

A Single-Cell Analysis of Myogenic Dedifferentiation Induced by Small Molecules

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Summary

An important direction in chemical biology is the derivation of compounds that affect cellular differentiation or its reversal. The fragmentation of multinucleate myofibers into viable mononucleates (called cellularization) occurs during limb regeneration in urodele amphibians, and the isolation of myoseverin, a trisubstituted purine that could apparently activate this pathway of myogenic dedifferentiation in mammalian cells, generated considerable interest. We have explored the mechanism and outcome of cellularization at a single-cell level, and we report findings that significantly extend the previous work with myoseverin. Using a panel of compounds, including a triazine compound with structural similarity and comparable activity to myoseverin, we have identified microtubule disruption as critical for activation of the response. Time-lapse microscopy has enabled us to analyze the fate of identified mononucleate progeny, and directly assess the extent of dedifferentiation.

Introduction

The most extensive regenerative ability in adult vertebrates is found among the various species of salamander, urodele amphibians that retain the tail after metamorphosis. These animals are the only vertebrates able to regenerate the limb, and this proceeds by formation of a blastema, a growth zone of mesenchymal stem cells at the end of the stump [1]. Regeneration in salamanders depends on the plasticity of differentiated cells at the site of tissue injury or removal, and one process implicated in the formation of the blastema is that of cellularization, the fragmentation of multinucleated myofibers into mononucleate cells that divide and contribute to the regenerate [2]. Cellularization has been studied in salamanders both by implantation of labeled myotubes into the limb blastema [3, 4] and by direct observation of microinjected myofibers in the context of the regenerating tail [5]. The cellular and molecular mechanisms underlying cellularization are unclear, and the discovery of a small molecule that could induce

this process in mammalian myotubes was therefore a significant step forward. The purine derivative 1 (Figure 1A), named myoseverin, was identified after screening a combinatorial library [6], and there is currently considerable interest in finding compounds that can induce cellular differentiation or its reversal, with attendant implications for regenerative biology and medicine [7–11].

Myoseverin is a molecule that binds to and depolymerizes microtubules, but several established depolymerizing agents were found not to induce reversible cellularization of mouse C2C12 myotubes in the manner of myoseverin [6, 12]. Following cellularization, an apparent increase in cell division could be observed in the cultures, and this suggested that the mononucleate progeny were able to return to the cell cycle, a further step in the reversal of myogenic differentiation. In addition to the morphological changes brought about in response to myoseverin, the authors reported a second aspect of its action. The cellularization events reminiscent of urodele regeneration were complemented by a transcriptional program indicative of tissue regeneration and wound healing. A microarray analysis revealed that over half of the genes whose expression was altered significantly by myoseverin were deemed to be involved in processes suggestive of a tissue response to injury, such as remodeling of the extracellular matrix, inflammation, and coagulation [6].

In this paper, we have reinvestigated the mechanism of cellularization in mammalian myotubes first by using a panel of compounds that induce this effect, second by introducing methods that follow the process in real time in single myotubes, and third by relating cellularization to other aspects of phenotypic dedifferentiation. Our results indicate a significant reappraisal of the activity of these compounds.

Results

A Triazine Compound Can Induce Cellularization of Mammalian Myotubes

In addition to myoseverin, our investigation included a second set of compounds, structurally related to myoseverin but based around a triazine scaffold [13–15]. We compared the effect of these compounds on differentiated cultures of pm128 cells, a murine myoblast cell line in which cells typically fuse to form elongated, multinucleate myotubes [16, 17] that are more responsive to cellularizing agents than C2C12 cells. A cellular assay based on detection of myosin heavy chain (MHC), a late marker of myogenic differentiation, was established to quantitate the ability of these compounds to induce myotube fragmentation. MHC is expressed only in differentiated muscle after postmitotic arrest and fusion, and it remained detectable by immunocytochemistry for 24 hr in myotube fragments generated by the action of myoseverin (Figure 1B). The number of MHC-positive mononucleates, expressed as the percentage of all mononucleates, was used as an index of myotube fragmentation. The triazine 2 (Figure 1A) had the same

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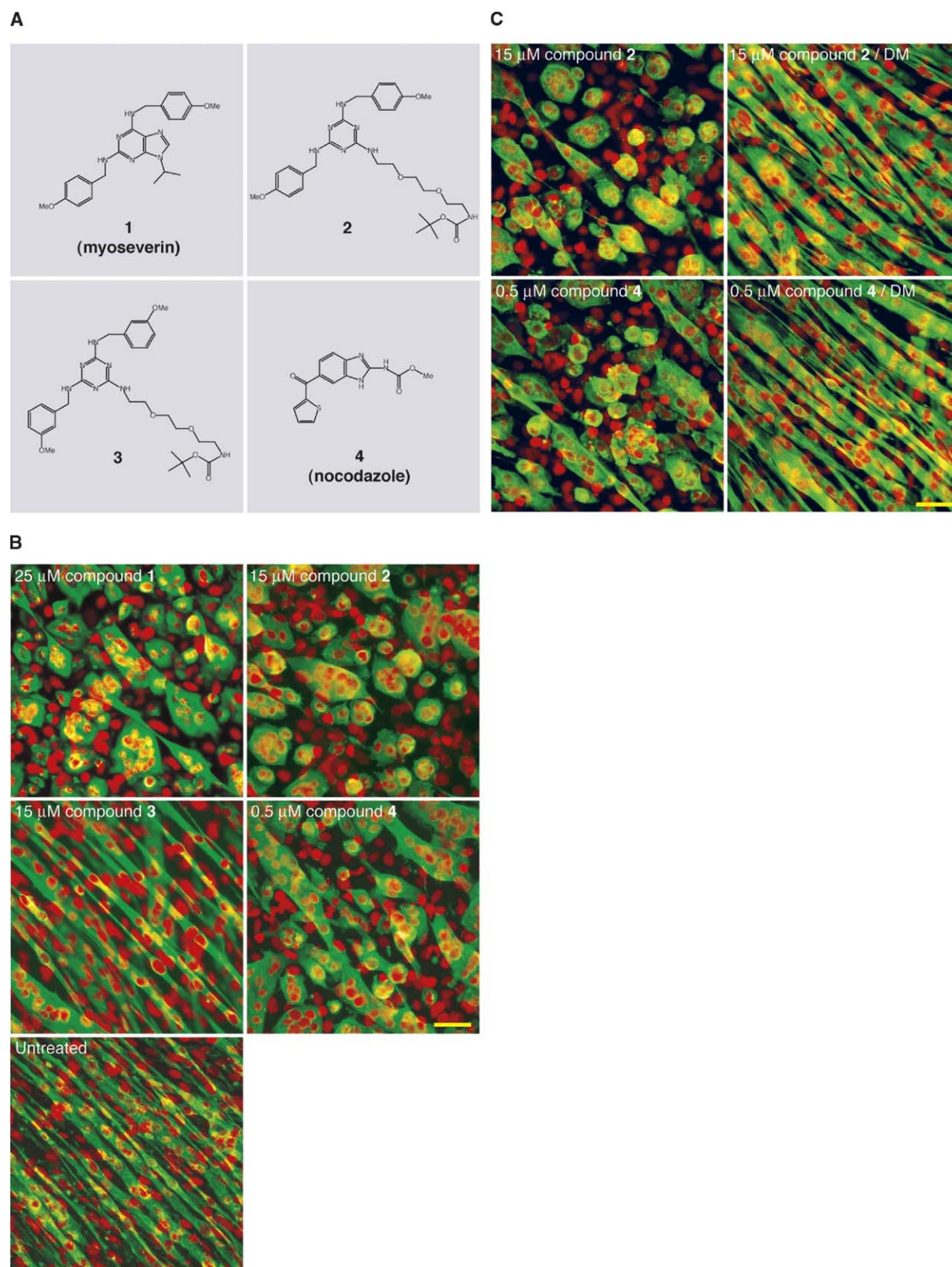


Figure 1. Myoseverin and Other Microtubule Binding Molecules Induce the Reversible Fragmentation of Mammalian Myotubes

(A) Structures of myoseverin, 1, the structurally related triazine compounds, 2 and 3, and the known microtubule-depolymerizing agent nocodazole, 4. Details of the synthesis of 2 and 3 will be published elsewhere.

(B) pmi28 myotube cultures were treated for 24 hr with a range of compounds and then stained with an antibody against MHC (green), and the nuclei were counterstained with propidium iodide (red). Compound 2 affects myotubes in the same way as myoseverin, causing the cells to

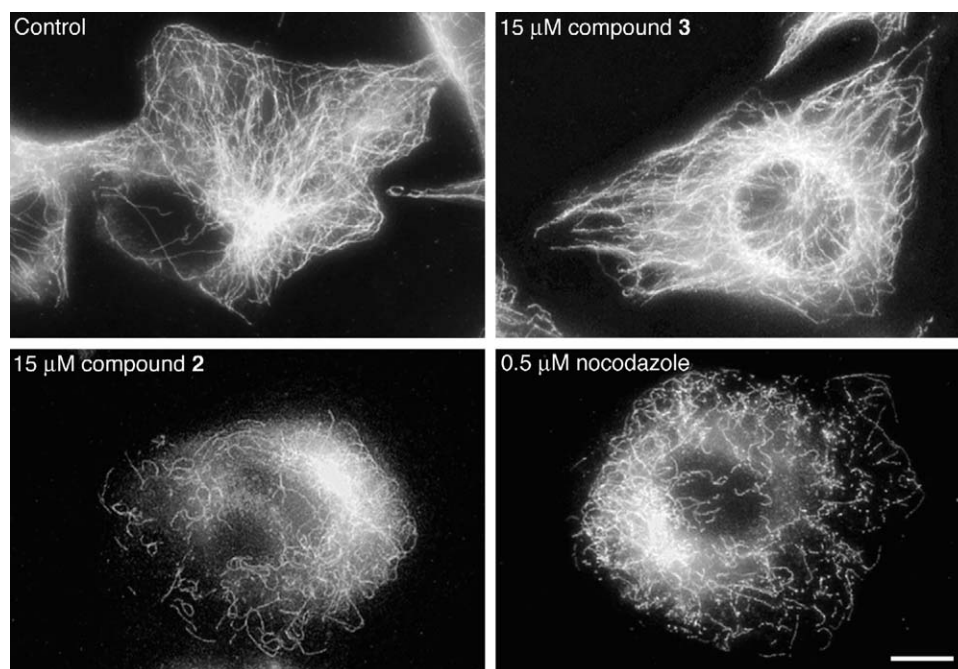


Figure 2. Compound 2 and Nocodazole Disrupt the Microtubule Cytoskeleton

Immunofluorescence images of β -tubulin in myoblasts treated with differentiation medium alone (Control), the inactive triazine compound 3, compound 2, or nocodazole for 24 hr. No effect on microtubule organization is seen in the presence of compound 3, but at concentrations sufficient to induce cellularization of myotubes, compound 2 and nocodazole have analogous effects on polymerized tubulin, causing microtubules to become broken and fragmented. The scale bar is 10 μ m.

ability as myoseverin to fragment mammalian myotubes in culture (Figure 1B); there was an average 5.2-fold increase in MHC-positive mononucleates (18,000 cells; $n = 4$), a number comparable to that seen with myoseverin. In contrast, compound 3 (Figure 1A) did not induce a measurable response, and cultures retained the appearance of control, untreated cells (Figure 1B). As a structural isomer of 2, the inactive triazine 3 is an ideal control compound.

Microtubule Disruption Can Induce Cellularization

While microtubules are the only known target of myoseverin, the specific role of the cytoskeleton in cellularization has not been examined in detail. We compared the effect of myoseverin and compound 2 with that of nocodazole (4, Figure 1A), the known microtubule binding agent and structurally distinct molecule, in order to determine the significance of microtubule disruption in cellularization. The effect of nocodazole at 1–10 μ M was previously reported as both toxic and irreversible [6, 12]; we found that 0.5 μ M nocodazole was able to mimic the action of myoseverin without toxicity, inducing an average 4.5-fold increase in the proportion of MHC-positive mononucleates (12,000 cells; $n = 3$). In addition, the effects of both 2 and nocodazole were shown to be readily reversible (Figure 1C), as had

been previously demonstrated for myoseverin [6]. When the compounds were removed and replaced with fresh differentiation medium, MHC-positive mononucleates returned to levels comparable to control cultures. This suggests that the mononucleates arising by cellularization were competent to fuse again to form myotubes under differentiation conditions. The reversible fragmentation of myotubes by compound 2 is shown in Movies S1 and S2 (see the Supplemental Data available with this article online).

Observation of the effects of compound 2 and nocodazole on the microtubule cytoskeleton also revealed analogous results for the two molecules. At concentrations sufficient to induce cellularization of myotubes, both compound 2 and nocodazole disrupted microtubule organization and polymerization, as reported previously for myoseverin and shown in Figure 2. The inactive triazine compound 3 retained an intact microtubule cytoskeleton comparable with that of untreated cells (Figure 2).

Imaging Cellularization of Single Myotubes

Previous work describing the cellularization of mammalian myotubes by myoseverin has been unable to directly demonstrate the events of cellularization since the high density within differentiated cultures has

fragment and round up, generating mononucleates and multinucleate fragments. Compound 3 is inactive, and the cells retain the appearance of an untreated culture in which myotubes tend to align with one another, with nuclei distributed along the length. Compound 4, nocodazole, is able to mimic the effects of myoseverin.

(C) The effects of nocodazole and compound 2 are reversible. Panels on the left show myotubes treated for 24 hr with compound 2 or nocodazole. The right-hand panels show fusion and restoration of cell morphology after removal of the compounds and incubation in fresh differentiation medium (DM) for an additional 24 hr. Scale bars are 50 μ m.

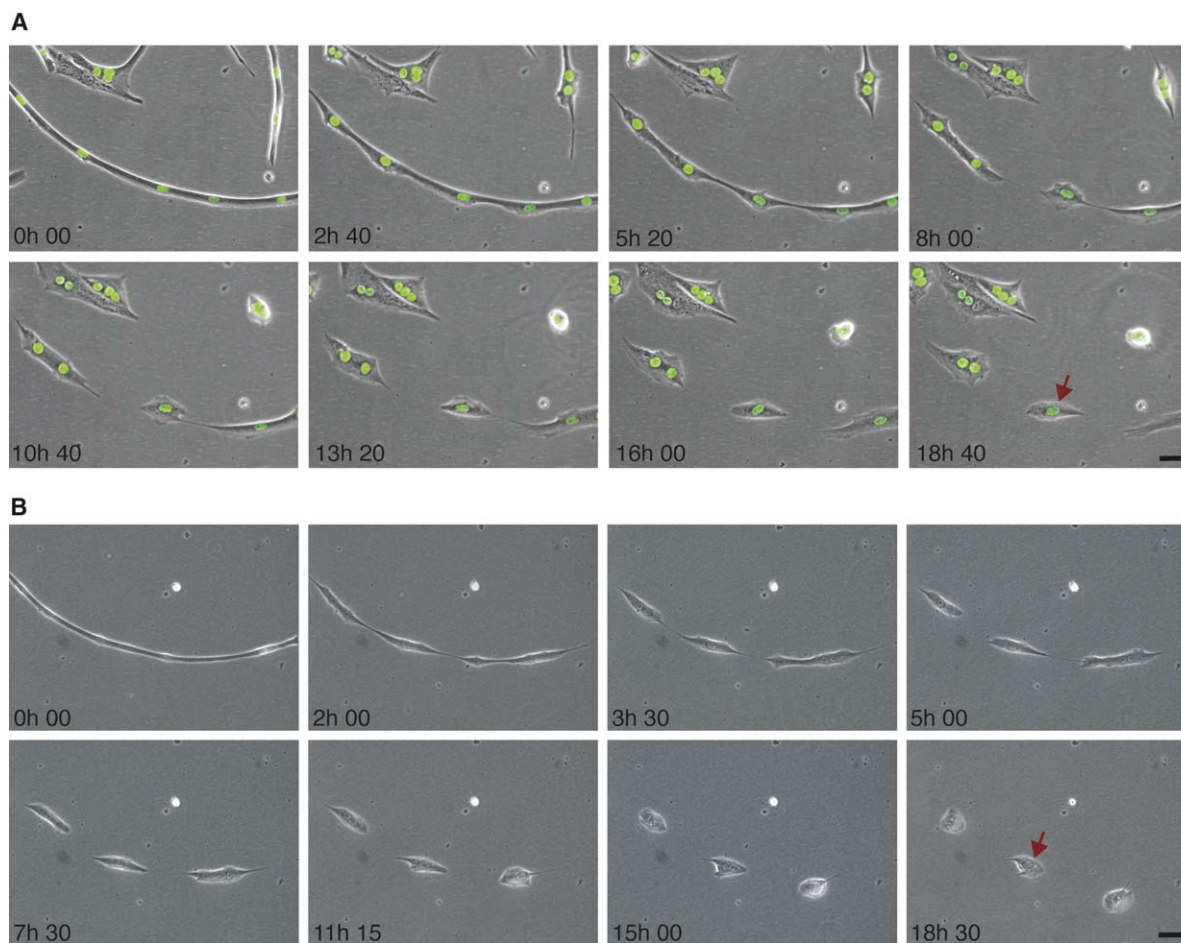


Figure 3. Demonstration of Cellularization of Mammalian Myotubes at a Single-Cell Level

(A and B) Selected frames from time-lapse sequences show the fragmentation of single myotubes over time in response to (A) 15 μ M compound 2 and (B) 0.5 μ M nocodazole. Cells were differentiated and purified to give isolated myotubes, and these cells were then imaged by time-lapse microscopy with phase contrast images captured at 5 min intervals. In each case, the resulting mononucleate cell with a single nucleus is highlighted by a red arrow in the final panel. Cells in (A) were previously transfected with a nuclear-localized eGFP, and fluorescent images are shown overlaid with phase contrast.

made it difficult to visualize individual cells. In order to study more clearly the morphological changes during cellularization, we used pmi28 cells stably expressing a fusion protein of human histone 2B and eGFP [18], which provided a convenient marker in the nucleus. The purification of differentiated cultures enabled us to isolate individual labeled myotubes and to follow their response to compound 2 by time-lapse microscopy. An example of a single labeled myotube undergoing cellularization is shown in Figure 3A and in Movie S3. Compound 2 is seen to induce cytoplasmic constrictions in the myotube on either side of a single nucleus. This is followed by severing of the cytoplasmic bridges connecting the emerging mononucleate to the myotube fragments. Cellularization of a single myotube by nocodazole is shown in Figure 3B and in Movie S4; the response is striking in its similarity to the action of compound 2 (Figure 3A). These images of cellularization are indicative of extensive reorganization of the cytoskeleton, and the comparable effects of nocodazole and compound 2 at this level suggest that microtubule depolymerization plays a critical role in this aspect of myogenic dedifferentiation.

Gene Transcription during Cellularization

We analyzed cellularization in the absence of de novo protein synthesis in order to assess the likely contribution of a transcriptional program in the response of myotubes to the triazine compound 2. Inhibition of protein synthesis within myotubes to a level of 95% was achieved with 10 μ M anisomycin, while cellularization with compound 2 was inhibited only by 57% (data not shown). Although the inhibition of protein synthesis in these experiments was less than 100%, the induction of significant levels of cellularization within 24 hr suggested that gene transcription might not be obligatory.

The finding that nocodazole can also bring about the reversible fission of mammalian myotubes has allowed us to investigate further the mechanism of cellularization by microarray analysis. The use of nocodazole to dissect the genes regulated by compound 2 enabled us to assess whether, in addition to its effects on microtubules, compound 2 may also be activating a specific pattern of gene expression. The number of genes altered by at least 1.5-fold with either compound 2 or nocodazole over a 24 hr period is shown in Figure 4A. The inactive isomer 3 was used as a control. Of specific

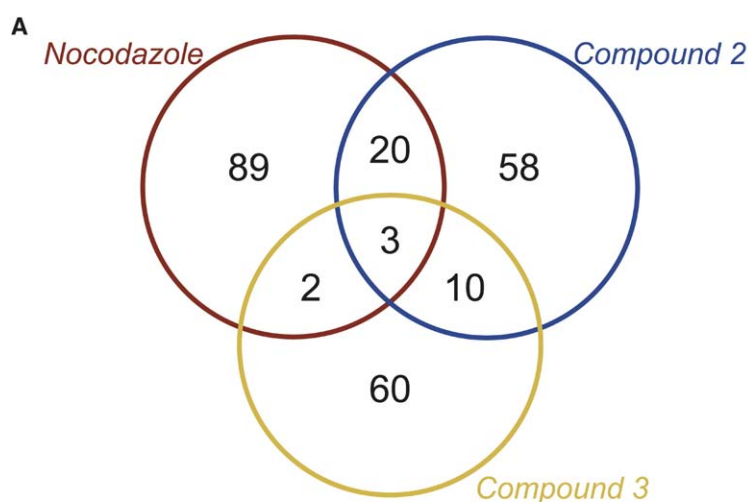


Figure 4. Gene Expression Analysis of Cellularization by Nocodazole and Compound 2

(A) Summary of genes altered greater than 1.5-fold with either compound 2, nocodazole, or the inactive compound 3. Note that 2 and nocodazole are active but structurally unrelated, whereas 3 is an inactive isomer of 2.

(B) List of 20 genes regulated in response to both compound 2 and nocodazole.

B

Accession no.	Gene name	Fold change compound 2	Fold change nocodazole
AI181132	Creatine kinase, mitochondrial 2	3.57	4.84
X70296	Serpine 2	2.88	2.74
U47737	Sca-2/TSA-1	2.80	1.97
U04541	Tropomyosin 3, gamma	2.71	3.08
M12571	Heat shock protein 1a	2.65	1.88
AJ001418	Pyruvate DH kinase 4	2.50	2.35
X83202	Hydroxysteroid 11-beta DH 1	2.08	2.09
D88994	AMP deaminase 3	2.02	1.88
L10244	Sat	1.96	2.40
X96737	Synaptobrevin like 1	1.67	1.77
AA270365	CLF-1	-1.61	-1.89
U61969	Wnt10a	-1.75	-2.13
J03750	RNA polymerase II transcriptional coactivator	-1.82	-1.92
X04663	Tubulin, beta 5	-1.82	-2.00
AA716963	Isopentenyl-diphosphate delta isomerase	-1.85	-2.44
AF060539	Cipp	-1.89	-2.78
AW122523	Fatty acyl elongase	-1.92	-2.56
M13443	Tubulin alpha 7	-1.96	-1.92
M33988	Histone H2A.1	-2.22	-1.92
M24377	Egr-2/Krox-20	-2.44	-2.44

interest are the 20 genes, shown in Figure 4A and listed in Figure 4B, that are regulated by compound 2 and nocodazole, structurally distinct molecules with the same activity of myogenic dedifferentiation. This list comprises genes covering a wide range of functions and includes the downregulation of α - and β -tubulin.

Time-Lapse Observations of Mononucleate Progeny

Reversal of the mononucleate-to-multinucleate transition is a significant and necessary step in myogenic dedifferentiation. In order to determine if this event was accompanied by a complete reversal of myogenesis resulting in proliferating mononucleate cells, as was originally suggested based on observations of dense and heterogeneous cultures [6], we again used time-lapse microscopy to follow individual mononucleates arising by cellularization of single myotubes in response to compound 2. The use of a motorized stage enabled

a higher-throughput approach, with the successive capture of images from up to 20 predetermined fields at each chosen time point for the duration of the experiment, and the assembly of 20 near-simultaneous movies from a single culture (Figure 5A). The cells were maintained in growth medium for up to 4 days after cellularization and washing to remove the compound. Under these conditions, preexisting mononucleate myoblasts in the culture were seen to proliferate, confirming that culture conditions were sufficient to induce division. In the majority of cases, the cells arising as the result of a cellularization event survived as mononucleates and regained the elongated morphology characteristic of larger multinucleate myotubes. A total of 115 movies from 13 independent experiments were assembled and analyzed, and no cases of division were observed in the population of 146 mononucleates formed by cellularization with compound 2 (summarized in Figure 5B). In addition, identified mononucleate

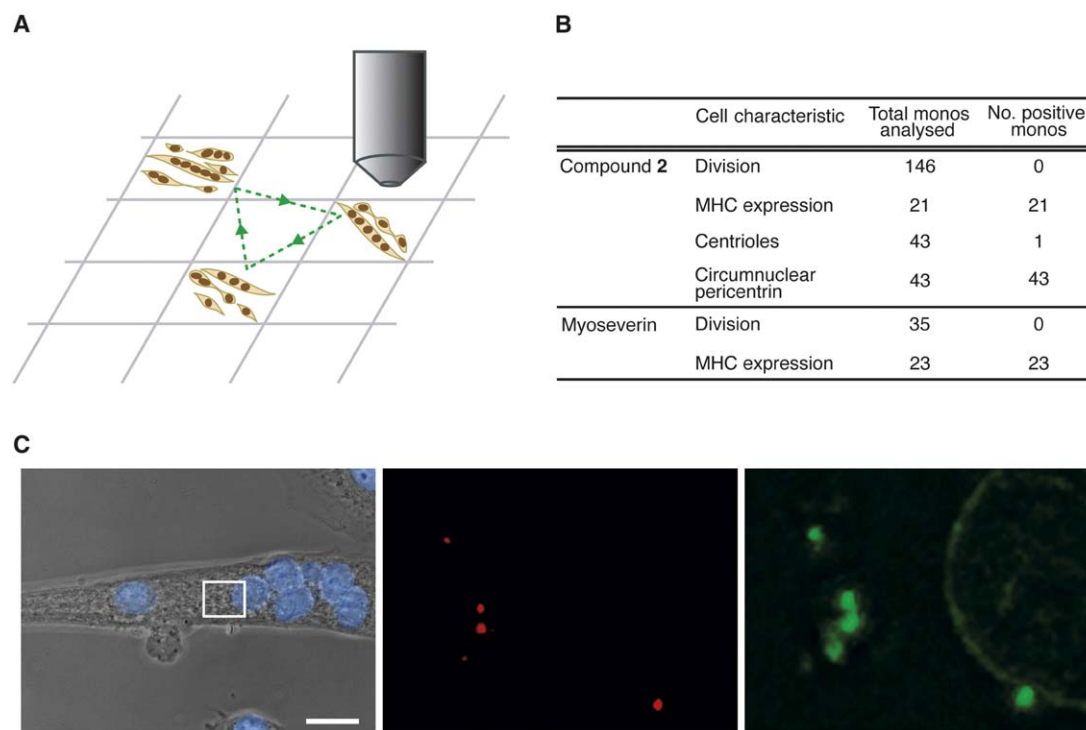


Figure 5. Determination of Cell Fate of Mononucleate Progeny

(A) Schematic of a time-lapse experiment to follow the behavior of individual mononucleates, generated by the cellularization of myotubes. Following purification, isolated myotubes were monitored over a 24 hr period of exposure to compound 2. In conjunction with a motorized stage, time-lapse microscopy enabled the observation of multiple cellularization events in parallel. The resulting mononucleate progeny were then followed for an additional 4 days in growth medium before fixation.

(B) Summary of characterization of mononucleate cells (monos) followed by time-lapse microscopy. Of 146 cells (34 followed for 3 days, and the remaining 112 followed for 5 days) arising by cellularization with compound 2, no cells were observed to undergo cell division. The same was true of 35 mononucleate cells arising by fragmentation of myotubes by myoseverin. All cells analyzed retained expression of MHC after 5 days in culture, while only a single mononucleate was positive for the presence of centrioles.

(C) Detection of centrioles in multinucleate myotubes by immunocytochemistry. The left panel is a phase contrast image of an isolated myotube; nuclei were stained with hoechst (overlaid in blue). The scale bar is 20 μ m. The remaining two panels represent a magnified view of the marked region on the phase contrast image: the central panel shows γ -tubulin staining, and the right panel shows pericentrin staining. γ -tubulin localizes to discrete points in the cytoplasm, the centrioles, while pericentrin also localizes to the centrioles but forms a diffuse cloud of pericentriolar satellites. Pericentrin is also redistributed to the nuclear surface in myotubes.

progeny arising by fragmentation of myotubes by myoseverin were also examined. Again, no cases of cell division were observed in the 35 mononucleates followed (in 2 independent experiments) (Figure 5B). This finding contrasts with that of earlier work with myoseverin in which cell division was reported [6, 12].

Cells Retain Properties of Parental Myotubes

In a number of cases, cells were fixed at the end of the time-lapse experiment and were stained for MHC, a marker of terminally differentiated myotubes. All mononucleate progeny analyzed were found to retain strong expression of the protein (Figure 5B). MHC-positive mononucleates were also analyzed for incorporation of BrdU (bromodeoxyuridine) after fragmentation of purified myotube cultures with compound 2, followed by washing of the cells and incubation for a 24 hr period in growth medium in the presence of BrdU. The results did not differ significantly between control untreated cultures in which an average of 2.6% of the MHC-positive mononucleates incorporated BrdU and cultures treated with compound 2 in which 1.3% of the cells staining positive for MHC were also positive for

incorporation of BrdU (in four independent experiments). This is consistent with the absence of division reported above.

In addition to MHC, identified mononucleates fixed at the conclusion of time-lapse experiments were stained for markers of centrioles. Centrosomes act as the primary microtubule organizing center (MTOC) of almost all mammalian somatic cell types. They comprise a pair of centrioles and a surrounding matrix, the pericentriolar material (PCM), from which microtubules are nucleated and anchored [19]. The centrioles replicate once every cell cycle, maintaining a 2:1 ratio with the nucleus, and, in addition to their role in microtubule organization, they have been shown to be required for cell cycle progression [20–22]. The progressive loss of centrioles from muscle cells after differentiation has been well documented [23–26], and, if they remain absent from the mononucleates generated by cellularization of myotubes, then this may contribute to the observed failure to undergo cell division. Pericentrin and γ -tubulin are both well-characterized components of the PCM [27, 28], and antibodies to these proteins were used here to detect centrioles in myotubes and in the

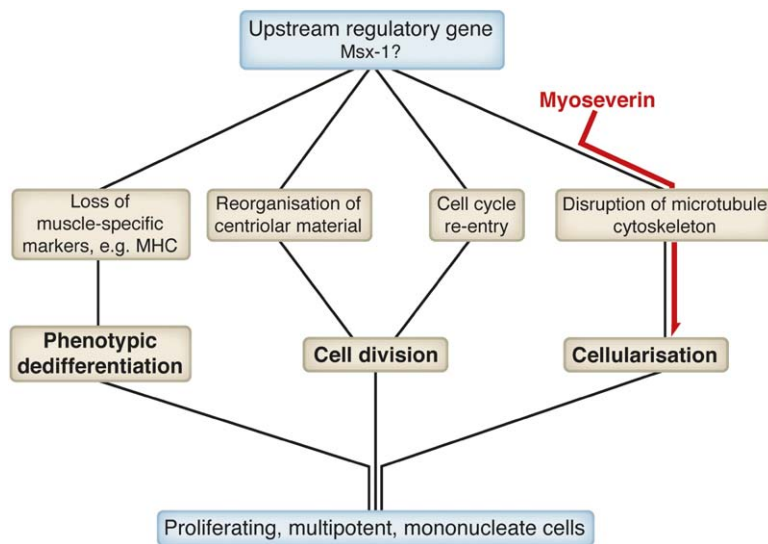


Figure 6. Schematic Diagram of Myogenic Dedifferentiation

A number of different aspects of myogenesis must be reversed in order to generate proliferating mononucleate cells from multinucleate, postmitotic myotubes. Cellularization is an essential process during myogenic dedifferentiation and can be induced by myoseverin, but it is not sufficient to activate the full program of dedifferentiation.

mononucleate progeny generated from them by the action of compound 2. A reduced number of centrioles was observed in myotubes, and they were no longer associated in pairs, but were instead seen as isolated foci or sometimes as clusters (Figure 5C). In all but one of the mononucleate cells followed by time-lapse microscopy and stained for pericentrin and γ -tubulin, no centrioles were detected (Figure 5B). All cells stained for pericentrin displayed circumnuclear localization of the protein, a distribution pattern associated with terminally differentiated myotubes [24, 26], as shown in Figure 5C.

Discussion

Our results with a panel of small molecules have defined a specific role for cellularization within the myogenic dedifferentiation program, and they significantly extend the conclusions drawn previously with myoseverin. We report the discovery of a triazine compound 2, with structural similarity to myoseverin, which is able to bring about the fragmentation of mammalian myotubes in culture. Compound 3, a structural isomer of compound 2, but inactive in our assay for cellularization, provides an ideal control for experiments with compound 2 to investigate the mechanism of cellularization in pmi28 cells. The pmi28 cell line [16, 17] was found to be more stable in culture and more responsive to cellularization with both myoseverin and compound 2 when compared with the C2C12 cell line, possibly as a result of the higher proportion of long, narrow, and unbranched myotubes, a morphology previously reported to favor cellularization [6]. The convergence of forward and reverse activities with nocodazole indicates that myoseverin brings about myotube fragmentation primarily through its effects on microtubules, and has confirmed microtubules as the critical target of myoseverin [6] and also of the related triazine compound 2.

The use of nocodazole and compound 2 to examine the gene expression changes that are specific to cellularization has extended the earlier microarray analysis carried out with myoseverin alone. We observed the downregulation of both α - and β -tubulin, but we did

not detect an abundance of transcripts involved in tissue repair and the response to injury as originally described for myoseverin [6]. It should be noted, however, that our microarray experiments did not attempt a direct comparison between myoseverin and compound 2, and it remains possible that, despite a common action on the microtubule cytoskeleton, structural differences between myoseverin and compound 2 may enable myoseverin to bind to additional targets within the cell that are not available to the larger triazine compound. We also note that the use of the pmi28 cell line in these experiments and the C2C12 cell line in the earlier work with myoseverin is an additional consideration. The microarray data presented here are consistent with the hypothesis that the cellularization of myotubes by compound 2 is brought about through effects on the microtubule cytoskeleton rather than by the activation of a program such as that involved in the response of fibroblasts to serum [29], or during urodele regeneration [2].

The reversal of the mononucleate-to-multinucleate transition is an essential step in the dedifferentiation program, and Figure 6 summarizes the different aspects of myogenic dedifferentiation, including cellularization, that are required to generate proliferating multipotent, mononucleate cells from terminally differentiated myotubes. Our experiments have addressed each of these aspects in response to our panel of small molecules. Analysis at the single-cell level has confirmed that myoseverin is able to generate mononucleate progeny from multinucleate myotubes, but time-lapse microscopy shows that these cells retain key properties of the parental myotubes, including expression of MHC and the postmitotic arrest. The application of time-lapse microscopy in our experiments has enabled the unambiguous identification of individual mononucleate progeny in the presence of contaminating preexisting myoblasts, and it is a significant improvement in methodology for determining cell fate and behavior in the context of mixed cultures. Using this technology, we did not observe division of mononucleate progeny, and one aspect of this inability to divide may be the absence of

centrioles. As indicated in the schematic, we propose that myoseverin is able to bring about cellularization by acting directly on the microtubule cytoskeleton without activating other elements of the dedifferentiation program.

Microtubules take part in many vital processes within the cell, and changes in microtubule dynamics play an important role in bringing about morphological changes. During myogenesis, the rearrangement of microtubules from a radial to a linear array is instrumental in the elongation and alignment of myoblasts prior to fusion. While the microtubule cytoskeleton participates in establishing the differentiated state, it seems unlikely that its integrity is solely responsible for maintaining it, as supported by our findings that microtubule disruption is unable to activate a full program of myogenic dedifferentiation. Studies of cellularization in other systems, such as *Drosophila*, in which the formation of single cells from a multicellular syncytium occurs during normal embryonic development, have yet to establish the role of microtubules in this context, and it remains to be seen whether there are mechanistic links with urodele regeneration [30, 31].

While it remains possible that extracts of the newt limb blastema, which have been reported to induce cellularization of mammalian myotubes in culture [32], may contain myoseverin-like molecules that act directly on microtubules, it appears more likely that the different aspects of dedifferentiation (Figure 6) are controlled by an upstream regulator such as the homeodomain protein *Msx-1*. Evidence from both urodele and mammalian systems has implicated *Msx-1* in the events of muscle dedifferentiation [33, 34]. Reversal of the mononucleate-to-multinucleate transition is an important and necessary aspect of myogenic dedifferentiation and is dependent on dramatic cytoskeletal rearrangements; we have shown, however, that this is not sufficient to activate the more complete reversal of the myogenic program seen during limb regeneration.

Significance

There is currently significant interest in mechanisms of dedifferentiation and their relevance for regenerative medicine, and an important direction is the identification of compounds that induce cellular differentiation or its reversal. The work described here demonstrates the value of using small molecules in the dissection of complex biological processes such as myogenic dedifferentiation. We have used myoseverin and the triazine compound 2 to analyze the role of one aspect of myogenic dedifferentiation in the context of a more complete program. The use of time-lapse microscopy to carry out an extensive analysis of the consequences of cellularization for identified mononucleate cells has enabled us to revise the current view of myoseverin and describe a role for cellularization as necessary, but not sufficient, to induce a full program of myogenic dedifferentiation. The complete reversal of myogenesis is likely to require regulatory elements, such as *Msx-1*, functioning upstream of cellularization. While the exact mechanism of cellularization awaits further clarification, the disruption of microtubules is clearly a critical event.

Experimental Procedures

Cell Culture

Pmi28 cells were kindly provided by Prof. Anna Starzynski-Powitz and were grown on BIOCOAT collagen I T-75 flasks (Beckton-Dickinson) as described [16]. Cells were transfected with a pseudotyped retroviral vector encoding an H2B-eGFP fusion protein as previously described [35]. Pmi28 cultures were allowed to differentiate for 3 days before being trypsinized and purified. The cell suspension was passed first through a 25 μ m filter (VWR), and cells retained on the filter were resuspended in differentiation medium, then passed through a 100 μ m filter (VWR). Cells in the flow-through were plated onto collagen-coated plastic dishes. BrdU was added to culture medium at a final concentration of 10 μ M for 24 hr, followed by extensive washing. Incorporation into cells was detected by immunocytochemistry.

Cellularization Assay

Cells were plated at high density on silane-coated glass coverslips and were differentiated for 3 days in pmi28 differentiation medium before addition of compounds. Myoseverin was used at 25 μ M, and the triazine compounds 2 and 3 were used at 15 μ M; nocodazole (Calbiochem) was used at 500 nM. All compounds were diluted in differentiation medium. For each independent experiment, duplicate coverslips were used for every treatment. After treatment, all cells were fixed and stained with an antibody against MHC; nuclei were counterstained with propidium iodide.

Immunocytochemistry

For detection of MHC, cells were fixed in 2% paraformaldehyde (PFA), followed by postfixation with cold 100% methanol. Cells were incubated with a monoclonal antibody to mouse MHC (A4.1025, Dr. Simon Hughes, Randall Institute, Kings College, London) for 1 hr, followed by incubation with FITC-conjugated rabbit anti-mouse (Dako) for 1 hr. For detection of BrdU, cells were fixed as for MHC staining, which was followed by the addition of 0.5 M hydrochloric acid. Cells were incubated with a mouse monoclonal antibody to BrdU (Amersham) overnight at 4°C, followed by incubation with Texas Red-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc.) for 1 hr. For detection of centrioles, cells were washed briefly in extraction buffer (EB; 60 mM PIPES [pH 6.9], 25 mM HEPES [pH 6.9], 10 mM EGTA, 1 mM MgCl₂), then fixed on ice in 4% PFA in EB for 10 min. Cells were then washed in EB + 0.2% Triton X-100 and were postfixed in cold 100% methanol. The cells were incubated with the two primary antibodies against pericentrin (rabbit antibody; Covance) and γ -tubulin (clone GTU-88, mouse monoclonal IgG1 isotype; Sigma), simultaneously, overnight at 4°C, followed by incubation with FITC-conjugated swine anti-rabbit for 1 hr (Dako) and then Texas Red-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc.) for 1 hr. For microtubule staining, cells were fixed in 100% cold methanol and incubated overnight with antibody against β -tubulin (clone TUB 2.1; Sigma). All antibody incubations and washes were carried out in D-PBS containing 10% goat serum (Sigma) and 0.05% sodium azide. Nuclei of fixed cells were stained with either Hoechst 33258 or propidium iodide at 1 μ g/ml. The cells were observed on a Zeiss Axioplan 2 microscope, and phase contrast or fluorescent images were captured with an AxioCam HRC digital camera (Zeiss), controlled through the Axiovision 3.1 software. Fluorescent images of centrioles and of microtubules were acquired at 0.2 μ m intervals, and deconvolution was applied to the z-stacks with Volocity and Openlab software (Improvision).

Microarray

Triplicate cultures of differentiated pmi28 cells were prepared for time zero, for 12 and 24 hr treatments with 15 μ M compound 2, and for 12 and 24 hr treatments with 500 nM nocodazole; duplicate cultures were prepared for 24 hr treatment with 15 μ M compound 3. Each individual culture gave rise to material for hybridization to a single Affymetrix GeneChip; all replicates were treated as independent samples. Total RNA was extracted by using Tri reagent (Sigma); cDNA synthesis and in vitro transcription was carried out according to the Affymetrix Expression Analysis Technical Manual and the manufacturer's instructions. For each independent sample, 15 μ g

biotin-labeled cRNA was hybridized to a single Affymetrix MG-U74Av2 chip containing 12,488 probe sets. Hybridization was carried out at 45°C for 16 hr. The GeneChip Fluidics Station 400 was used to wash and stain the probe array. The antibody amplification protocol was followed according to the Affymetrix manual. During the course of sample preparation, RNA degradation and contamination was checked periodically by gel electrophoresis, spectrophotometry, and an Agilent bioanalyzer. At all stages, RNA was of high quality and was within Affymetrix recommended parameters.

Initial processing of the raw data files for each chip was carried out by implementation of a number of statistical algorithms in the Affymetrix Microarray Suite 5.0. A detection call of Present, Marginal, or Absent was assigned to each probe set, and a signal was calculated to provide a measure of the abundance of the transcript. GeneSpring was used to compare the transcription profiles of the individual array and calculate expression ratios; values less than 1.5-fold were considered unchanged. Affymetrix-supplied gene names were updated by crossreferencing with GenBank accession numbers, and where more than one name existed, the most common or most descriptive was applied.

Time-Lapse Microscopy

Cells in a 35 mm culture dish was placed under an Axiovert S100 (Zeiss) inverted microscope fitted with an incubation chamber to provide a controlled environment. The microscope was equipped with a motorized stage and focus (Prior Scientific), controlled through Image Pro Plus software and an AFA module (Media Cybernetics). Phase contrast or fluorescence images were sequentially acquired through an FDI (Photonic Sciences) or Hamamatsu Orca ER Camera integrated with the imaging system. The culture dish under study was secured on a dish holder on the stage and covered with a heated quartz glass lid that allowed for long-term imaging without any condensation. Humidified 5% CO₂ was supplied to the chamber through a flex pipe. The cells were treated for 24 hr with cellularizing agents, and images were captured at each predefined stage position and focus plane on the dish every 4–5 min through a 10× objective. After 24 hr of treatment, the medium was removed; cells were washed and replaced with growth medium. Thereafter, time-lapse imaging was continued at the same positions and focus plane for 4 days at 20 min intervals. Image sequences were created from each stage position and were analyzed in Image Pro Plus. The image frames from the sequence of interest were archived and further processed in Adobe Photoshop 7.0 and Adobe Illustrator CS.

Supplemental Data

Supplemental Data including movie sequences of myotubes undergoing cellularization in response to compounds are available at <http://www.chembiol.com/cgi/content/full/12/10/1117/DC1/>.

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Accession Numbers

The microarray data was deposited in ArrayExpress at the European Bioinformatics Institute (accession number [E-MEXP-401](#)).