Modulation of acto-myosin contractility in skeletal muscle myoblasts uncouples growth arrest from differentiation

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Summary

Cell-substratum interactions trigger key signaling pathways that modulate growth control and tissue-specific gene expression. We have previously shown that abolishing adhesive interactions by suspension culture results in G_0 arrest of myoblasts. We report that blocking intracellular transmission of adhesion-dependent signals in adherent cells mimics the absence of adhesive contacts. We investigated the effects of pharmacological inhibitors of acto-myosin contractility on growth and differentiation of C2C12 myogenic cells. ML7 (5-iodonaphthalene-1-sulfonyl homopiperazine) and BDM (2,3, butanedione monoxime) are specific inhibitors of myosin light chain kinase, and myosin heavy chain ATPase, respectively. ML7 and BDM affected cell shape by reducing focal adhesions and stress fibers. Both inhibitors rapidly blocked DNA synthesis in a dose-dependent, reversible fashion. Furthermore, both ML7 and BDM suppressed expression of MyoD and myogenin, induced $p27^{kip1}$ but not $p21^{cip1}$, and inhibited differentiation. Thus, as with suspension-arrest, inhibition of acto-myosin contractility in adherent cells led to arrest uncoupled from differentiation. Over-expression of inhibitors of the small GTPase RhoA (dominant negative RhoA and C3 transferase) mimicked the effects of myosin inhibitors. By contrast, wild-type RhoA induced arrest, maintained MyoD and activated myogenin and p21 expression. The Rho effector kinase ROCK did not appear to mediate Rho's effects on MyoD. Thus, ROCK and MLCK play different roles in the myogenic program. Signals regulated by MLCK are critical, since inhibition of MLCK suppressed MyoD expression but inhibition of ROCK did not. Inhibition of contractility suppressed MyoD but did not reduce actin polymer levels. However, actin depolymerization with latrunculin B inhibited MyoD expression. Taken together, our observations indicate that actin polymer status and contractility regulate MyoD expression. We suggest that in myoblasts, the Rho pathway and regulation of acto-myosin contractility may define a control point for conditional uncoupling of differentiation and the cell cycle.

Key words: ML7, BDM, Adhesion-dependent signaling, Acto-myosin contractility, Rho GTPase, Reversible arrest

Introduction

Adult muscle precursor cells known as satellite cells uncouple differentiation from cell cycle exit when dormant, but re-couple these programs when activated, during regeneration of damaged muscle (reviewed in Seale and Rudnicki, 2000). To model the entry and exit of satellite cells from G_0, we have studied reversibly arrested myoblasts in culture (Sachidanandan et al., 2002). C2C12 myogenic cells enter an undifferentiated G_0 state when deprived of adhesion, despite the presence of mitogens, and synchronously re-enter the cell cycle when replated on adhesive surfaces (Milasincic et al., 1996).

Adhesive interactions of cells with the extracellular matrix (ECM) play an important role in regulating motility, proliferation, differentiation and survival (Geiger et al., 2001). Trans-membrane focal adhesion complexes serve as integration sites for signaling between the ECM and the cytoskeleton. Indeed, dynamic cytoskeletal reorganization resulting from adhesive alterations is a key regulator of cell configuration and gene expression (Assoian, 1997; Assoian and Schwartz, 2001).

Cell-ECM interactions form the molecular basis of anchorage-dependent proliferation, and loss of anchorage-dependence is the hallmark of tumorigenesis (Stoker et al., 1968). Unlike epithelial cells, where detachment from the substratum leads to cell death or anoikis (Frisch and Francis, 1994), mesenchymal cell types such as fibroblasts (Benecke et al., 1978) and myoblasts (Milasincic et al., 1996) respond to non-adherent conditions by reversible arrest in G_0. Cell cycle re-entry requires the cooperation of adhesion molecules and receptors for soluble mitogens (Assoian, 1997). In myoblasts, where cell cycle exit is normally associated with differentiation into multinucleated myotubes (Nadal-Ginard, 1978; Andres and Walsh, 1996), reversible arrest allows analysis of the uncoupling of cell cycle control from activation of muscle-specific genes (Milasincic et al., 1996; Yoshida et al., 1998; Kitzmann et al., 1998; Sachidanandan et al., 2002). Such reversible uncoupling is a pre-requisite for progenitor cell function during muscle regeneration (Seale and Rudnicki, 2000).

Myogenic differentiation is accompanied by induction of a

battery of muscle-specific genes, many of which encode cytoskeletal proteins (Nadal-Ginard, 1978; Olson, 1992; Lassar et al., 1994). Assembly of a differentiated cytoskeleton is critical for muscle contraction. However, the cytoskeleton and adhesion complexes of undifferentiated myoblasts are largely composed of non-muscle elements. For example, unlike skeletal muscle myosins that undergo stable sustained contractions, non-muscle myosins regulate rapid cellular events (Citi and Kendrick-Jones, 1987; Bresnick, 1999). The contractility of non-muscle myosin II is regulated by myosin light chain kinase (MLCK), a Ca^{2+}-calmodulin-dependent enzyme. Phosphorylation of the myosin regulatory light chain (RLC) promotes filament assembly and actin-activated ATPase activity of the myosin heavy chain (Bresnick, 1999).

Pharmacological inhibitors of myosin II function have been used to investigate acto-myosin involvement in cell motility (Small et al., 1999; Cramer and Mitchison, 1995) and signal transduction pathways that link cytoskeletal function with mitogen-induced pathways (Chrzanowska-Wodnicka and Burridge, 1996). Inhibition of myosin II function affects cell shape and adhesion, as a result of effects on microfilament dynamics (Chrzanowska-Wodnicka and Burridge, 1996). Stress fibers, the most prominent acto-myosin arrays in cultured cells arise as a consequence of contractility (Heath and Dunn, 1978; Chrzanowska-Wodnicka and Burridge, 1996), which also results in focal adhesion assembly (Lamb et al., 1988; Fernandez et al., 1990; Burridge and Chrzanowska-Wodnicka, 1996).

Attachment of cells to the substratum rapidly activates structural changes (focal adhesion formation, microfilament bundling, integrin clustering), signal transduction (tyrosine phosphorylation of focal adhesion kinase and paxillin) and cell cycle regulators (Assoian, 1997; Geiger and Bershadsky, 2001). Adhesion-dependent signals cooperate with mitogen-activated pathways whose effects are mediated by the Rho family of small GTPases (Ridley and Hall, 1992; Hotchin and Hall, 1995). An important target of Rho activation is the Rho-associated kinase ROCK, which activates acto-myosin contractility via direct phosphorylation of MLC as well as indirectly, by inactivation of MLC phosphatase (Kimura et al., 1996; Van Aelst and D'Souza-Schorey, 1997).

In this study, we show that pharmacological inhibition of myosin II function reversibly modulates cell adhesion, the cell cycle and MyoD expression. Our results also implicate the Rho pathway in mechanisms that uncouple arrest from differentiation.

Materials and Methods

Cell culture

C2C12 mouse myoblasts (Blau et al., 1983) were obtained from H. Blau, Stanford University; a strictly anchorage-dependent subclone (Sachidanandan et al., 2002) was used in all experiments. Adherent cells were cultured in growth medium [GM; Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum]. To synchronize cells in G_0, suspension culture in methyl cellulose was performed as described previously (Milasincic et al., 1996; Sachidanandan et al., 2002). Cell culture reagents were from Invitrogen.

Treatment of cells with myosin inhibitors

For asynchronous cultures, myoblasts (3000 cells per 13 mm coverslip) were plated in GM for 12 hours, then incubated in low serum differentiation medium (DMEM with 2% horse serum) containing either dimethyl sulfoxide vehicle, 3-30 mM 2,3 butane dione monixime (BDM) (Sigma) or 1.5-15 μM 5-iodonapthalene sulfonic acid (ML7) (Calbiochem) for 24 hours. Cells synchronized in G_0 by suspension were replated (3000 cells per coverslip), myosin inhibitors (MIs) was added 30 minutes later and they were cultured for 24 hours.

DNA synthesis

Cells were exposed to 5-bromodeoxyuridine (BrdU) (100 μM for 15 minutes for pulse labeling; 10 μM for 2-28 hours for cumulative labeling) and BrdU was detected using an anti-BrdU monoclonal antibody (Sigma) 1:200, and the Vectastain ABC reaction (Vector) (Sachidanandan et al., 2002). Controls excluding primary antibody or BrdU were negative. The frequency of S phase was determined by counting ~250 nuclei/sample.

Cell cycle analysis

DNA content was measured using FACS analysis. Cells (2×10^6 per 100 mm dish) were treated with control medium or medium containing BDM (15 mM) or ML7 (15 μM) for 24 hours, stained with propidium iodide (50 μg/ml) and analyzed on a FACS Calibur (Becton Dickenson) using CelQuest software.

Immunofluorescence

Cells plated on coverslips were fixed with 3.5% formaldehyde, permeabilized in PBS-0.2% Tween 20. Primary antibodies were diluted in PBS, 10% horse serum (HS), 0.02% Tween 20: anti-MyoD (Novocastra) 1:80; anti-myogenin (Santa Cruz) 1:100; anti-p21 (Pharmingen) 1:500; anti-p27 (Transduction, 1:250); anti-paxillin (Transduction, 1:200); anti-vinculin (Sigma, 1:250). Secondary antibodies were goat anti-mouse Alexa Fluor 594 or goat anti-rabbit Alexa Fluor 488 (Molecular Probes), 1:500. Secondary antibody controls were negative; no cross reactivity of secondary reagents was detected. Nuclei were stained with Hoechst 33342 (1 μg/ml). Actin was detected using Oregon Green-phalloidin (Molecular Probes, 1:50, 4 hours) in cells fixed with 4% paraformaldehyde prepared in cytoskeletal buffer (CB) containing 0.2% Triton X-100 (Herzog et al., 1994). Staining was recorded on a CCD camera using an Olympus microscope equipped with epifluorescence.

Differentiation

Cells were plated in GM (10,000 cells per coverslip) for 24 hours, rinsed with PBS and shifted to differentiation medium (DMEM + 2% HS) containing vehicle or different doses of BDM or ML7. Medium was replaced daily for 3 days. Cells were fixed with 4% paraformaldehyde, 0.3% Triton X-100 in cytoskeletal buffer (CB), and stained with A41025 anti-myosin monoclonal (Blau et al., 1983) and goat anti-mouse Alexa Fluor 594, 1:500. The fusion index (number of nuclei in myotubes of >2 nuclei/total number of nuclei×100) was estimated by counting ~500 nuclei per sample.

Transfection

Myoblasts were plated on coverslips for ~12 hours prior to transfection with plasmids encoding wild-type RhoA (HA tagged), dominant-negative Rho (RhoA N19) (Ridley and Hall, 1992), C3 transferase or GFP [pKA23, encoding nlsEGFP (Murphy et al., 2002)]. Transfections used 1.25 μg DNA (1 μg of test plasmid (wild-type RhoHA, RhoAN19, C3 transferase or pBS) plus 0.25 μg of GFP plasmid as marker) and 2.5 μl of Lipofectamine 2000 (Invitrogen) in serum-free DMEM for 6 hours, incubation in GM for 24 hours, pulsing with BrdU (100 μM for 15 minutes) and fixation with 3.5% formaldehyde. Anti-BrdU was detected with biotinylated anti-mouse (Vector, 1:200) and

streptavidin-Alexa Fluor 350 (Molecular Probes, 1:100). Transfected cells were detected with an anti-HA monoclonal (for RhoHA) or by the co-transfected nlsGFP. At least 100 GFP-positive cells were scored for each sample. Transfection efficiencies were 30-50%.

Treatment with other inhibitors

Cells plated on coverslips were treated with Y27632 (10 μM), latrunculin B (0.5 μM) or cytochalasin D (0.5 μM) in DMEM + 2% HS for 18-24 hours.

FACS quantification of actin polymer

Actin polymer status was assessed as described previously (Howard and Meyer, 1984; Copeland and Treisman, 2002) after trypsinization, fixation and staining of cells with rhodamine-phalloidin (33 nM final), and quantified using flow cytometry after gating for forward and side scatter.

Image analysis

Digital images were captured and processed using identical settings within an experiment using Image Pro Plus; composites and overlays were prepared using Adobe Photoshop 5.0.

Results

Abolishing adhesion in C2C12 myoblasts leads to reversible G_0 arrest (Milasincic et al., 1996; Sachidanandan et al., 2002). We sought to explore whether blocking adhesion-dependent signals by perturbation of specific cytoskeletal functions in adherent cells, would have similar effects. Cell-permeable inhibitors of myosin II function have been used to probe the involvement of acto-myosin systems in motility and signaling (Cramer and Mitchison, 1995; Chrzanowska-Wodnicka and Burridge, 1996; Kaverina et al., 1999; Bresnick, 1999). We used two inhibitors with distinct modes of action to block myosin contraction: ML7 inhibits MLCK-mediated phosphorylation of myosin light chain (Nakanishi et al., 1990), while BDM inhibits the intrinsic ATPase activity of myosin heavy chain (Higuchi and Takemori, 1989).

Inhibitors of non-muscle myosin II affect cell shape in undifferentiated C2C12 myoblasts

To establish whether doses of MIs known to be biochemically active in vitro (Saitoh et al., 1987; Cramer and Mitchison, 1995) and to affect other cell types (Chryzanowska-Wodnicka and Burridge, 1996; Olazabal et al., 2002) were effective on myoblasts, asynchronously growing C2C12 cells were treated with serial dilutions ranging from 0.12 μM to 30 μM (ML7) or 0.12 mM to 30 mM (BDM) for 24 hours. Doses between 1.5 and 15 μM ML7 and 3-30 mM BDM had acute effects on cell shape (observable in minutes) that were sustained over 24 hours but did not cause significant detachment. In cytotoxicity assays (Mosmann et al., 1983), the highest doses resulted in some cell death (data not shown). Fig. 1 shows the reduction in stress fibers and alteration of cell shape in drug-treated cells.

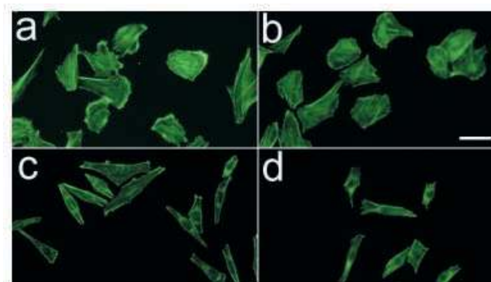


Fig. 1. Myosin inhibitors affect cell shape and stress fiber organization. Asynchronous cultures of myoblasts were treated for 24 hours with low serum medium alone (a), or in the presence of DMSO (b), 30 mM BDM (c) or 15 μM ML7 (d). Actin stress fibers (Oregon Green-phalloidin) and cell spreading are markedly reduced in drug-treated cells. Scale bar: 20 μm.

Myosin inhibitors induce growth arrest in asynchronous myoblasts

To examine whether MIs affect proliferation, asynchronous adherent myoblasts were exposed to ML7 (1.5-15 μM) or BDM (3-30 mM) for 24 hours and DNA synthesis monitored by pulse-labeling with BrdU. A dose-dependent inhibition of DNA synthesis was observed in cultures treated with MIs (Fig. 2A,B). Interestingly, while BDM was able to inhibit DNA synthesis in either low or high serum (Fig. 2A,B respectively), ML7's action was not observed in high serum (Fig. 2B; >5% FBS inhibited ML7's suppression of DNA synthesis, not shown). The addition of the vehicle DMSO alone had little effect (Fig. 2A, compare bars D and C), while the extent of S phase inhibition correlated with the concentration of the MI.

BDM blocks adhesion-dependent entry of G_0 myoblasts into S phase

To determine whether MIs affected cell cycle re-entry, synchronized myoblasts were used. Cells synchronized in G_0 by suspension re-enter S phase in an adhesion- and mitogen-dependent manner (Milasincic et al., 1996; Sachidanandan et al., 2002). When replated in low serum less than 2% of G_0 cells progressed to S phase, whereas in growth medium ~40% of cells had synthesized DNA at 24 hours (Fig. 2C, bar D). As in asynchronous cells, ML7 did not prevent entry into S phase when serum was present. However, reattachment-induced DNA synthesis was suppressed by BDM, despite the presence of serum. Taken together, these observations indicate that in addition to mitogens and adhesive contacts, progression to S phase requires a contractile cytoskeleton.

Kinetics of arrest by myosin inhibitors

To determine the kinetics of MI action, asynchronous myoblasts were treated with 15 μM ML7 or 30 mM BDM in low serum for 2-30 hours, and DNA synthesis measured (Fig. 2D). Whereas control cultures remained proliferative throughout this period, both MIs rapidly caused arrest. Six hours of treatment were sufficient for >50% reduction in the percentage of S phase cells, reaching >90% inhibition by 24 hours. Inhibition by ML7 was more severe than by BDM. The time taken by asynchronous myoblasts to enter G_0 when placed in suspension is 36-48 hours (J. Dhawan, unpublished). Thus, drug-induced inhibition of contractility acts more rapidly to suppress proliferation than detachment from adhesive contacts.

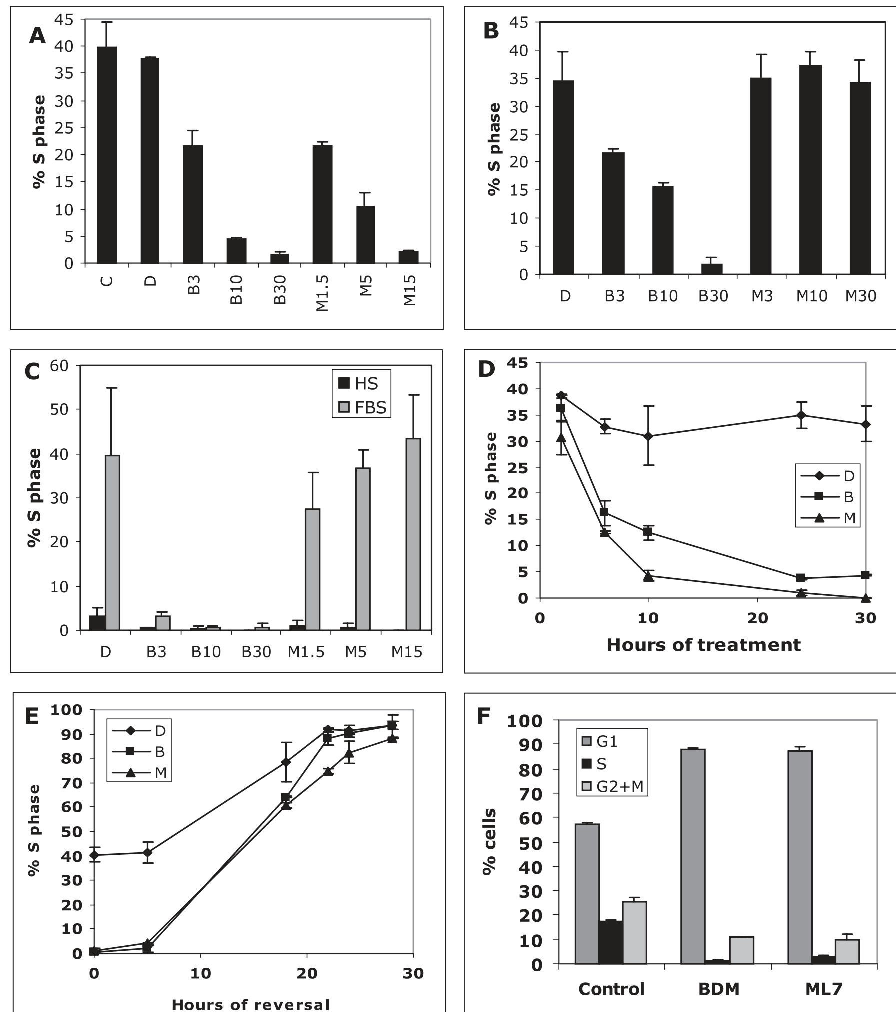


Fig. 2. Myosin inhibitors reversibly arrest myoblast proliferation. (A) Dose response of DNA synthesis in asynchronous myoblasts in low serum. Asynchronous myoblasts were treated for 24 hours with medium containing 2% HS alone (C), or 2% HS + vehicle (D) or BDM at 3, 10 or 30 mM (B3, B10, B30 respectively) or ML7 at 1.5, 5 or 15 μM (M1.5, M5, M15, respectively). Cells were pulsed with BrdU and labeled nuclei detected by immunostaining. Both BDM and ML7 elicited a dose-dependent decrease in the frequency of S phase cells (mean ± s.e.m. *n*=2). (B) Dose response of DNA synthesis in asynchronous myoblasts in high serum. Asynchronous myoblasts were treated with MI in high serum (20% FBS) and DNA synthesis measured as described in A. Whereas BDM effectively inhibited DNA synthesis, ML7's action was not evident in the presence of serum (mean ± s.e.m. *n*=2). (C) MIs prevent progression to S phase. Myoblasts were synchronized in G_0 by suspension culture, replated in either 2% HS (black bars) or 20% FBS (gray bars) for 24 hours, and DNA synthesis measured as described in Fig. 2A. Vehicle-treated cells (D) entered S phase in a serum-dependent manner. BDM (but not ML7) inhibited S phase entry in a dose-dependent manner in 20% FBS. (mean ± s.d. *n*=4). (D) Growth arrest by MI is rapid. The kinetics of arrest by MI were measured by treating asynchronous myoblasts for 2-30 hours with 2% HS + DMSO (D), 30 mM BDM (B) or 15 μM ML7 (M). Inhibition of DNA synthesis was evident within 6 hours of MI addition. (mean ± s.e.m. *n*=2). (E) Arrest is reversible. Myoblasts were arrested by 24 hours treatment (0 hours after drug removal) in 15 μM ML7 or 30 mM BDM compared to vehicle alone. Cumulative BrdU-labeling of cells entering S from 5-28 hours after addition of growth medium showed that ~90% were labeled by 22-28 hours. MI-treated cells took 4-8 hours longer to achieve 90% labeling. (F) MI treatment arrests cells in G_0. Asynchronous myoblasts were treated for 24 hours with vehicle, 30 mM BDM or 15 μM ML7 and DNA content measured by FACS. Whereas control cells were distributed in all phases of the cell cycle, ~90% of MI-treated cells possessed a G_1 DNA content consistent with arrest in G_0. (mean ± s.e.m. *n*=2).

Myosin inhibitors induce reversible G_0 arrest

Both ML7 and BDM are reversible inhibitors of their respective target enzymes in vitro (Saitoh, 1987; Herrmann et al., 1992). To determine whether the effects of MIs on DNA synthesis were also reversible, asynchronous cultures were treated with low serum medium containing 15 μM ML7, 30 mM BDM or vehicle alone for 24 hours, replaced with inhibitor-free growth medium for 0 to 24 hours and DNA synthesis measured (Fig. 2E). 24 hours after drug treatment (0 hours after medium replacement), whereas >40% of control cells incorporated BrdU in a 2 hour pulse, <3% of MI-treated cells were found to be in S phase by incorporation of BrdU. Effects of drug removal on cell shape were evident within 30 minutes and the rounded/elongated cells spread to produce control-like morphologies by ~18 hours (not shown).

MI removal permitted serum-stimulated cell-cycle re-entry. Cumulative labeling with BrdU for 5-28 hours after medium replacement revealed that whereas ~90% of control cells had entered S phase by 18 hours, BDM-treated and ML7-treated cells did not reach this level till 4-8 hours later. Labeled mitoses were detected at 18 hours after medium change in control cells, but not until 24 hours post MI removal (not shown). These results demonstrate that the effects of MIs on arrest are reversible. Indeed, the kinetics of cell cycle re-entry upon removal of MIs resembled the adhesion-induced return to S phase from suspension-synchronized (G_0) myoblasts (Sachidanandan et al., 2002). The additional period taken to achieve 90% labeling is consistent with the G_0/G_1 transition and suggests that MI treatment, like suspension culture, arrests cells in G_0.

To directly measure the DNA content of MI-treated cells, we used flow cytometry (Fig. 2F). FACS analysis revealed that whereas control cells showed a distribution typical of an asynchronous population, treatment with either BDM or ML7 resulted in the accumulation of ~90% of cells in G_1, with a concomitant reduction in S and G_2+M. Thus, MI treatment arrests cells in G_0.

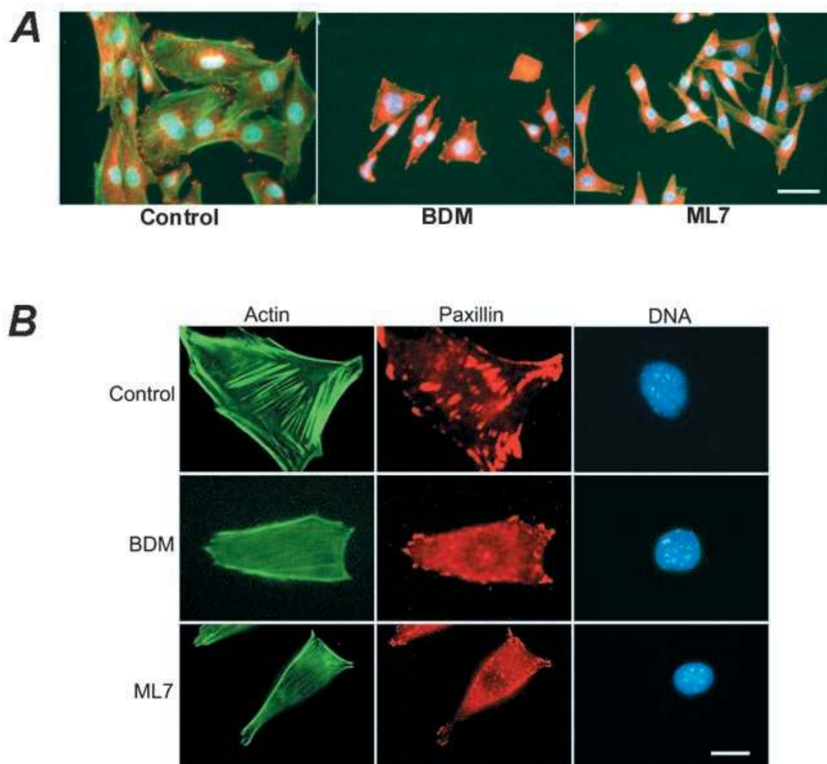


Fig. 3. Disruption of focal adhesions in myoblasts treated with MIs. Asynchronous myoblasts were incubated for 24 hours in 2% HS + DMSO, 30 mM BDM or 15 μM ML7. (A) Cells were triple-stained to reveal actin (green), the focal adhesion component vinculin (red) and DNA (blue). Vinculin-positive focal adhesions were located at the ends of stress fibers in control cells. Stress fibers and focal adhesions were disrupted in MI-treated cells, accompanied by an increase in diffuse cytoplasmic staining. Scale bar: 20 μm. (B) Immunodetection of the focal adhesion component paxillin (red). Both the number and size of paxillin^{+} focal contacts were reduced by MI treatment. Scale bar: 10 μm.

Myosin inhibitors affect cytoskeletal organization

To assess the alterations in cell shape and cytoskeletal organization resulting from inhibition of acto-myosin contractility we used fluorescent probes for actin and focal adhesion components vinculin and paxillin (Fig. 3). The effect of MIs on stress fibers and focal contacts was dose-dependent and paralleled the suppression of DNA synthesis (data not shown). ML7 had a pronounced effect and at the highest doses resulted in cell rounding, loss of stress fibers and the accumulation of clumps of actin in the cytoplasm. BDM-treated cells showed few longitudinally oriented stress fibers, and were spindle shaped or markedly triangular as compared to control cells that had spread morphologies and extensive stress fibers. Furthermore, MI treatment led to a disruption of focal adhesion complexes as evidenced by detection of vinculin (Fig. 3A) and paxillin (Fig. 3B). The number of focal contacts containing paxillin was reduced from an average of 85 in control cells (range 56-151) to 25 in ML7-treated cells (range 6-45) (n=20). Staining for other focal adhesion components (phosphotyrosine, focal adhesion kinase) showed similar results (not shown). Thus, focal adhesions and the actin cytoskeleton, major determinants of cell configuration and signaling were disrupted by doses of MIs that inhibited DNA synthesis.

Arrest does not induce differentiation

Cell cycle exit triggered by mitogen depletion of adherent myoblasts is coupled to differentiation, marked by a defined sequence of early myogenic genes (Andres and Walsh, 1996). Induction of the muscle regulatory factor (MRF) myogenin precedes that of the cyclin-dependent kinase inhibitor (CKI) p21, terminal cell cycle arrest and activation of muscle structural genes. By contrast, in suspension-arrested myoblasts, quiescence is uncoupled from differentiation and myogenic markers are not induced (Milasincic et al., 1996; Sachidanandan et al., 2002). To determine the expression of early markers of differentiation, we used immunofluorescence assays (Fig. 4A,B). We found that differentiation does not accompany MI-induced arrest. Control cultures showed expectedly low frequencies of myogenin-positive and p21-positive cells (~5%) that rose to ~30% within 24 hours of serum withdrawal. Neither myogenin nor p21 were induced during treatment with MIs at doses that caused arrest. However, the CKI p27, a quiescence-associated protein, was up-regulated in MI-arrested cells (Fig. 4C,D). These observations are consistent with the finding that MI-activated arrest is reversible, since induction of myogenin and p21 in myoblasts has been associated with permanent cell cycle exit.

In suspension arrest, not only is differentiation not activated, even the expression of the determination factors MyoD and Myf5 is suppressed (Milasincic et al., 1996; Sachidanandan et al., 2002). To examine the expression of MyoD in MI-treated myoblasts, we used immunofluorescence assays (Fig. 5). As MyoD is cell cycle regulated (Kitzmann et al., 1998), ~50% of cells in control asynchronous cultures in growth medium or in low serum medium (+/– vehicle) expressed MyoD (Fig. 5A). By contrast, cells that were treated with MI for 24 hours exhibited an inhibition of MyoD expression at the single cell level (Fig. 5A,B), and suppression of MyoD transcripts (not shown). Furthermore, MyoD was re-expressed on removal of the inhibitors (Fig. 5C). BrdU incorporation monitored in parallel samples confirmed arrest and reactivation (not shown). The recovery of MyoD was expectedly more rapid than S phase re-entry. Recovery from BDM treatment led to control levels of MyoD expression in G_1, 6 hours after drug removal, that were sustained thereafter. As with DNA synthesis, recovery of MyoD expression from ML7 inhibition was slower than BDM reversal. Removal of MIs led to a return of MyoD expression regardless of whether the medium contained serum or not (not shown), suggesting that the recovery of acto-myosin contractility is sufficient for MyoD activation. Taken together, these observations demonstrate that MI-induced growth arrest is not accompanied by differentiation, because of a suppression of MRF expression.

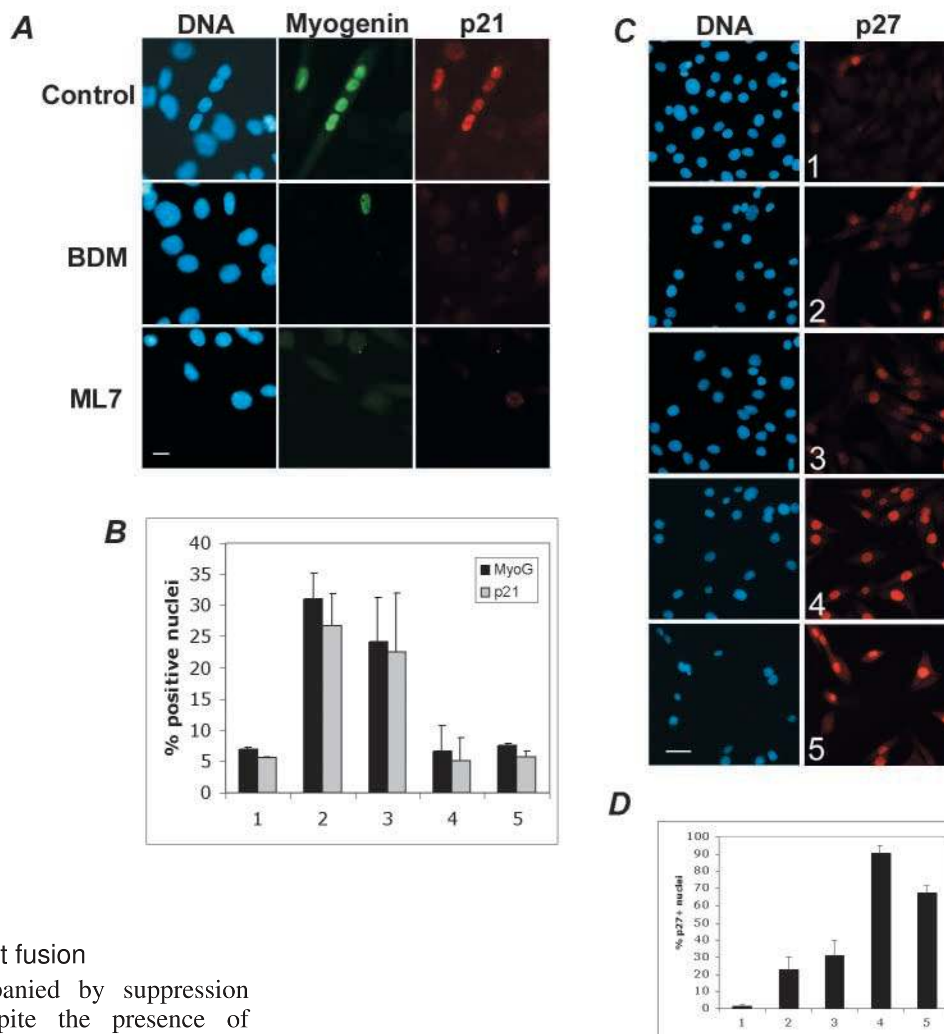


Fig. 4. MIs do not induce myogenin and p21. Asynchronous myoblasts were incubated in control medium or 30 mM BDM or 15 μM ML7 for 24 hours. Myogenin (green) and p21 (red) were simultaneously detected using antibodies. (A) Staining of both early myogenic markers was readily detected in control cells but not in MI-treated cells. Scale bar: 10 μm. (B) The frequency of myogenin and p21 expression (mean ± s.e.m. *n*=2). The low level of myogenin and p21 expression in growth medium (1), rises after 24 hours in differentiation medium alone (2) or with DMSO vehicle (3). Both markers are suppressed by BDM and ML7 (4,5). (C,D) Induction of quiescence-related protein $p27^{kip1}$ after BDM and ML7 treatment. Serum withdrawal caused some induction of p27 as expected (compare 1 with 2 or 3; numbers indicate conditions as in panel B), but MI treatment (4,5) led to increased frequency and intensity of p27 staining (mean ± s.e.m. *n*=2). Scale bar in C: 25 μm.

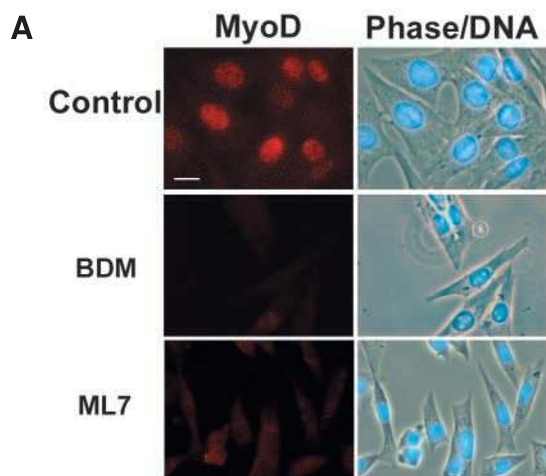
Myosin inhibitors suppress myoblast fusion

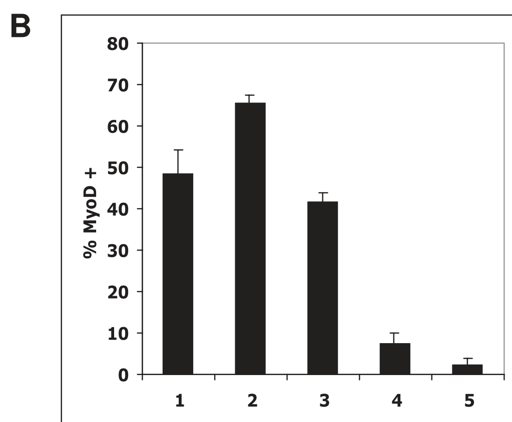
MI-induced quiescence was accompanied by suppression of early myogenic regulators, despite the presence of differentiation-inducing conditions (2% HS). To determine the effect of prolonged treatment with MIs on fusion, high-density cultures were placed in differentiation medium containing vehicle alone, 15 μM ML7 or 15 mM BDM for 3 days. As expected, control cultures began to fuse within 24 hours and by 72 hours, large multinucleated myosin-expressing myotubes were observed (Fig. 6). By contrast, MI-treated cultures remained mononucleate and at 72 hours a few myosin-positive 'needles' were seen. The fusion index was 41.3±7.1 for control cells, 0.5±0.1 for BDM-treated cells and 0.8±0.2 for ML7-treated cells (mean ± s.d., *n*=3). Thus, non-muscle myosin function is required for fusion and myogenic differentiation.

Transfection of active and dominant interfering Rho induces arrest

Rho GTPases act as molecular switches that regulate key signal transduction pathways (Ridley and Hall, 1992; Van Aelst, 1997). In particular, RhoA stimulates acto-myosin contractility that drives formation of stress fibers and focal adhesions and tyrosine phosphorylation of focal adhesion components in fibroblasts (Chrzanowska-Wodnicka and Burridge, 1996; Helfman et al., 1999). Furthermore, expression of MyoD and other myogenic genes is regulated via a Rho-dependent pathway by the transcriptional regulator, serum response factor (SRF) (Carnac et al., 1998; Wei et al., 1998). To determine whether Rho is involved in growth control in myoblasts, we transiently transfected constructs encoding the wild-type (WT), dominant-negative RhoA (RhoAN19) or the Rho inhibitor C3 transferase (C3T). Interestingly, over-expression of both active and dominant-negative (DN) forms of Rho, as well as C3T inhibited DNA synthesis compared to transfection of a GFP construct (Fig. 7).

We hypothesized that over-expression of WT RhoA, resulting in up-regulation of MyoD, would lead to both arrest and differentiation. However, DN RhoA might arrest proliferation but as MyoD expression also requires active Rho signaling, transfected cells would not differentiate. To test this hypothesis, we assessed the expression of MyoD, myogenin and p21 in transfected cells (Fig. 8). In myoblasts transfected with WT Rho, the frequency of MyoD-expressing cells was not much increased, consistent with previous reports (Carnac et al., 1998). However, myogenin and p21 were expressed at significantly higher frequency in WT Rho transfectants than in GFP transfectants. By contrast, cells transfected with DN Rho or C3T showed no increase in myogenin- or p21-positive cells but significantly fewer cells expressed MyoD. Taken together, these observations suggest that while over-expression of WT Rho leads to arrest accompanied by differentiation, inhibition





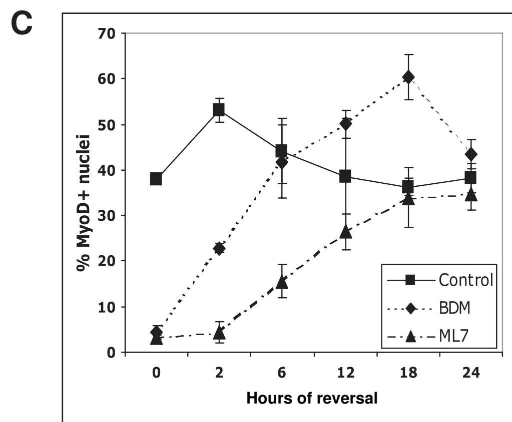


Fig. 5. MIs reversibly inhibit MyoD expression. Asynchronous myoblasts were incubated in 20% serum alone (1), 2% HS alone (2), 2% HS + vehicle (3), 30 mM BDM (4) or 15 μM ML7 (5) for 24 hours. (A) MyoD (red) was detected in ~50% of control cells, but was reduced in both BDM and ML7 treated cells (Scale bar: 10 μm). (B) The frequency of MyoD expression (mean ± s.e.m., *n*=2). (C) Replacement of MIs with growth medium leads to a rapid return of MyoD expression (mean ± s.e.m., *n*=2). The differences in the kinetics and plateau of the recovery curves may reflect differences in extent of cell cycle synchrony, the enzymatic properties of the two target enzymes or their effects on upstream regulators of MyoD.

of the Rho pathway, like MI treatment, compromises both proliferation and differentiation. Thus, increased Rho signaling in myoblasts is accompanied by irreversible cell cycle exit, while inactivation of the Rho pathway probably results in reversible arrest, as differentiation is not induced.

Inhibition of the Rho-associated kinase ROCK does not affect MRF expression

To further dissect the role of the Rho pathway in regulation of MyoD expression, we investigated the effects of Y27632, a pharmacological inhibitor of the Rho effector kinase ROCK. Cells exposed to 10 μM Y27632 (Fig. 8) showed altered cell shape and suppression of DNA synthesis, but expressed both MyoD and Myogenin. Thus, while RhoA regulates both proliferation and MyoD, the downstream effector ROCK appears to mediate cell cycle progression but not MyoD expression.

Over-expressed RhoA can over-ride the effect of BDM on the cytoskeleton

To determine whether increased Rho signaling could over-ride the effects of MI, we transfected cells with GFP alone, WT Rho or RhoAN19, treated with BDM and assessed the organization of paxillin and actin. Transfection of Rho led to a marked reduction of cell size and agglomeration of paxillin-positive focal adhesions, consistent with increased contractility (Fig. 9). DNRho produced less obvious changes, but appeared to slightly increase cell size and focal adhesion dispersal, consistent with decreased contractility. GFP-transfectants and untransfected cells in BDM-treated cultures were elongated, with reduced paxillin and actin staining. However, in ~70% of RhoA-transfected cells (*n*=161), BDM treatment did not prevent the Rho-induced cell shrinkage and paxillin accumulation. Thus, increased Rho signaling may counteract the reduced contractility induced by BDM.

BDM does not reduce actin polymer levels

Actin polymer status has been implicated in mediating Rho signaling to SRF in non-muscle cells (Sotiropoulos et al., 1999; Copeland and Treisman, 2002). Since SRF is a regulator of MyoD (Carnac et al., 1998), and since MI affect stress fiber organization, we asked whether actin polymerization was also affected by MIs. Stress fiber bundles were reduced in MI-treated cells (Figs 1, 3), but actin polymer status is not evident from microscopy. Therefore, F-actin levels were estimated by FACS quantitation of rhodamine-phalloidin binding (Howard and Meyer, 1984; Copeland and Treisman, 2002). Control cells were treated with vehicle or latrunculin B (latB), an actin depolymerizing agent. As expected, latB-treated cells showed ~70% of the mean fluorescence intensity of cells treated with vehicle alone, consistent with decreased F-actin levels (Fig. 10A). However, BDM treatment led to increased phalloidin fluorescence, indicating that despite decreased stress fiber bundling, overall F-actin levels increased.

Actin depolymerizing drugs differentially affect MyoD expression

To directly address whether actin polymer status affects MyoD

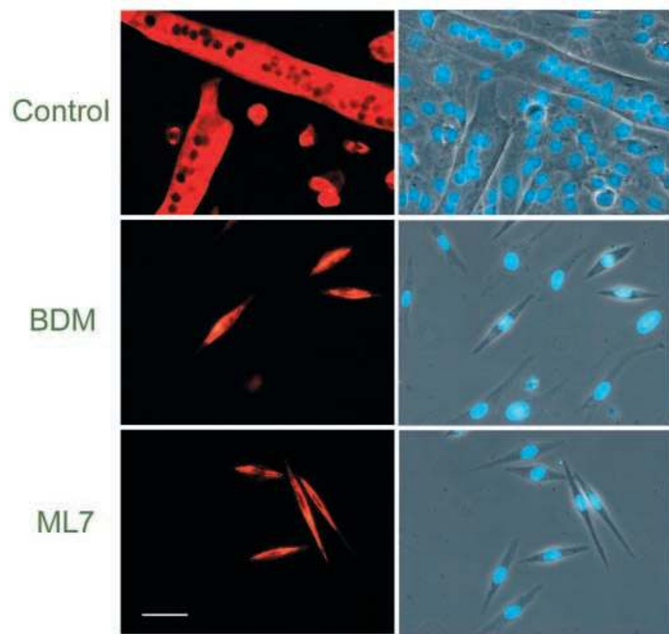


Fig. 6. MI treatment inhibits differentiation. Dense asynchronous cultures were treated with 2% HS containing DMSO (control), 15 mM BDM or 15 μM ML7 for 3 days, and stained for muscle-specific myosin expression. Control cultures fused into large multinucleated myotubes, but BDM and ML7 inhibited fusion. Scale bar: 25 μm.

expression, we tested the effects of actin depolymerizing drugs. As expected, both latB and cytochalsin D (cytoD) acutely affected cell shape and inhibited BrdU incorporation (not shown). However, latB inhibited MyoD expression but cytoD did not (Fig. 10B), consistent with their differential effects on SRF (Sotiropoulos et al., 1999; Copeland and Treisman, 2002).

Taken together, our results suggest that MyoD expression and S-phase entry are responsive to alterations of actin filament organization (polymer status), as well as contractile function. Since RhoA regulates cytoskeletal dynamics, MyoD expression and proliferation, we conclude that in myogenic cells, acto-myosin contractility and the Rho pathway play a central role in coupling quiescence to tissue-specific gene activation.

Discussion

In this report we describe the uncoupling of myogenic differentiation from cell cycle arrest by modulation of signaling pathways that are dependent on acto-myosin contractility.

Inhibitors of acto-myosin contractility rapidly induce reversible G_0 arrest

We have previously shown that non-adherent culture conditions led to reversible growth arrest despite the presence of soluble mitogens, whereas cell cycle re-entry was dependent on both growth factors and adhesion (Milasincic, 1996; Sachidanandan et al., 2002). Here, we tested the hypothesis that intracellular blockade of adhesion-dependent signals in adherent myoblasts would result in a similar synchronization in G_0.

Focal adhesions, the predominant cell-substrate contacts in cultured cells, act as trans-membrane signaling centers, because of the presence of molecules such as integrins, src, FAK and paxillin. Regulated interaction of actin filaments with focal adhesions underlies the dynamic interdependence of cell configuration, signal transduction and adhesion (Geiger and Bershadsky, 2001). A key component of the actin cytoskeleton in undifferentiated myoblasts is myosin II, whose ATPase activity drives contraction of the acto-myosin complex (reviewed by Bresnik, 1999).

Acto-myosin contractility is regulated by signals that converge at two sites – phosphorylation of myosin light chain by MLCK, and modulation of the ATPase activity of myosin heavy chain. We perturbed these enzymatic activities using cell-permeable inhibitors of contractility that have been widely used to investigate its role in focal adhesion assembly and signaling (Chrzanowska-Wodnicka and Burridge, 1996), cell motility (Kaverina et al., 1999; Rottner et al., 1999; Cramer and Mitchison, 1995), phagocytosis (Olazabal et al., 2002) and neurite retraction (Amano et al., 1998). ML7, a selective inhibitor of MLCK (Saitoh et al., 1987) inhibits MLC phosphorylation at Ser19, a residue critical for actin binding, filament assembly and myosin contraction. BDM, a less specific uncompetitive inhibitor of the myosin ATPase, acts via stabilization of (Myosin.ADP.Pi) complexes (Herrmann et al., 1992; Higuchi and Takemori, 1992). Despite reports of effects not directly related to myosin ATPase activity (Blanchard et al., 1990; Ferreira et al., 1997; Castillo, 2002), BDM continues to be accepted as the myosin inhibitor of choice (Chrzanowska-Wodnicka and Burridge, 1996; Olazabal et al., 2002). Importantly, doses of BDM that inhibit purified myosins in vitro also inhibit myosin-dependent spreading of cultured cells (Cramer and Mitchison, 1995).

Both ML7 and BDM suppressed proliferation in adherent asynchronous myoblasts, as well as adhesion-induced entry of pre-synchronized myoblasts into S phase, at doses that disrupted stress fibers and altered cell shape but caused little detachment. The effects of MIs on actin organization and cell shape were rapid and preceded inhibition of DNA synthesis. G_0 arrest by MI was completely reversible, as were effects on stress fibers and cell shape, consistent with reports that ML7 and BDM are reversible inhibitors of their respective target enzymes (Saitoh, 1987; Herrmann et al., 1992). The observation that drugs with distinct biochemical modes of action have similar effects on stress fiber organization and focal adhesion number (Fig. 3) and tyrosine phosphorylation of focal contacts (Chrzanowska-Wodnicka and Burridge, 1996) argues that they inhibit DNA synthesis by mechanisms that depend on these features of attached cells.

Non-adherent myoblasts show few microfilaments: stress fibers rapidly assemble during re-attachment, well before DNA synthesis (C. Sachidanandan and J.D., unpublished). Adhesion in serum-free medium can activate the G_0/G_1 transition, marked in myoblasts by the return of MyoD expression (S. Gopinath and J.D., unpublished), but S-phase re-entry requires co-operation with mitogens. BDM inhibited the adhesion-activated, serum-dependent S-phase re-entry of suspension-synchronized myoblasts, while ML7 did not inhibit progression when serum was present. This observation is

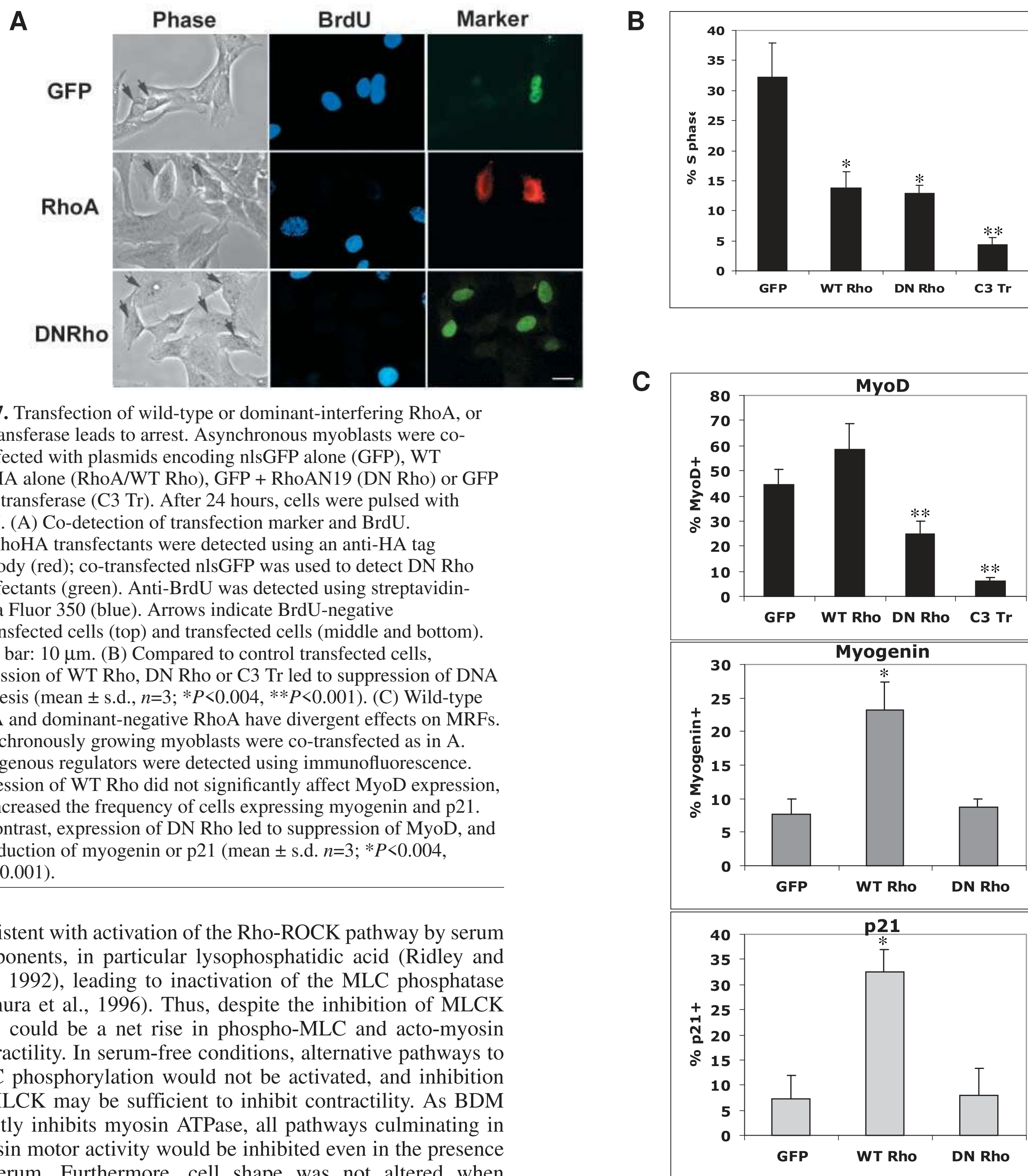


Fig. 7. Transfection of wild-type or dominant-interfering RhoA, or C3 transferase leads to arrest. Asynchronous myoblasts were co-transfected with plasmids encoding nlsGFP alone (GFP), WT RhoHA alone (RhoA/WT Rho), GFP + RhoAN19 (DN Rho) or GFP + C3 transferase (C3 Tr). After 24 hours, cells were pulsed with BrdU. (A) Co-detection of transfection marker and BrdU. WTRhoHA transfectants were detected using an anti-HA tag antibody (red); co-transfected nlsGFP was used to detect DN Rho transfectants (green). Anti-BrdU was detected using streptavidin-Alexa Fluor 350 (blue). Arrows indicate BrdU-negative untransfected cells (top) and transfected cells (middle and bottom). Scale bar: 10 μm. (B) Compared to control transfected cells, expression of WT Rho, DN Rho or C3 Tr led to suppression of DNA synthesis (mean ± s.d., n=3; *P<0.004, **P<0.001). (C) Wild-type RhoA and dominant-negative RhoA have divergent effects on MRFs. Asynchronously growing myoblasts were co-transfected as in A. Endogenous regulators were detected using immunofluorescence. Expression of WT Rho did not significantly affect MyoD expression, but increased the frequency of cells expressing myogenin and p21. By contrast, expression of DN Rho led to suppression of MyoD, and no induction of myogenin or p21 (mean ± s.d. n=3; *P<0.004, **P<0.001).

consistent with activation of the Rho-ROCK pathway by serum components, in particular lysophosphatidic acid (Ridley and Hall, 1992), leading to inactivation of the MLC phosphatase (Kimura et al., 1996). Thus, despite the inhibition of MLCK there could be a net rise in phospho-MLC and acto-myosin contractility. In serum-free conditions, alternative pathways to MLC phosphorylation would not be activated, and inhibition of MLCK may be sufficient to inhibit contractility. As BDM directly inhibits myosin ATPase, all pathways culminating in myosin motor activity would be inhibited even in the presence of serum. Furthermore, cell shape was not altered when ML7 was added in serum, but BDM elicited similar changes whether or not serum was present (J.D., unpublished). Taken together, these data demonstrate that acto-myosin contractility is required for myoblast proliferation and that these pharmacological inhibitors are a useful means of establishing experimental control over the process.

Acto-myosin contractility is required for differentiation of arrested myoblasts

Of the MRFs, the determination factors MyoD and Myf5 are expressed in proliferating myoblasts, whereas myogenin is induced early during differentiation (Olson, 1992). In myotubes, MyoD and myogenin expression is sustained, but Myf5 is suppressed (Kitzmann et al., 1998; Sachidanandan et al., 2002). Furthermore, even in growing myoblasts, MyoD and Myf5 are regulated by distinct mechanisms; in particular, inhibition of RhoA suppresses MyoD but not Myf5 expression (Carnac et al., 1998). RhoA modulates MyoD expression through SRF: both DN SRF and DN RhoA suppressed MyoD (Gauthier-Rouviere et al., 1996; Carnac et al., 1998), although neither proliferation nor the differentiation of transfected cells was assessed.

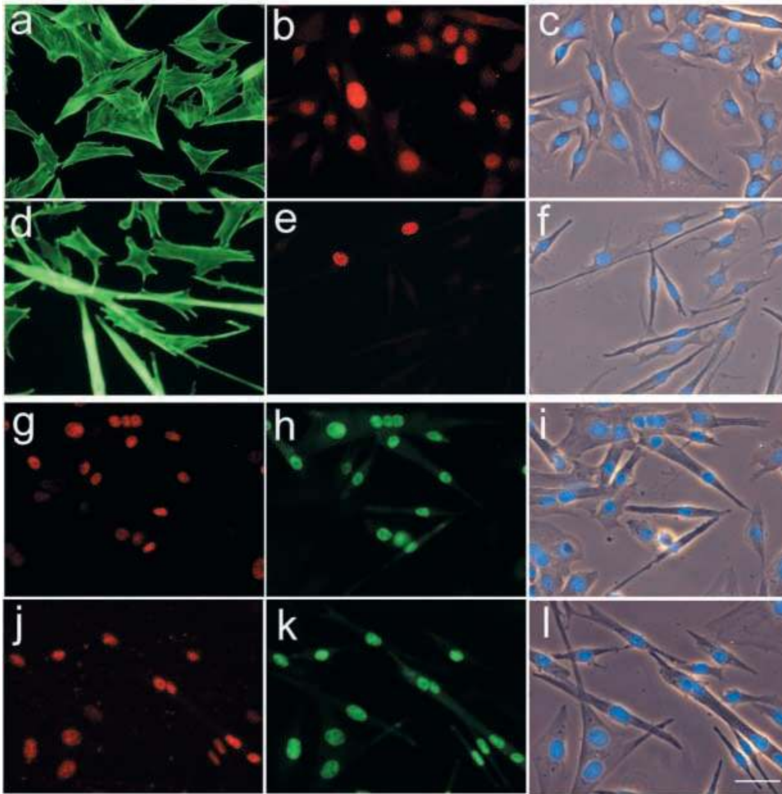


Fig. 8. ROCK inhibitor Y27632 blocks proliferation but not MRF expression. (A) Asynchronous myoblasts were treated with control medium (a-c, g-i) or 10 μM Y27632 (d-f, j-l) for 24 hours. Oregon Green-phalloidin staining reveals a distinct morphology in Y27632-treated cells (d) marked by elongated processes (compare to control in a). Pulse labeling with BrdU is inhibited by ROCK inhibitor (compare e with b); (c,f) phase contrast + DNA stain of the same fields as in b and e, respectively. MyoD (j) and myogenin (k) staining are undiminished in Y27632-treated cells compared to control cells (g) and (h) respectively, (i,l) phase contrast + DNA stain of the same fields as in g,h and j,k, respectively. Scale bar: 25 μm.

MyoD's role in embryonic myoblasts can largely be replaced by Myf5 (Rudnicki et al., 1992; Rudnicki et al., 1993). However, MyoD is required for complete differentiation of adult myoblasts or satellite cells (Megeney et al., 1996) from which the C2 cell line was derived (Yaffe and Saxel, 1977), indicating developmental stage-specific functions for this MRF. In C2 cells, MyoD is cell cycle dependent, such that low levels during G_0 rise to a peak in mid-G_1, correlating with the timing of induction of competence for differentiation (Kitzmann et al., 1998). Adhesion-dependent cell cycle controls also operate in G_1 (Assoian, 1997).

Our finding that MyoD expression is suppressed by MI doses that also inhibit proliferation, extends these observations to suggest that the coupling of cell cycle exit with differentiation requires acto-myosin contractility. A direct test of this hypothesis was to assess early differentiation markers in MI-arrested myoblasts. The sequential activation of myogenin and p21 (Andres and Walsh, 1996) is disrupted in suspension-induced arrest and re-activation (Layne and Farmer, 1999) (J.D., unpublished), as it is by drugs that inhibit acto-myosin contractility. MI also inhibited myoblast fusion: sarcomere assembly is critically dependent on contractility, but inhibition of differentiation by MI occurred at a much earlier stage, by suppression of MyoD, whose expression confers competence to differentiate.

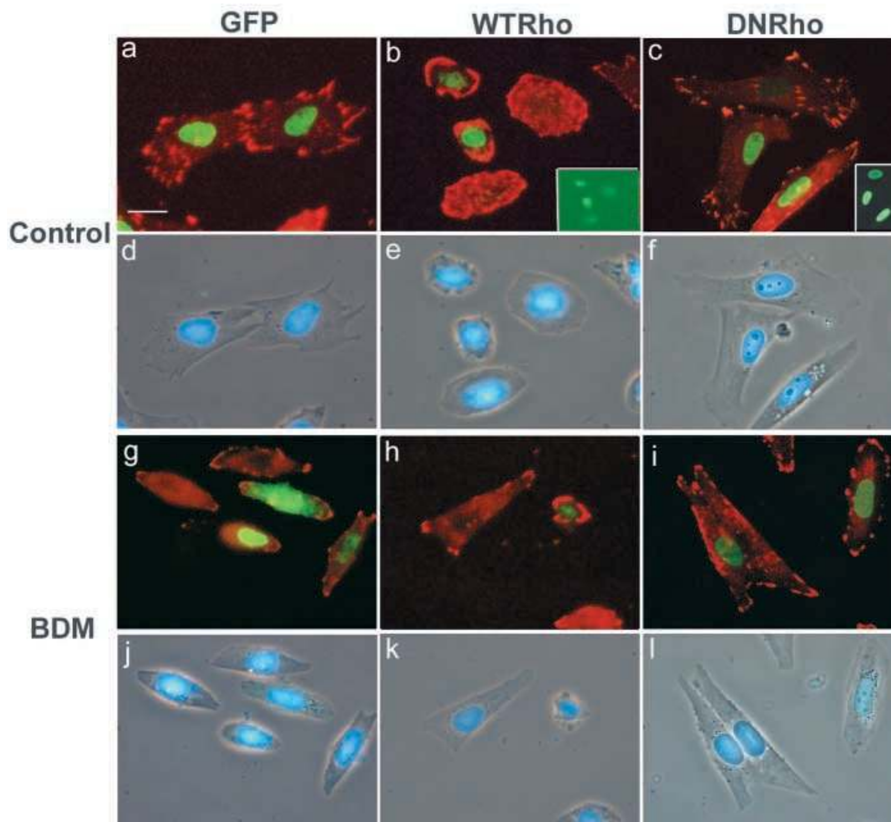


Fig. 9. Over-expression of RhoA overrides the effect of BDM on cytoskeletal organization. Asynchronous cultures were transfected as in Fig. 7 and 12 hours later were treated with vehicle (a-f) or 30 mM BDM (g-l) for a further 12 hours, (d-f) phase contrast + DNA stain of a-c. Control GFP cells were well spread with distributed paxillin staining; WT Rho-transfected cells were smaller with increased paxillin-positive focal contacts. Insets in b and c show cells expressing low levels of GFP marker, not visible in the merged images. DNRho transfection suggested a reciprocal effect to WTRho, with dispersed focal contacts. BDM treatment led to cell elongation and reduction of paxillin in GFP transfected cells (g), but did not reverse Rho-mediated cell shrinkage (h), (j-l) phase contrast + DNA stain of g-i. Scale bar: 10 μm.

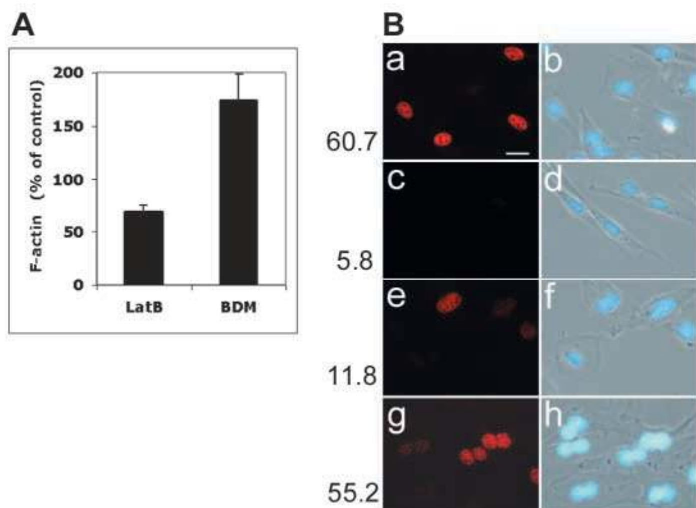


Fig. 10. (A) BDM does not decrease actin polymer levels. Actin polymer status was measured using FACS analysis of rhodamine-phalloidin binding in myoblasts treated with vehicle, latB (0.5 μM) or BDM (15 mM). (Expressed as a percentage of vehicle control, mean ± s.e.m.; *n*=2). (B) MyoD expression responds differentially to actin depolymerizing drugs. Myoblasts were treated with vehicle (a,b), 15 mM BDM (c,d), 0.5 μM latB (e,f) or 0.5 μM cytoD (g,h) for 18 hours before staining for MyoD. Numbers represent the percentage of MyoD-positive cells. Scale bar: 10 μm.

Acto-myosin contractility regulates coupling of arrest with differentiation

The reversible inhibition of MyoD expression by MIs paralleled the reversible inhibition of DNA synthesis. Yet, antagonistic interactions between MyoD and cell cycle activators regulate the coupling of differentiation with cell cycle exit (Wei and Paterson, 2001). Over-expression of MyoD inhibits the cell cycle in fibroblasts, independent of its ability to activate differentiation (Crescenzi et al., 1990; Sorrentino et al., 1990). Direct activation of cell cycle inhibitors such as p21 by MyoD (Halevy et al., 1995; Guo et al., 1995) and the buffering of CDK4 activity (Zhang et al., 1999a; Zhang et al., 1999b) have been proposed to mediate MyoD's growth inhibitory function. Induction of p21 by MyoD may be relevant to coupling quiescence and differentiation, since direct induction of p21 by a p53-mediated route leads to a failure of irreversible arrest (Puri et al., 1997). However, endogenous levels of MyoD expression are compatible with entry into S phase (S. Gopinath and J.D., unpublished) and this MRF may not be required for quiescence per se, as myoblasts can arrest without inducing MyoD (Milasinsic et al., 1996; Yoshida et al., 1998; Kitzmann et al., 1998). Importantly, in G_0 satellite cells in adult muscle, MyoD is absent and only induced upon injury-mediated cell cycle activation (Grounds et al., 1992; Cornelison and Wold, 1997). Thus, MyoD's role in cell cycle exit appears to be restricted to situations where quiescence and myogenic gene expression are coupled. Since MIs suppress both MyoD expression and cell cycle progression, we infer that acto-myosin contractility is required for coupling these processes.

The RhoA GTPase pathway is involved in the coupling of quiescence and differentiation

Rho signaling is known to promote myogenesis as RhoA GTPase activity increases during differentiation (Charrasse et al., 2002) and inhibition of Rho suppresses expression of MyoD (Carnac et al., 1998) and myogenin (Takano et al., 1998). Furthermore, the Rho-dependent transcription factor SRF activates expression of MyoD and dominant-negative SRF blocks myogenesis (Gauthier-Rouviere et al., 1996; Wei et al., 1998). The Rho pathway has also been implicated in the determination of mesenchymal precursors, as p190B RhoGAP-deficient mice show defective adipogenesis and a propensity for precursor cells to differentiate into muscle in response to IGF-I (Sordella et al., 2003). IGF-I-mediated induction of myogenesis was blocked by Y27632, implicating ROCK. In our studies, and other reports (Wei et al., 2001), Y27632 did not affect differentiation in response to serum deprivation. This discrepancy may reflect a specific role for ROCK downstream of IGF-I.

In fibroblasts, RhoA is a key regulator of acto-myosin contractility whose effects are inhibited by BDM (Chrzanowska-Wodnicka and Burridge, 1996). Our finding that MI treatment of myoblasts suppresses MyoD expression ties together these earlier observations to suggest that MyoD expression is dependent on acto-myosin contractility. Furthermore, over-expression of RhoA in myoblasts results in quiescence, maintenance of MyoD expression and induction of myogenin and p21. We infer that RhoA's ability to sustain MyoD expression is conducive to differentiation coupled to arrest. As our experiments were performed in growth medium, forced expression of RhoA appears to override the mitogen-mediated suppression of differentiation normally seen in proliferating cultures. The inability of over-expressed RhoA alone to increase MyoD expression is consistent with earlier reports (Carnac et al., 1998) and may suggest a signaling threshold. More interestingly, inhibition of Rho signaling by transfection of RhoAN19 led not only to the suppression of MyoD, but also quiescence without the induction of myogenin and p21, probably reflecting the absence of MyoD, as both genes are targets of this MRF. Thus, Rho signaling is required not only for proliferation and MyoD expression but also for differentiation, supporting earlier results (Takano et al., 1998) where over-expression of RhoGDI led to inhibition of differentiation.

Forced expression of C3 transferase (which inactivates Rho GTPase by ADP-ribosylation) had similar consequences to expression of RhoAN19 – inhibition of both proliferation and MyoD expression. Interestingly, treatment of cells with the ROCK inhibitor Y27632, led to cell shape changes mimicking those elicited by C3 transferase, and suppressed DNA synthesis but not MyoD or myogenin expression. Thus, interference with the Rho pathway at the level of Rho itself (C3 transferase) or Rho's ability to interact with its effectors (RhoAN19) inhibited both DNA synthesis and MyoD expression. By contrast, interference with the downstream Rho kinase only inhibited proliferation, suggesting that ROCK does not mediate MyoD suppression. In non-muscle cells, at least two pathways downstream of Rho affect the activity of SRF: ROCK mediates one pathway that involves LIM kinase and cofilin, but a

ROCK-independent pathway involving mDiaphanous also exists (Geneste et al., 2002). As ROCK can directly phosphorylate MLC, the differential effects of the MLCK-specific inhibitor ML7 and the ROCK inhibitor Y27632 on MyoD indicate that the regulation of MyoD by Rho is ROCK independent.

In non-muscle cells, Rho-dependent pathways culminating in increased actin polymer levels activate SRF (Sotiropoulos et al., 1999). Since MIs affect both stress fibers and MyoD expression, it was important to determine whether MIs reduce actin polymer levels, indirectly leading to inhibition of MyoD by inhibition of SRF. Interestingly, doses of BDM that suppressed MyoD led to increased actin polymer levels, suggesting an added specificity of the inhibitor for contractility rather than polymer status. Importantly, microfilament disrupting drugs latB and cytoD that have opposing effects on SRF activity in non-muscle cells (Geneste et al., 2002), have opposing effects on MyoD expression, consistent with the interpretation that actin polymer status also affects MyoD through SRF.

Over-expression of Rho in myoblasts led to markedly reduced cell size and increased focal adhesions. The effect on cell size is consistent with reports that increased Rho signaling in RhoGAP p190B mutant cells leads to reduced cell size (Sordella et al., 2002). Interestingly, BDM treatment (which normally leads to reduced focal contacts) did not diminish annular paxillin accumulation in the majority of WT Rho transfectants, suggesting that enhanced Rho signaling mitigates the inhibition of acto-myosin contractility. Rho transfectants resisted BDM-induced elongation and focal adhesion disruption. Taken together, these observations suggest that the ability of Rho to sustain MyoD expression is dependent on pathways that increase contractility and adhesive contacts.

Rho signaling may define a control point for myogenic differentiation in G_1

Several lines of evidence point to a critical period for myogenic differentiation in G_1. Early studies (Nadal-Ginard, 1978; Clegg et al., 1987) suggested that competence for differentiation is restricted to the G_1 phase of the cell cycle. Subsequently, the demonstration that both MyoD and Myf5 are tightly cell-cycle controlled, with a period during mid-G_1 corresponding to maximal MyoD levels and minimal Myf5 levels, provided a molecular logic for the G_1 checkpoint for differentiation (Kitzmann et al., 1998). The finding that MyoD expression is dependent on RhoA signaling (Carnac et al., 1998) and that Rho is crucial for other G_1-specific events such as cyclin D1 expression (Welsh and Assoian, 2000) lends credence to this argument. Finally, the demonstration that switching between active and inhibited states of Rho leads to a switch between G_0 (quiescence uncoupled from differentiation) and G_D (arrest linked to differentiation) suggests that Rho signaling plays a key role in regulating the decision between reversible and irreversible arrest. Such signaling events may also be involved in the choice between self-renewal and differentiation that occurs in activated satellite cells during the recovery of muscle tissue from damage.

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