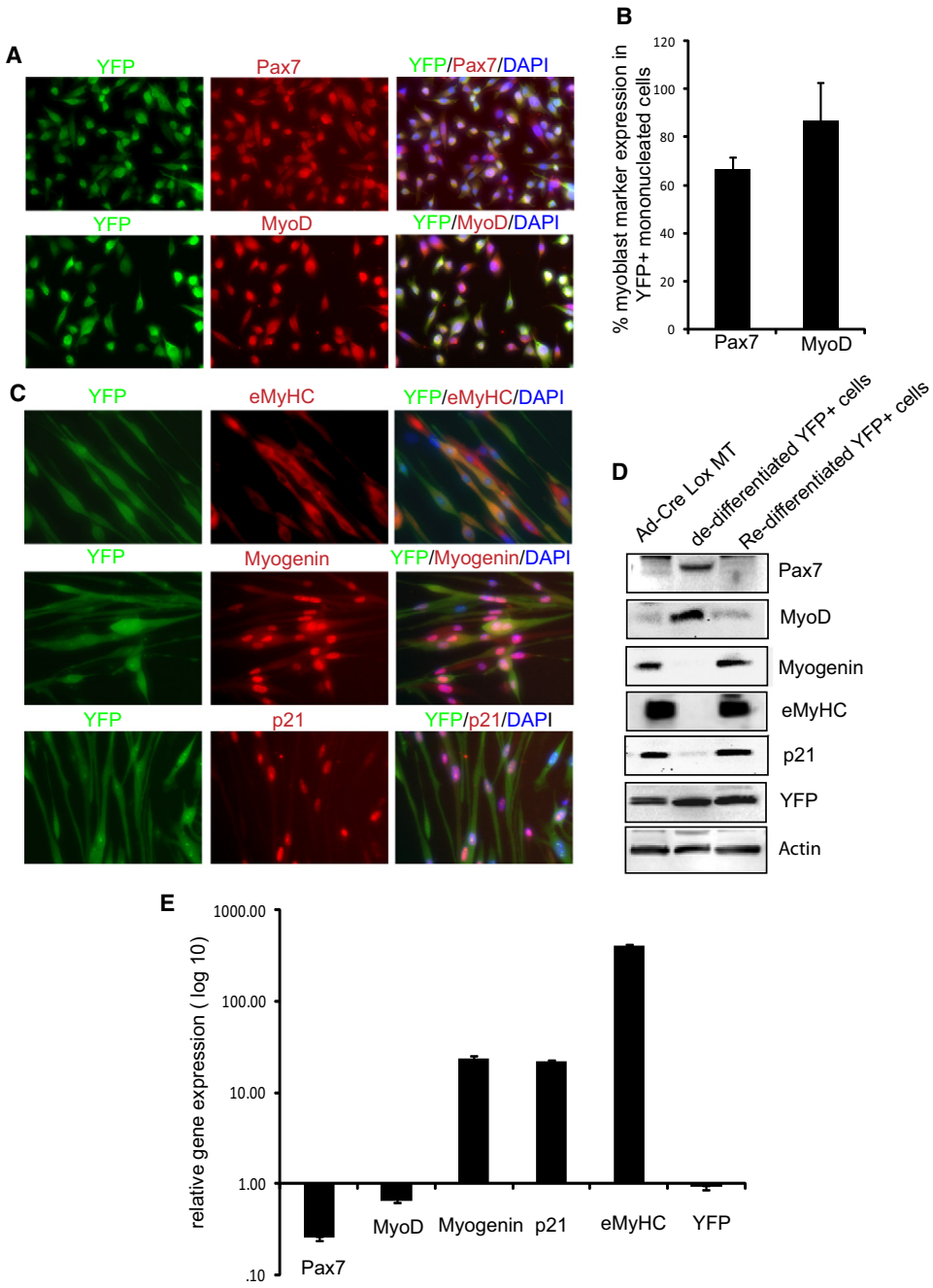

Figure 4. Genetically Labeled Progeny of Dedifferentiated Myotubes Have Functional and Genetic Attributes of Muscle Progenitor Cells

(A) Coimmunostaining of FACS-sorted, proliferating YFP$^+$ mononucleated cells for (1) Pax7 and (2) MyoD along with anti-YFP antibody was performed, and representative images are shown.

(B) Histogram quantifies Pax7- and MyoD-expressing YFP$^+$ mononucleated cells, which represents mean and standard deviation of three independent experiments.

(C) Dedifferentiated, FACS sorted, YFP$^+$ cells were expanded in GM and cultured in DM for 96 hr where myoblasts typically form myotubes; cultures were coimmunostained with antibodies specific to YFP and to myotube specific marker (1) eMyHC, (2) myogenin, as well as (3) the CDK inhibitor p21.

(D) Western blotting with antibodies specific for Pax7, MyoD, eMyHC, p21, myogenin, and YFP was performed using protein extracts from Cre-Lox-YFP myotubes, dedifferentiated YFP$^+$ mononucleated cells, and redifferentiated YFP$^+$ cells as indicated. Actin served as loading control.

(E) Gene expression analysis of muscle differentiation markers. qRT-PCR data in log scale for Pax7, MyoD, myogenin, p21, and eMyHC depicts the relative gene expression of redifferentiated myotubes to dedifferentiated YFP$^+$ cells. The data represent the mean and standard error for three independent experiments.

See also Figure S6.

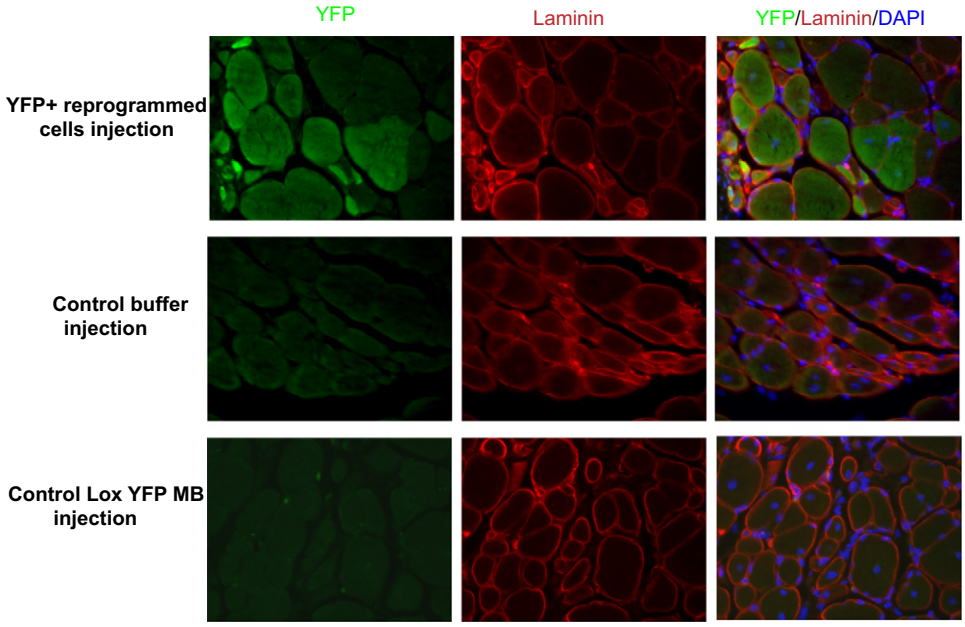

Figure 5. Reprogrammed YFP$^+$ Proliferative Cells Contribute to In Vivo Muscle Regeneration

FACS sorted YFP$^+$ proliferating mononucleated cells were expanded in GM and injected in cardiotoxin injured tibialis anterior (TA) immunocompromised NOD-SCID mice. Two to three weeks later, TA muscles were dissected out, sectioned at 10 μm, and costained with YFP and laminin to visualize YFP$^+$ myofibers. Control buffer and Lox YFP myoblast injected TA muscle did not show any YFP$^+$ myofibers.

McKinsey et al., 2002; Palacios and Puri, 2006; Sartorelli and Caretti, 2005). Because a subset of labeled myotubes enters cell cycle and proliferates in GM, we reasoned that inhibitor mix has a global effect and that perturbation of signaling pathways would, in turn, affect chromatin remodeling, thereby facilitating reprogramming of myotubes to their progenitor cells. To analyze these changes during inhibitor mix treatment, PCR arrays for chromatin enzymes and chromatin remodeling factors were performed on untreated and inhibitor mix-treated Cre-Lox YFP myotube cultures (Figure 7). As summarized in Figures 7C and 7D, Carm1, Suv39h1, and SWI/SNF complex components, which have been earlier shown to promote myogenic differentiation (Ait-Si-Ali et al., 2004; Chen et al., 2002; de la Serna et al., 2001), were down-regulated upon inhibitor mix treatment along with other histone methyltransferases. Tables S1 and S2 provide a complete list of chromatin factor and enzyme genes modulated by inhibitor mix treatment. These findings suggest that inhibitor mix down-regulates the chromatin factors and enzymes dedicated to the maintenance of differentiated state in primary myotubes, enabling them to respond to the growth factors present in serum and dedifferentiate to YFP$^+$ proliferating progenitor cells.

DISCUSSION

Our studies explored the small molecule pharmacological approach to dedifferentiation and reprogramming that does not involve overexpression of exogenous genes, which is the currently searched for method in the field of cell reprogramming. The use of a broad pharmacological inhibitor of tyrosine phosphatases simultaneously with the inhibition of apoptosis was, in our hands, sufficient to induce actual reprogramming of terminally differentiated postmitotic multinucleated skeletal muscle cells into their progenitors. The use of small molecule inhibitors for reprogramming studies has high translational significance. The apoptosis inhibitors used in our studies are a reversible treatment, have a short half life, and are not present in cells when expanded in vitro for transplantation experiments. Hence, the reprogrammed myogenic progenitor cells transplanted with the aim to alleviate myopathic conditions will not be resistant to apoptosis and, consequently, will not pose the risk of cancer. In this work, the irreversible cell-fate lineage marking of myotubes was based on the fact that terminally differentiated skeletal muscle cells are normally produced by the fusion of myoblasts. Thus, Lox-YFP Rosa 26 nuclei and Cre-containing nuclei at some point coexisted in a multinucleated cell in order to produce YFP$^+$ myotubes. The Cre-Lox myotube labeling method efficiently distinguishes reserve cells from multinucleated myotubes. Our data show that 4-day-old cultures of YFP$^+$ primary myotubes express typical muscle differentiation markers, such as myogenin and eMyHC, express high levels of CDK inhibitor p21, and do not incorporate BrdU, which strongly suggests that these YFP-marked cells are indeed terminally differentiated (Figures 2A–2D). The observation that dividing YFP$^+$ mononucleated myogenic progeny were obtained from YFP$^+$ myotubes unambiguously establishes the reprogramming step toward a less-differentiated precursor cell. Importantly, such genetic labeling for the first time demonstrates dedifferentiation of mature 4-day-old multinucleated primary myotubes into proliferating fusion-competent myoblasts that expand in vitro and repair muscle in vivo.

The calculation of reprogramming efficiency from terminally differentiated myotubes to the muscle progenitor cells is complicated by the fact that heterogenous Cre and Lox YFP myoblasts

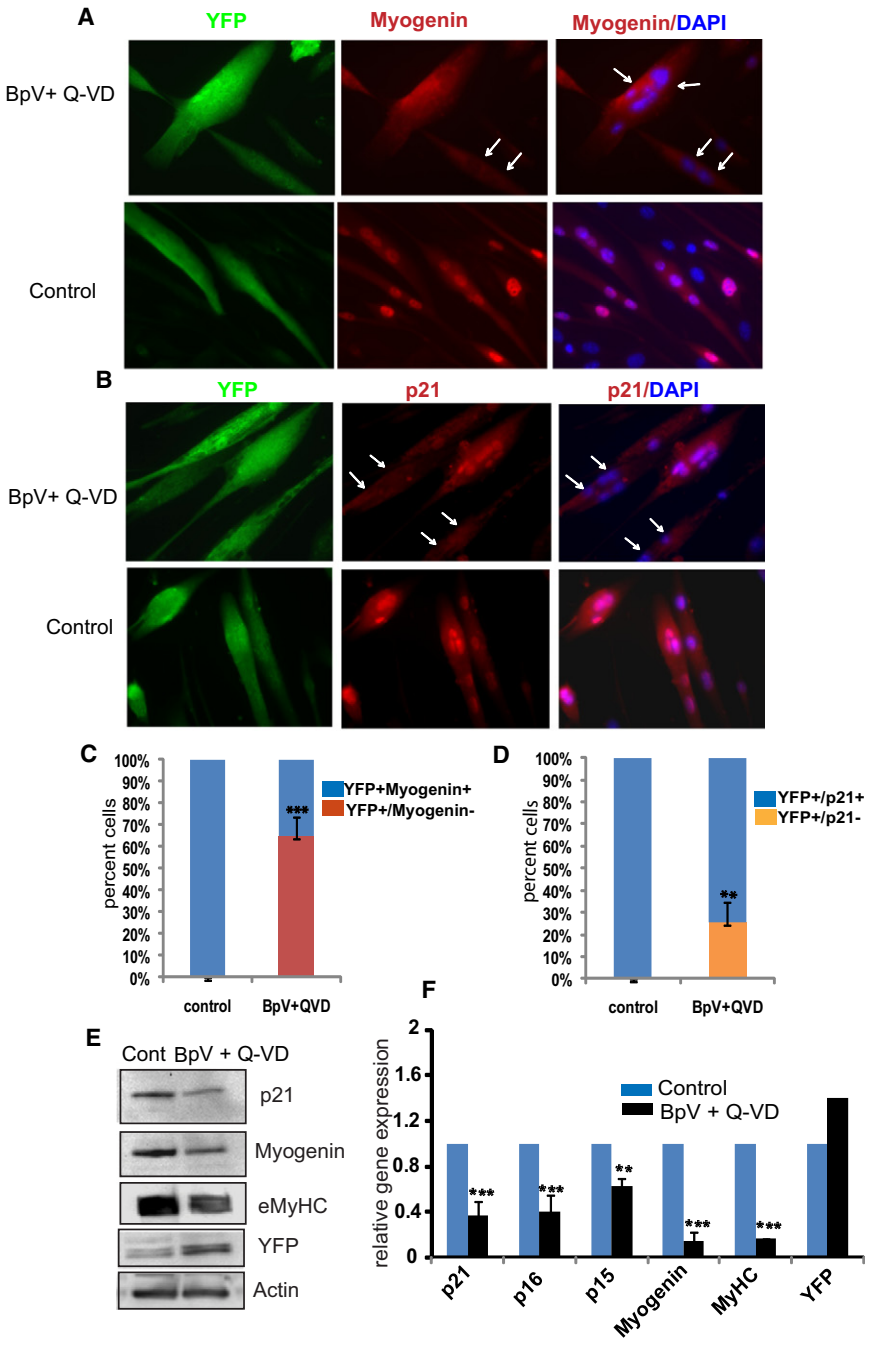

Figure 6. Molecular Analysis of Reprogramming in Genetically Labeled Myotubes

Inhibitor mix treatment down-regulates muscle differentiation marker in Cre Lox-YFP myotubes.

(A and B) Four-day old Ad-Cre-Lox-YFP myotubes were untreated/treated with inhibitor mix for 48 hr, followed by immunodetection of myogenin (A), p21 (B), and YFP (green), using antibodies specific for these proteins. Myogenin and p21 were down-regulated in a subset of YFP^+ myotubes (shown by white arrows). Control myotubes did not change expression of muscle differentiation markers.

(C and D) The histogram quantifies the percentage of YFP^+/myogenin$^+$, YFP^+ myogenin$^-$ cells and $YFP^+/p21^+$ and $YFP^+/p21^-$ in the experiment shown in (A) and (B) ($n = 3 \pm SD$; $p^{***} < 0.001$, $p^{**} < 0.05$).

(E and F) Ad-Cre- Lox-YFP myotubes untreated/treated with BpV plus Q-VD for 48 hr were analyzed for protein and mRNA levels. Protein lysates were subjected to Western blotting for antibodies against p21, myogenin, and eMyHC. Actin served as a loading control. q-RT-PCR was performed on RNA lysates for gene expression of p21, p15, p16, myogenin, and eMyHC. Data were normalized to GAPDH and represent mean and standard deviation of three independent experiments each done in triplicates ($n = 3 \pm SD$; $p^{***} < 0.001$, $p^{**} < 0.05$). Untreated sample was taken as 1.

form YFP^+ myotubes, where varying numbers of both myonuclei coexist in the same multinucleated myotube. Furthermore, only Lox YFP myonuclei, and not Ad-Cre MB nuclei, coexisting in YFP^+ myotubes will give rise to YFP^+ mononucleated cells when labeled myotube dedifferentiates. Moreover, the dedifferentiation of myotubes that are produced by syngeneic fusion events was not accounted for in our YFP-labeling method. Hence, we estimated efficiency by two different methods. By method 1, the total number of YFP^+ mononucleated cells was divided by the total number of YFP^+ myotubes before inhibitor treatment. According to this method, efficiency was estimated as ~12%–13% in the presence of inhibitor mix as compared to BpV alone, which was~1.18% (Figure S2D). By method 2, the total number of YFP^+ mononucleated cells was divided by an estimated number of Lox YFP myonuclei present in all labeled YFP^+ myotubes before inhibitor treatment (see Experimental Procedures). This method gave an estimate of ~5% in the presence of inhibitor mix and ~0.4% in the presence of BpV alone (Figure S2D; Experimental Procedures). For the above reasons, we think that these calculations give very conservative estimates of dedifferentiation. No matter the method of quantification, BpV alone gave poor reprogramming efficiency and apoptotic inhibitor was needed to augment the dedifferentiation likely by facilitating the survival of those myotubes that undergo reprogramming in the presence of phosphatase inhibitor.

Recent reports have shown that cells expressing higher levels of antiproliferative genes and those involved in senescence are indeed difficult to reprogram (Li et al., 2009; Utikal et al., 2009). Because myotubes are postmitotically arrested cells that express high levels of CDK inhibitors, these may have low reprogramming efficiency. Studies have indicated that experimental down-regulation of CDK inhibitors in postmitotic myotubes results in accumulation of DNA damage, hinders cell cycle reentry, and cause DNA fragmentation and apoptosis

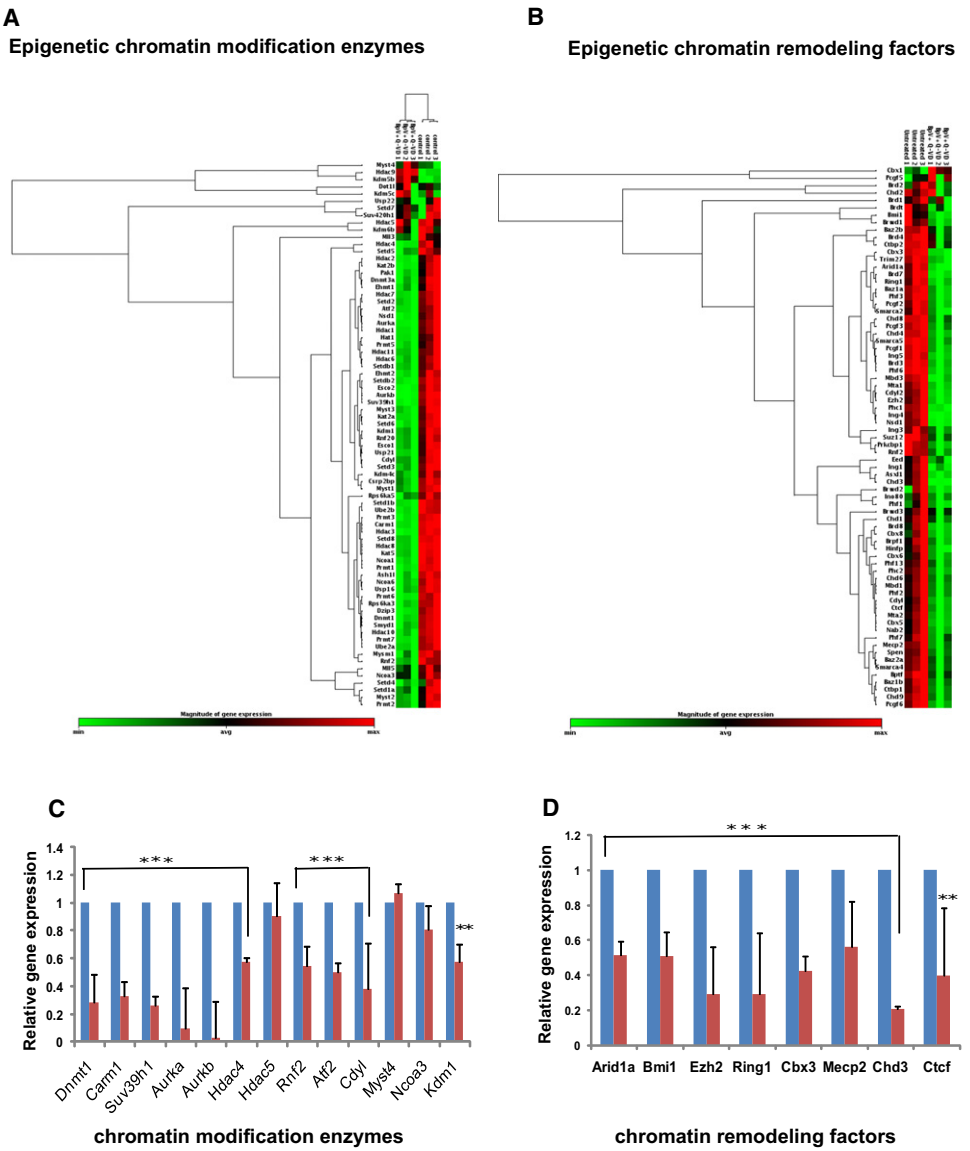

Figure 7. Inhibitor Mix Treatment Modulates Chromatin Remodeling Factors and Enzymes

(A and B) Clustergram analysis of chromatin remodeling factors and enzymes for Ad-Cre-Lox YFP myotubes treated and untreated with BpV plus Q-VD (inhibitor mix) for 48 hr using SA Biosciences/QIAGEN PCR arrays; 0.5 μg of RNA isolated from three independent set of experiments of Ad-Cre-Lox YFP myotubes were reverse transcribed, and gene expression profile was monitored.

(C and D) Histogram representation for few set of genes normalized by Hprt gene levels. Control untreated was taken as 1 (n = 3 ± SD; $p^{***} < 0.001$, $p^{**} < 0.05$). See also Tables S1 and S2.

(Pajalunga et al., 2010) It has been shown that dividing cells robustly repair DNA damage (Nouspikel and Hanawalt, 2002), and because our dedifferentiated cells are cultured in mitogen-high growth medium (following the BpV and apoptosis inhibitor treatments) and are actively dividing, any DNA damage accumulated in myotubes is likely to be repaired during the process of DNA replication. Notably, our results definitively demonstrate that reprogrammed myotubes give rise to functional muscle progenitor cells which form new muscle in vitro and in vivo; hence, no irreversible DNA damage or mutations occurred to compromise the myogenic properties of the dedifferentiated cells. Recent studies have also shown that inhibition of Rb and p16/p19 can induce cell-cycle entry in postmitotic myocytes (Pajcini et al., 2010). Our work conducted on 4-day-old mature myotubes not only down-regulated CDK inhibitors and muscle differentiation markers but also decreased gene expression of chromatin remodelers that maintain differentiated state. The role of chromatin organization in establishment and maintenance of cell fates has been well defined. It has also been shown to play a role in the commitment of myoblast to terminally differentiated myotubes, because different signaling pathways have been shown to modulate chromatin signaling in muscle progenitor cells upon differentiation (Caretti et al., 2004; de la Serna et al., 2001; McKinsey et al., 2002; Palacios and Puri, 2006; Saccone

and Puri, 2010). Recent work has also demonstrated the stage-specific role of Ezh2 in muscle regeneration, where Ezh2 occupies the Pax7 regulatory sequences in a differentiated state (Palacios et al., 2010). This suggests that, in spite of down-regulation of Ezh2 upon differentiation, as reported earlier (Caretti et al., 2004), a certain level of Ezh2 along with other polycomb members would be required to maintain muscle progenitor genes in a repressed state. Our PCR arrays on Cre-Lox myotubes after inhibitor mix treatment demonstrate significant down-regulation of Ezh2 and other polycomb members, as compared to untreated myotubes, suggesting the creation of sensitized background in myotubes that may force them to reexpress muscle progenitor genes and enter the cell cycle. The conversion of cell fates due to enhanced cell fate plasticity in polycomb compromised backgrounds has been documented in other model systems, such as flies (Lee et al., 2005) and worms (Yuzyuk et al., 2009). In this regard, our work highlights the fact that key players in maintaining and establishing a terminally differentiated state need to be erased transiently at the epigenetic level in order to switch a differentiated state to a less differentiated state within the same cell lineage.

Because we used a generic tyrosine phosphatase and apoptosis inhibitor, which is expected to modulate signaling in many biochemical pathways, the down-regulation of specific chromatin remodeling factors predisposed primary myotubes to respond to the mitogens of GM, thereby changing their cell fate to that of proliferating myogenic precursor cells. Interestingly, although upon the addition of inhibitor mix there were profound changes in the morphology of many myotubes, only a subset of these myotubes dedifferentiated into proliferating mononucleated precursor cells. As there is a temporal progression toward the degree of terminal differentiation in myotubes, there might be a specific time window when myotubes would be more responsive to the treatment and capable of undergoing cell fate reversal. Future work would determine the exact role of chromatin remodeling factors in acquiring muscle progenitor cell fate from the multinucleated postmitotic differentiated state. Since there is sequential gene expression upon myotube differentiation, it would be interesting to study in the future whether the inhibitor mix is sufficient to induce dedifferentiation in mature myofibers formed in vivo or indeed whether other factors are required to yield regenerative cells.

SIGNIFICANCE

Use of pharmacological inhibitors to modulate different signaling pathways without gene overexpression is therapeutically relevant in coaxing differentiated cells to yield regenerative cells. Our data highlight the combinatorial use of tyrosine phosphatase and apoptosis inhibitors for primary myotube dedifferentiation to yield myogenic proliferating cells, which aided in muscle regeneration both in vitro and in vivo in SCID mice. The myotube labeling technique developed for this study served as a powerful tool to clearly show the origin of reprogrammed cells and to sort them away from reactivated reserve myoblasts. Importantly, small molecule inhibitors induced changes in myotubes at epigenetic level and facilitated them to enter proliferative state in mitogen rich medium. All together, the labeling technique employed, along with the use of small molecule inhibitors, advanced the ongoing research in regenerative medicine and would enable unique clinical strategies for enhancing tissue regeneration.

EXPERIMENTAL PROCEDURES

Animal Strains

B6;129-*Gt(ROSA)26Sor*$^{tm1(rtTA*M2)Jae}$ *Col1a1*$^{tm2(tetOPou5f1)Jae}$/J strain (stock number: 006911), B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J strain (stock number: 006148), NOD.CB17-*Prkdcscid*/J (stock number: 001303), and C57BL6/J (2–3 months old) mice were obtained from pathogen-free breeding colonies at The Jackson Laboratories. Animals were housed at the Northwest Animal Facility, University of California, Berkeley, and procedures were performed in accordance to administrative panel on the Office of Laboratory Animal Care, UC Berkeley.

Reagents, Antibodies, Western Blotting, and Immunofluorescence

BpV(phen) (Alexis Biochemicals), doxycycline (Sigma), and apoptosis inhibitor (Q-VD-OPh, Non O methylated Cat No. 551476, Calbiochem), BrdU (Sigma), ECM (Sigma), Hoechst 33342 (Sigma), DNase1 (Sigma), propidium iodide (molecular probes), Rnase A (Fermentas), and mouse bFGF (R&D) were purchased. Antibodies to BrdU (ab6326), GFP (ab6556 and ab13970), Ki67 (abcam ab155800), and Oct4 (ab18976) were from Abcam. Antibodies against Actin (rabbit polyclonal) were from Sigma, Pax7 was from DSHB (Developmental Studies Hybridoma Bank), eMyHC was from DSHB and Upstate, and mouse monoclonal antibodies against myogenin, MyoD, and p21 were from Santa Cruz Biotechnology. For Western blotting, the cells were lysed in RIPA buffer (50 mM Tris-Cl [pH7.6], 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 1 mM EDTA, and 1× Protease inhibitor, Sigma), and protein concentration was determined by Bradford Assay. Thirty micrograms of protein was run on precast 4%–20% Gels (BioRad) and then was transferred to nitrocellulose membrane for 2 hr, and protein expression was detected by the BioRad Gel Doc/Chemi Doc imaging system and Quantity One Software. For immunofluorescence, cells were fixed with 4% PFA for 15 min at room temperature, followed by permeabilization with 0.25% Triton X-100 for 12 min, and were blocked for 1 hr in blocking buffer (1% BGS plus 0.1% Na-Azide in 1× PBS) followed by primary antibody incubation in blocking buffer for 2 hr or overnight and secondary antibody incubation for 1 hr in blocking buffer with Alexa fluorophore-conjugated species-specific secondary antibody (Invitrogen). For BrdU labeling, cells were pulsed with 10 μM BrdU for either 2 or 24 hr, fixed with 4% PFA for 15 min, and permeabilized with 0.25% Triton X-100 for 12 min, followed by DNase1 treatment (0.2 units/μL) for 30 min at room temperature. For all immunofluorescence assays, cells were mounted with mounting media containing DAPI (Prolong Gold Antifade, Invitrogen) to visualize nuclei in all immunostaining experiments.

Primary Myoblast Isolation

Primary myoblasts were obtained by isolation of satellite cells from these mice, as described previously (Conboy and Conboy, 2010). TA and gastrocnemius muscle of the different transgenic mice were injected with a total of 5 μg of cardiotoxin (Sigma) dissolved in 1× PBS, and muscle was dissected out after 3 days of injury, as described elsewhere (Conboy and Conboy, 2010). In brief, muscle underwent enzymatic digestion at 37°C in DMEM (Cellgro) plus 1% penicillin/streptomycin plus 250 units/ml of Collagenase Type II (Sigma) solution for 1.5 hr on a rocker with slight agitation. Bulk myofibers were purified by repeated rounds of trituration, sedimentation, and washing to remove interstitial cells, tendons, and so froth. Satellite cells were purified from these bulk myofibers by incubation in 0.5 units/ml dispase at 37°C for 0.5 hr, followed by sedimentation, washing, and fine mesh straining. The satellite cells were then cultured on plates coated with 1:500 ECM (Sigma) in myoblast growth medium containing Ham's F-10 nutrient mixture (GIBCO) plus 20% bovine growth serum (BGS; Hyclone), 9 ng/ml bFGF (R&D), and 1% penicillin/streptomycin. Later, proliferating fusion-competent myoblasts were preplated to remove fibroblasts from culture.

Adenovirus Infection and Primary Myotube Labeling

Wild-type MB or Tet-Oct4 MB obtained were infected with Ad-CMV-Cre or Ad-RFP control virus (Vector BioLabs) for 4–6 hr, washed off, and cultured in GM for 24 hr. Ad-Cre MB were then lifted off the plates, counted, and cocultured with Lox-YFP myoblasts in differentiation-inducing medium DMEM (Cellgro) plus 2% horse serum (Sigma) and 1% penicillin/streptomycin (BD Falcon) on 12-well ECM-coated plates. The 96-hr-old myotubes were visualized for YFP expression, treated with different experimental conditions, and photographed every day using a Zeiss epifluorescence microscope Axio Observer A.1 fitted with a Zeiss EYFP filter (BP 500/20 FT515 BP 535/30) at 10× and 20× objective.

Dedifferentiation Assay

WT myoblasts or Tet-Oct4 (3×10^5) myoblasts, upon 4–6 hr infection with 300 MOI of Ad-Cre virus, were washed with growth medium twice to get rid of any residual virus and were incubated for another 24 hr in growth medium at 37°C in 5% CO_2 incubator. These myoblasts were then lifted off the plates by the addition of 1× PBS, counted (3×10^4 cells/well), and cocultured with 7×10^4 Lox-YFP myoblasts (obtained from B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J mice strain) per well in differentiation-inducing medium DMEM (Cellgro) plus 2% horse serum and 1% penicillin/streptomycin on 12-well plates (BD Falcon) coated with ECM (1:500). Later, 96-hr-old YFP$^+$ myotube cultures were treated with BpV (phen) (10 μM) along with apoptosis/caspase inhibitor (Q-VD-OPh) (10 μM) daily for 2 days with and without doxycycline (2 μg/ml) in differentiation medium. The inhibitors were then withdrawn, and cells were switched to growth medium containing bFGF (9 ng/ml). The treated cells were fed fresh growth medium and bFGF every day over a period of time, and the morphology of YFP-expressing myotubes was routinely visualized by epifluorescence microscope using a YFP filter and were photographed using 10× and 20× objectives. For live Hoechst staining, 4 μM of Hoechst was added every day to YFP-labeled myotube cultures, incubated for 10 min at 37°C in 5% CO_2 incubator, washed off to remove unlabeled Hoechst, and photographed.

Calculation of Myotube Reprogramming Efficiency

The labeled YFP$^+$ myotubes per well in 4-day-old Cre-Lox myotube cultures were determined to be ~2600. After inhibitor treatment, ~350 YFP$^+$ mononucleated cells were found in culture. Hence, reprogramming efficiency was calculated as the percentage of YFP$^+$ mononucleated cells out of the total number of labeled YFP$^+$ myotubes, which was determined to be ~13.46%. Another method was based on the determination of Lox YFP myonuclei in labeled myotubes. Because an average number of myonuclei present in each YFP+ myotube is ~4 and for myotube labeling, Cre and Lox YFP myoblasts were cultured in a ratio of 1:2, hence Lox YFP myonuclei were estimated to be ~6940 [(2600 × 4) × 2 / 3]. Therefore, reprogramming efficiency calculated was the percentage of YFP$^+$ mononucleated cells out of an estimated total number of Lox YFP myonuclei in YFP$^+$ myotubes and was determined to be ~5.04% [(350 / 6940) × 100] (see Figure S2D).

Cell Transplantation

Reprogrammed YFP$^+$ mononucleated cells (1×10^6) expanded in GM in culture were resuspended in medium containing Ham's F-10 with 2% BGS and were injected in 24-hr cardiotoxin preinjured TA muscles of NOD-SCID mice (4 weeks old) obtained from Jackson Laboratories. Control injections were performed with medium alone or with Lox YFP myoblasts resuspended in medium. After 2–3 weeks of cell injections, TA muscles were dissected out and fixed in 4% paraformaldehyde (PFA) for a minimum of 2 hr and subsequently were washed three times with 1× PBS for total of 45 min. The muscles were then sequentially transferred to 2%, 5%, and 10% sucrose in PBS with slight agitation at room temperature for 30 min to 1 hr. Finally, muscles were left overnight in 20% sucrose at 4°C and were frozen in liquid nitrogen-cooled iso-pentane in OCT embedding medium. Muscle sections (10 μm) were cut and mounted on superfrost slides; 300 μm serially spaced muscle sections from whole block were postfixed with 4% PFA for 10 min, permeabilized for 10 min with 0.25% Triton X-100, and blocked in goat serum. Primary antibodies against YFP (chicken polyclonal GFP, Abcam) and laminin (rat laminin from Sigma) were added overnight, followed by 1 hr of incubation in the respective fluorochrome-conjugated secondary antibodies (goat anti-chicken 488 and goat anti-rat 546) from Molecular probes.

RNA Isolation and qRT-PCR

RNA isolation was performed with an RNAeasy Kit (QIAGEN) according to the manufacturer's recommendations. For real-time qPCR, transcribed cDNA was diluted 1:5 for every sample, and 1 μl of this diluted cDNA was used for a 25-μl PCR containing 80 nM forward and reverse primers, and 12.5 μl of 2× iQ Syber Green mix (Bio-Rad) was run on an iQ5 cycler (Bio-Rad); data were analyzed by iQ5 optical system software. The values were normalized against internal control GAPDH and were plotted using the ΔΔCt method. The primers against specific genes for qRT-PCR are as follows: GAPDH-F, 5′-GGGAAGCCCATCA CCATCT-3′ and GAPDH-R, 5′-GCCTCACCCCATTTGATGTT-3′; Myogenin-F, 5′-GACCCTACAGACGCCCACAA and Myogenin-R, 5′-CCGTGATGCTGTCC ACGAT-3′; MyoD-F, 5′-CGGCTCTCTCTGCTCCTTTG-3′ and MyoD-R, 5′-GA GTCGAAACACGGGTCATCA-3′; Pax7-F, 5′-CCCTCAGTGAGTTCGATTAGC-3′ and Pax7-R, 5′-CCTTCCTCGTCGTCCTCTTTC-3′; p21-F, 5′GAACATCT CAGGGCCGAAAA-3′ and p21-R, 5′-TGCGCTTGGAGTGATAGAAATC-3′; eMyHC-F, 5′-AGAGGACGTGTATGCCATGA-3′ and eMyHC-R, 5′-TGGCCA TGTCCTCAATCTTGT-3′; Cre-recombinase-F, 5′-GCCGGGTCAGAAAAAAT GG and Cre recombinase-R, 5′-AGGGCGCGAGTTGATAGCT-3′; eYFP F, 5′-GCACGACTTCTTCAAGTCCGCCATGCC-3′ and eYFP R, 5′-GCGGATCTT GAAGTTCACCTTGATGCC-3′. Primers for p15 and p16 have been described elsewhere (Li et al., 2009). For SA biosciences/QIAGEN PCR arrays, 0.5 μg RNA was reverse transcribed using SA biosciences RT kit, and PCR arrays were performed according to the manufacturer's instructions. The Web-based PCR array data analysis tool of SA biosciences was utilized for data analysis.

Flow Cytometry

De-differentiated YFP$^+$ mononucleated cells were lifted off the plates by the addition of PBS, resuspended in PBS plus 5% BSA, filtered through a 40-μM filter (BD Falcon) to remove any aggregates, and placed on ice. Cell sorting was performed on a Cytopeia Influx sorter with gating on YFP$^+$ population set by a comparison with a negative control sample of Lox-YFP myoblasts. The sorted cells were replated on ECM-coated tissue culture dishes containing myoblast growth medium with 9 ng/ml bFGF and were assessed for different markers. For cell-cycle analysis, dedifferentiated cells were expanded in culture, pelleted down, and fixed in ice-cold 70% ethanol overnight at −20°C. The next day, cells were pelleted down at 1400 rpm for 5 min at 4°C, and ethanol was removed. Cells were washed once with ice-cold 1× DPBS and were resuspended in DNA staining solution (RNase, 0.1 mg/ml; propidium iodide, 2 μg/ml; and 0.05% Triton X-100 in 1× DPBS) for 30 min at room temperature. The cells were pelleted down, washed once with ice-cold DPBS, and resuspended in DPBS plus 3% BSA for FACS analysis.

Statistical Analysis

p values were determined using student's t test (2 samples equal variance, 2 tailed).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables, two movies, six figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.07.012.

ACKNOWLEDGMENTS

We thank Hector Nolla for FACS sorting (UC Berkeley flow cytometry facility). We acknowledge the CHPS Imaging core and Paul Herzmark for assistance with time lapse imaging microscopy. We also thank Mike Conboy, Conboy laboratory members, and Vivek Chopra for critical feedback, help, and support. This work was supported by the National Institutes of Health, National Institute on Aging (grants AG 027252 and CIRM RN1-00532 to I.M.C.) and a CIRM posdoctoral fellowship (CIRM TG2-01164 to P.P.). The authors declare no competing financial interests. P.P. conceived the project; designed and performed the experiments; collected, assembled, analyzed, and interpreted data; and wrote the manuscript. I.M.C. conceived the project, directed the study, interpreted the data, and wrote the manuscript.

Received: March 9, 2011
Revised: July 11, 2011
Accepted: July 13, 2011
Published: September 22, 2011

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