ACS chemical biology

ARTICLES

pubs.acs.org/acschemicalbiology

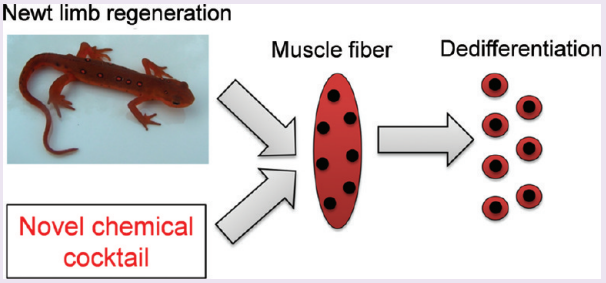
Novel Chemically Defined Approach To Produce Multipotent Cells from Terminally Differentiated Tissue Syncytia

Da-Woon Jung and Darren R. Williams*

Small Molecule Regulators and Biosystems Laboratory, School of Life Sciences, Gwangju Institute of Science and Technology, 1 Oryong-Dong, Buk-Gu, Gwangju 500-712, Republic of Korea

Supporting Information

ABSTRACT: In urodele amphibians, a critical step in limb regeneration is the cellularization and dedifferentiation of skeletal muscle. In contrast, mammalian skeletal muscle does not undergo this response to injury. We have developed a novel simple, stepwise chemical method to induce dedifferentiation and multipotency in mammalian skeletal muscle. Optimal muscle fiber cellularization was induced by the trisubstituted purine small molecule, myoseverin, compared to colchicine, nocodazole, or myoseverin B. The induction of a proliferative response in the cellulate was found to be a crucial step in the dedifferentiation process. This was achieved by down-regulation of the cyclin-dependent kinase inhibitor, p21 (CDKN 1A, CIP1). p21 was found to be a key regulator of this process, because down-regulation of the cyclin-dependent kinase inhibitors p27 (CDKN1B/KIP1) or p57 (CDKN1C/KIP2) or the tumor suppressor p53 (TP53/LFS1) failed to induce proliferation and subsequent dedifferentiation. Treatment with the small molecule reversine (2-(4-morpholinoanilino)-6-cyclohexylaminopurine) during this proliferative "window" induced the muscle cellulate to differentiate into non-muscle cell types. This lineage switching was assessed using a relatively stringent approach, based on comparative functional and phenotypic assays of cell-type specific properties. This showed that our chemical method allowed the derivation of adipogenic and osteogenic cells that possessed a degree of functionality. This is the first demonstration that mammalian muscle culture can be induced to undergo cellularization, proliferation, and dedifferentiation, which is grossly similar to the key early steps in urodele limb regeneration. These results, based solely on the use of simple chemical approaches, have implications for both regenerative medicine and stem cell biology.



Dedifferentiation describes the phenomenon where differentiated cells acquire a "stem cell"-like or multipotent state, before differentiating into one or more alternative tissue types.[1] All species possess a natural capacity for dedifferentiation as part of their ability to regenerate tissues.[2] For example, acinar or duct cells in the pancreas of mammals can dedifferentiate to produce insulin-expressing β-cells.[3] However, the capacity for dedifferentiation differs between species, with phylogenetically primitive vertebrates retaining a greater capacity for dedifferentiation. For example, cells of the central nervous system in amphibians and fish can dedifferentiate (reviewed in ref 4). Moreover, the retina, lens, and limb of urodele amphibians dedifferentiate during the entire regeneration of these tissues.[4] However, the greatest capacity for dedifferentiation is found in plants. For example, mesophyll protoplasts of tobacco (*Nicotiana tabacum*) undergo dedifferentiation and generate entire plants.[5]

During the 1990s, advances in combinatorial chemistry facilitated the large scale production of small molecule libraries based on the molecular scaffolds of known bioactive compounds.[6] Recently, we and others have used this approach to identify novel regulators of diverse biological processes.[7,8] The use of small molecules to manipulate cellular processes has a number of advantages (reviewed in 9). Small molecules typically provide a high degree of temporal control over protein function; they are often reversible and their effects can be finely tuned by varying the concentrations of the small molecule. In addition, a single small molecule has the potential to simultaneously modulate multiple specific targets within a cell. This approach can allow the production of a desirable phenotype in a synergistically favorable manner.

Phenotype-based screening of small molecule libraries has been employed to discover new regulators of cellular dedifferentiation.[10,11] A prominent example was the discovery of the tubulin-binding molecule myoseverin, identified from a library of 2,6,9-trisubstituted purines.[10,12] The molecular scaffold of myoseverin is based on olomoucine, a known inhibitor of the cell cycle regulatory protein cyclin-dependent kinase 2. Myoseverin was reported to promote dedifferentiation in the C2C12 mouse myoblast model of skeletal muscle differentiation. This result was significant because the fragmentation of multinucleated myofibers into proliferating mononucleate cells is an important feature of limb regeneration in urodele amphibians.[13] However, a

Received: October 10, 2010
Accepted: February 15, 2011
Published: February 15, 2011

subsequent study in the pmi28 mouse myoblast model of skeletal muscle differentiation suggested that myoseverin induces muscle fiber cellularization.[14] The cellularized, mononuclear cells remained refractory and did not dedifferentiate.

During limb regeneration in urodele amphibians, the nuclei of multinucleate skeletal muscle fibers reenter the cell cycle.[15] In contrast, nuclei in terminally differentiated mammalian skeletal muscle are relatively refractory to experimentally induced reactivation of the cell cycle.[16] A number of cyclin-dependent kinase inhibitors (CDKN) are implicated in the maintenance of skeletal muscle terminal differentiation, including p18 (CDKN2C/INK4C), p21 (CIP1/CDKN1A), p27 (CDKN1B/KIP1), and p57 (also known as CDKN1C/KIP2).[17−19] Recent research has suggested that sole knock-down of p21 is sufficient to reactivate the cell cycle in mammalian skeletal muscle, even in the absence of exogenous growth factors. [18]

In this research paper, we describe our attempt to develop a relatively simple, "gentle", and reversible chemically defined method for inducing the dedifferentiation of mammalian skeletal muscle, including cell cycle re-entry. We show that the sequential use of small molecule treatment and temporary p21 suppression allows the derivation of proliferating, mononuclear cells from differentiated mammalian skeletal muscle. These cells could be induced to act as multipotent stromal cells by further, stepwise treatment with the small molecule, reversine (2-(4-morpholinoanilino)-6-cyclohexylaminopurine)[11] and simple chemical modifications of the culture media.

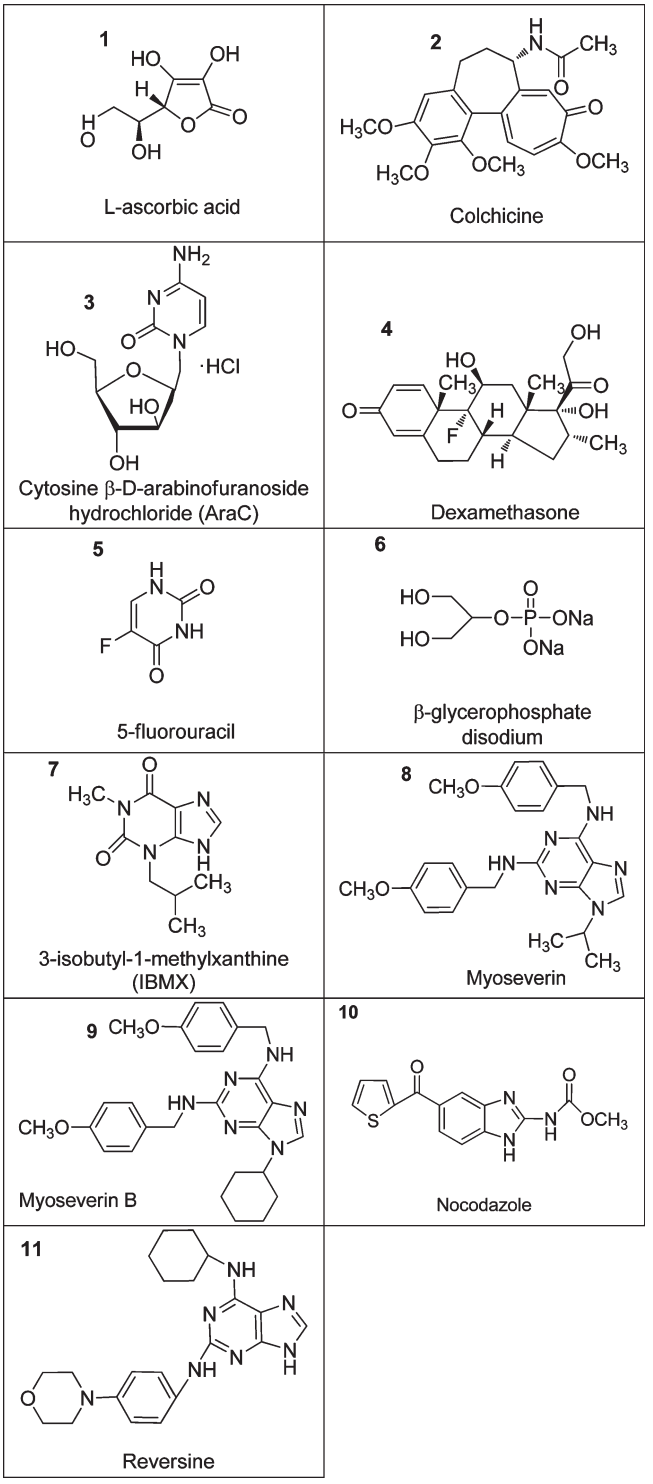
RESULTS AND DISCUSSION

Establishment of Culture Conditions. A list of the small molecules used in this study is shown in Table 1. To study effectively the process of myotube cellularization, a high degree of muscle differentiation should be obtained. However, a problem is contamination of the cultures with undifferentiated myoblasts, which has produced conflicting results in previous studies of cellularized myotube cultures.[10,14] To enhance myogenesis in C2C12 myoblast cultures, the antimitotic drugs AraC[20] and 5-FU[21] were tested, with the aim of eliminating proliferating myoblasts. It was found that treatment with AraC during the differentiation process could significantly increase the fusion index, which is a measure of the number of nuclei incorporated into myotubes (Figure 1a). In addition, sieving of trypsinized myotube cultures with a 40 μm mesh allowed the derivation of myotubes cultures with a fusion index of close to 100%, indicating an absence of single cell myoblasts (Figure 1a). Images of purified myotubes are shown in Supplementary Figure 1.

To study effectively the process of myotube cellularization, the optimum cellularization-inducing agent was identified. Four tubulin-binding small molecules were tested for their ability to induce cellularization, while causing relatively low cytotoxicity: colchicine, nocodazole, myoseverin, and myoseverin B. The induction of cellularization with myoseverin was found to produce the largest number of mononuclear cells that could exclude the dye, trypan blue, which is a marker of cell viability (Figure 1b). Thus, myoseverin was used for studies of C2C12 cellularization and transdifferentiation.

p21 Down-regulation Can Induce Cellularized Myotubes To Proliferate. Mononuclear cells generated by the cellularization of C2C12 myotubes were treated with siRNA targeting either p21 (CDKN 1A, CIP1), p27 (CDKN1B/KIP1), p57 (CDKN1C/KIP2), or the tumor suppressor p53 (TP53/LFS1)

Table 1. Structures of Small Molecules Used in This Study to Promote Skeletal Muscle Dedifferentiation and Redifferentiation[a]



[a] In alphabetical order; structures are numbered in bold font.

(shown schematically in Figure 2a). A decrease in protein expression was observed 48 h after transfection of the cellulate with each siRNA (Figure 2b). Gene knock-down was also confirmed by RT-PCR (Supplementary Figure 2).

dx.doi.org/10.1021/cb2000154 |ACS Chem. Biol. 2011, 6, 553–562

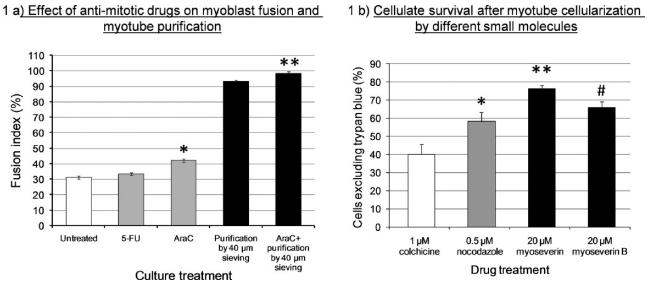


Figure 1. Establishment of culture conditions. (a) Treatment of differentiating C2C12 myoblast cultures with 50 μM AraC for 72 h increased the fusion index of the differentiated culture (*i.e.*, the number of cell nuclei incorporated into multinucleate myotube syncytia). Treatment of differentiating C2C12 myoblast cultures with another antimitotic drug, 10 μM 5-FU for 72 h, did not affect the fusion index. Sieving of the myotube culture to remove undifferentiated myoblasts, followed by replating in a new culture dish, dramatically increased the fusion index. Treatment with AraC during the differentiation step further increased the fusion index of the myotube cultures after sieving. Error = SD; $^{*}P < 0.05$ compared to untreated, differentiated C2C12 culture. $^{**}P < 0.05$ compared to purification by 40 μm sieving alone. Data is representative of three independent experiments. (b) Myotube cultures underwent cellularization by treatment with either 1 μM colchicine, 0.5 μM nocodazole 20 μM myoseverin, or 20 μM myoseverin B for 24 h. Viable cells were classified as those that excluded the dye, trypan blue. Myoseverin was found to induce cellularization that produced the least amount of cytotoxicity. Error = SD; $^{*}P < 0.05$ compared to colchicine treatment; $^{**}P < 0.05$ compared to nocodazole, colchicine and myoseverin B treatment; $^{\#}P = 0.05$ compared to myoseverin treatment. Data is representative of three independent experiments.

Propidium iodide (PI) staining of cell nuclei indicated that only p21 down-regulation induced an increased nuclei content of DNA, indicative of cell cycle progression (Figure 2c). This result was validated by the observation that only p21 down-regulation caused an increase in the portion of cells incorporating BrdU, an indicator of increased DNA synthesis (Figure 2d). This effect could also be seen in a count of the cell number, with p21 down-regulation producing a small but significant increase in cell number (Figure 2e).

p21 Down-regulation Overcomes the Negative Effect of Reversine on Myogenic Potential. Previous research has indicated that cellularization of myotubes does not produce a change in the differentiated state of the mononuclear cell compared to the parent myotube.[14] The effect of p21 down-regulation on the differentiated state of the mononuclear cells derived from cellularized myotubes was assessed using FITC-conjugated α-bungarotoxin, which binds to the acetylcholine receptor (a marker of myotube formation). p21 down-regulation reduced α-bungarotoxin labeling of mononuclear cells (Figure 3a and b) indicating a shift in the differentation status away from the myotube and toward the myoblast cell. The effect of p21 down-regulation was similar to that achieved by the small molecule reversine, which has been shown to increase the developmental plasticity of muscle cells.[11] However, reversine treatment reduced the ability of muscle cells and cellularized myotubes to re-enter myogenesis (Figure 3c). This reduction in myogenesis was overcome by the knock-down of p21 (Figure 3d and e). This result indicates that p21 down-regulation increases the developmental plasticity of muscle cells without affecting their ability to re-enter myogenesis.

p21 Down-regulation and Reversine Treatment Together Facilitates Adipogenic Conversion. The small molecule reversine has been shown to induce greater plasticity in myoblasts, allowing their subsequent differentiation into adipocytes by culture in a cocktail of adipogenic factors.[11] However, cellularized myotubes treated with reversine and adipogenic factors did not differentiate into adipocytes, as shown by their relatively low lipid accumulation compared to adipocytes derived from myoblasts (Figure 4a). Down-regulation of p21 in cellularized myotubes prior to reversine treatment promoted subsequent conversion into the adipogenic lineage, as shown by increased lipid accumulation (Figure 4a). Down-regulation of p21 and treatment with adipogenic factors (without reversine treatment) did not induce lipid accumulation. In addition, down-regulation of p27 or control siRNA could not replace p21 in the chemical treatment to induce lipid accumulation. This result could also be observed by staining with Oil Red O (Figure 4b). Insulin-stimulated uptake of glucose is a characteristic of adipogenic cells and can be measured by the fluorescent glucose analogue 6-NBDG.[22−24] Muscle cellulate treated with p21 siRNA, reversine, and adipogenic factors showed increased insulin-stimulated glucose uptake (Figure 4c), which could also be visualized by microscopy (Figure 4d). However, the degree of glucose uptake was less than that observed in the 3T3-L1 adipocyte cell line (Figure 4c). Replacing p21 siRNA with p27 siRNA or control siRNA failed to confer insulin-stimulated glucose uptake. Removal of reversine or p21 siRNA from the chemical treatment also failed to confer insulin-stimulated glucose uptake. A further test of adipocyte function is the insulin-mediated prevention of free fatty acid release after epinephrine stimulation.[25,26] Treatment of the muscle cellulate with both p21 siRNA and reversine, followed by adipogenic factors, was necessary to confer this adipocyte function to the muscle cellulate, identical to the result observed for the acquisition of insulin-stimulated glucose uptake (Figure 4e). However, the degree of fatty acid accumulation and release was less than that observed in the 3T3-L1 adipocyte cell line.

p21 Down-regulation and Reversine Treatment Together Facilitates Osteogenic Conversion. Myoblasts treated with reversine can be differentiated into osteoblasts by culture in a cocktail of osteogenic factors.[11] The acquisition of alkaline phosphatase activity has been used as an indicator of osteogenic conversion in C2C12 myoblasts transfected with regulators of osteogenesis, such as Wnt-1-induced secreted protein 1 (followed by treatment with bone morphogenetic protein 2).[27] Muscle cellulate treated with p21 siRNA, reversine, and osteogenic factors promoted differentiation into cells with markedly increased alkaline phosphatase activity (Figures 5a). Alkaline phosphatase activity was similar to that of osteogenic cells derived from C2C12 myoblasts. Replacing p21 siRNA with p27 siRNA or control siRNA failed to induce the same degree of alkaline phosphatase activity. Down-regulation of p21 and treatment with osteogenic factors (without reversine treatment) also induced markedly less alkaline phosphatase activity. ATDC5 cells induced to undergo endochondral bone formation was used as a positive control.

Mineralization is a feature of ossification that occurs during bone formation.[28] Calcification was assessed by Alizarin Red S staining in the C2C12 cellulate after p21 knock-down, reversine treatment, and culture in osteogenic media. Light miscroscopic analysis showed that mineralization occurred in the cellulate after p21 knock-down, reversine treatment and culture in osteogenic media (Figure 5b). The degree of mineralization was similar to that of osteogenic cells derived from C2C12 myoblasts.

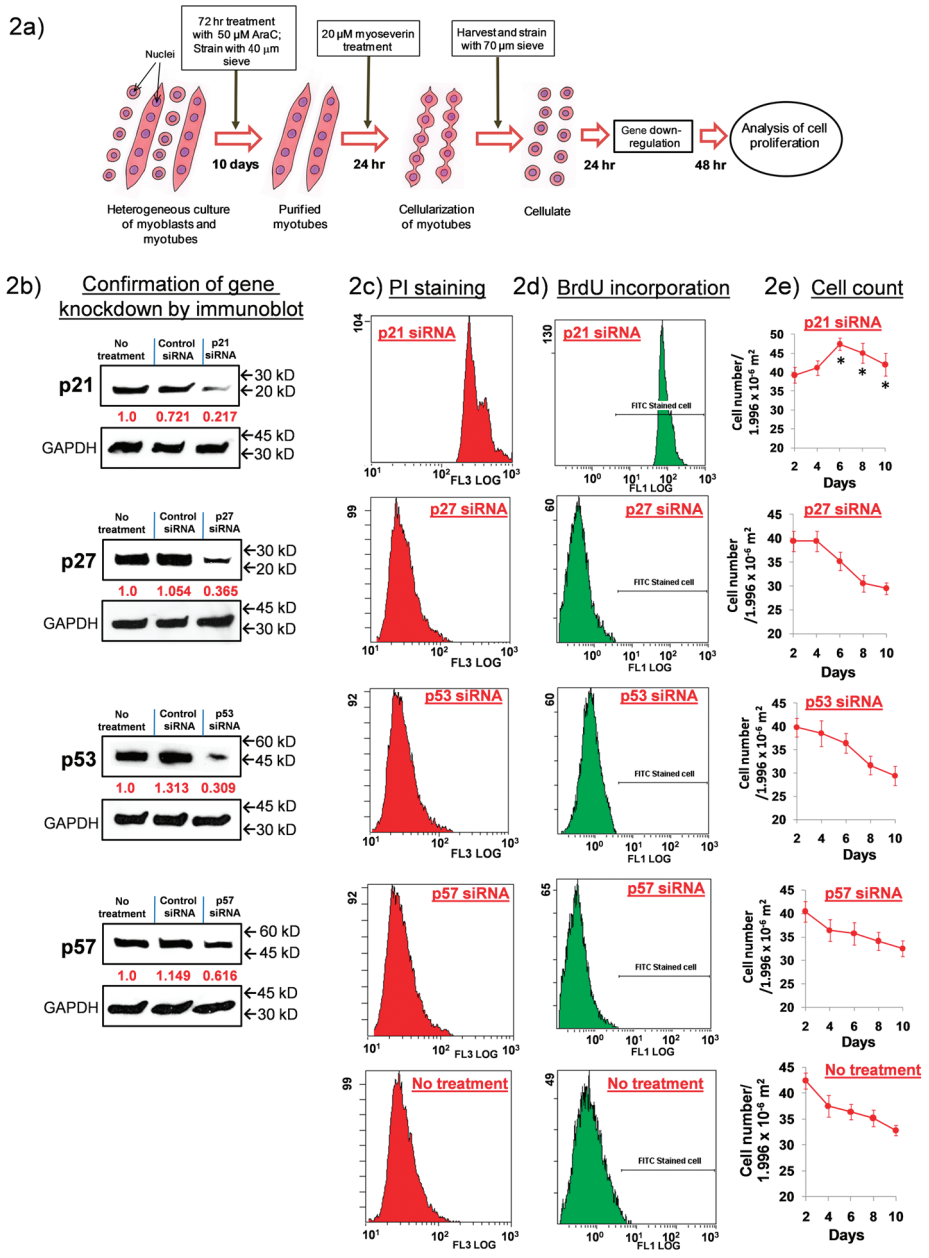


Figure 2. Induction of a proliferative response in the muscle cellulate. (a) Schematic diagram of the experimental protocol to induce proliferation in cellularized myotubes. (b) Immunoblot analysis to confirm gene knockdown in the C2C12 myotube cellulate. Numbers in red below each immunoblot show densitometric analysis of band intensity after normalization for the corresponding GAPDH housekeeping gene. (c) Flow cytometry analysis of propidium iodide (PI) stained cellularized C2C12 myotubes indicated that p21 down-regulation for 48 h induced re-entry into the cell cycle, as indicated by increased PI staining. (d) Down-regulation of p27, p53, or p57 did not affect PI staining. Flow cytometry analysis of BrdU incorporation in cellularized C2C12 myotubes showed that p21 down-regulation for 48 h induced DNA synthesis. Down-regulation of p27, p53, or p57 did not affect BrdU incorporation. Flow cytometry histograms are representative of three independent experiments. (e) Cell counting showed that p21 down-regulation for 48 h induced short-term cell division in cellularized C2C12 myotubes. The area of 1.996×10^{-6} m^2 (y-axis of graph) corresponds to a microscopic image obtained under 40X magnification. Error = SD; *$P < 0.05$ compared to 4 d post-siRNA treatment.

Mineralization was quantified using a microplate reader-based assay (Figure 5c).[28] C2C12 cellulate after p21 knock-down, reversine treatment, and culture in osteogenic media showed an increase in mineralization, similar to that of C2C12 myoblasts cultured in identical conditions. The level of mineralization was lower than that observed in ATDC5 cells after osteogenic culture. Replacing p21 siRNA with p27 siRNA or control siRNA failed to induce the same degree of mineralization in the muscle cellulate. Down-regulation of p21 and treatment with osteogenic factors (without reversine treatment) also induced markedly less mineralization.

The results presented in this paper are the first description of a chemical-based protocol that grossly follows the early step of limb regeneration in amphibians. Previously, a number of experimental approaches have been developed to induce adult cells to form multipotent cells or stem cells, including transfection with transcription factors (reviewed in ref 29). However, these approaches have not utilized terminally

dx.doi.org/10.1021/cb2000154 |ACS Chem. Biol. 2011, 6, 553–562

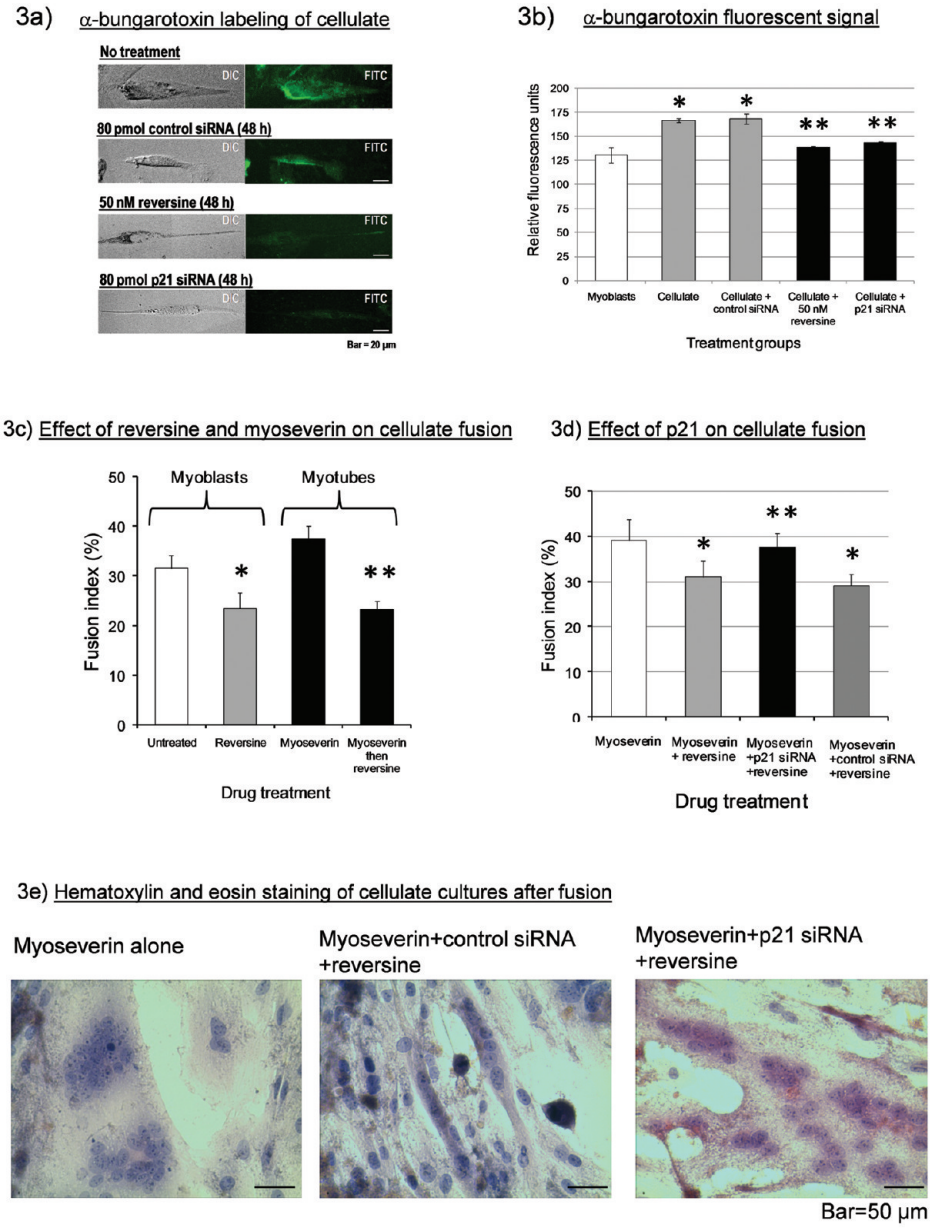


Figure 3. Effects of reversine and p21 down-regulation on the myogenic potential of the muscle cellulate. (a) p21 down-regulation or treatment with the small molecule, reversine, induced dedifferentiation in cellularized C2C12 myotubes, as shown by reduced labeling with FITC-α-bungarotoxin, a ligand for the acetylcholine receptor. (b) Microplate reader analysis to quantify FITC-α-bungarotoxin toxin labeling in cellularized C2C12 myotubes. p21 down-regulation or treatment with the small molecule, reversine, induced dedifferentiation in cellularized C2C12 myotubes. Error = SD; $^{*}P < 0.05$ compared to myoblasts; $^{**}P < 0.05$ compared to the C2C12 cellulate treated with control siRNA. Data is representative of three independent experiments. (c) Reversine treatment inhibited the myogenic potential of C2C12 myoblasts and C2C12 cellulate, as shown by a reduction in the fusion index after culture in differentiation media for 10 d. Error = SD; $^{*}P < 0.05$ compared to untreated myoblasts; $^{**}P < 0.05$ compared to the C2C12 cellulate treated with myoseverin alone. (d) p21 knock-down in the C2C12 cellulate reversed the inhibitory effect of reversine. Error = SD; $^{*}P < 0.05$ compared to the C2C12 cellulate treated with myoseverin alone; $^{**}P < 0.05$ compared to the C2C12 cellulate treated with myoseverin and reversine. Data is representative of three independent experiments. (e) Light microscopic analysis of myogenesis in C2C12 cultures derived from cellularized myotubes treated with 50 nM reversine and 80 pmol control siRNA, or 50 nM reversine and 80 pmol p21 siRNA. Hematoxylin and eosin staining shows that more nuclei reside within myotube syncytia from the C2C12 cellulate treated with reversine and p21 siRNA, as compared to treatment with reversine and control siRNA.

differentiated adult cells, which form the majority cellular population in humans.[30] Also, these approaches are hampered by technical issues, such as low efficiency. The chemical approach to achieve multipotency described in this paper can drive dedifferentiation and cell lineage conversion in mammalian skeletal muscle tissue, which is considered to be highly refractory.[16]

We considered the key first step in the induction of multipotency to be the cellularization of the skeletal muscle syncytia, because a similar phenomenon occurs in urodele skeletal muscle

dx.doi.org/10.1021/cb2000154 |ACS Chem. Biol. 2011, 6, 553–562

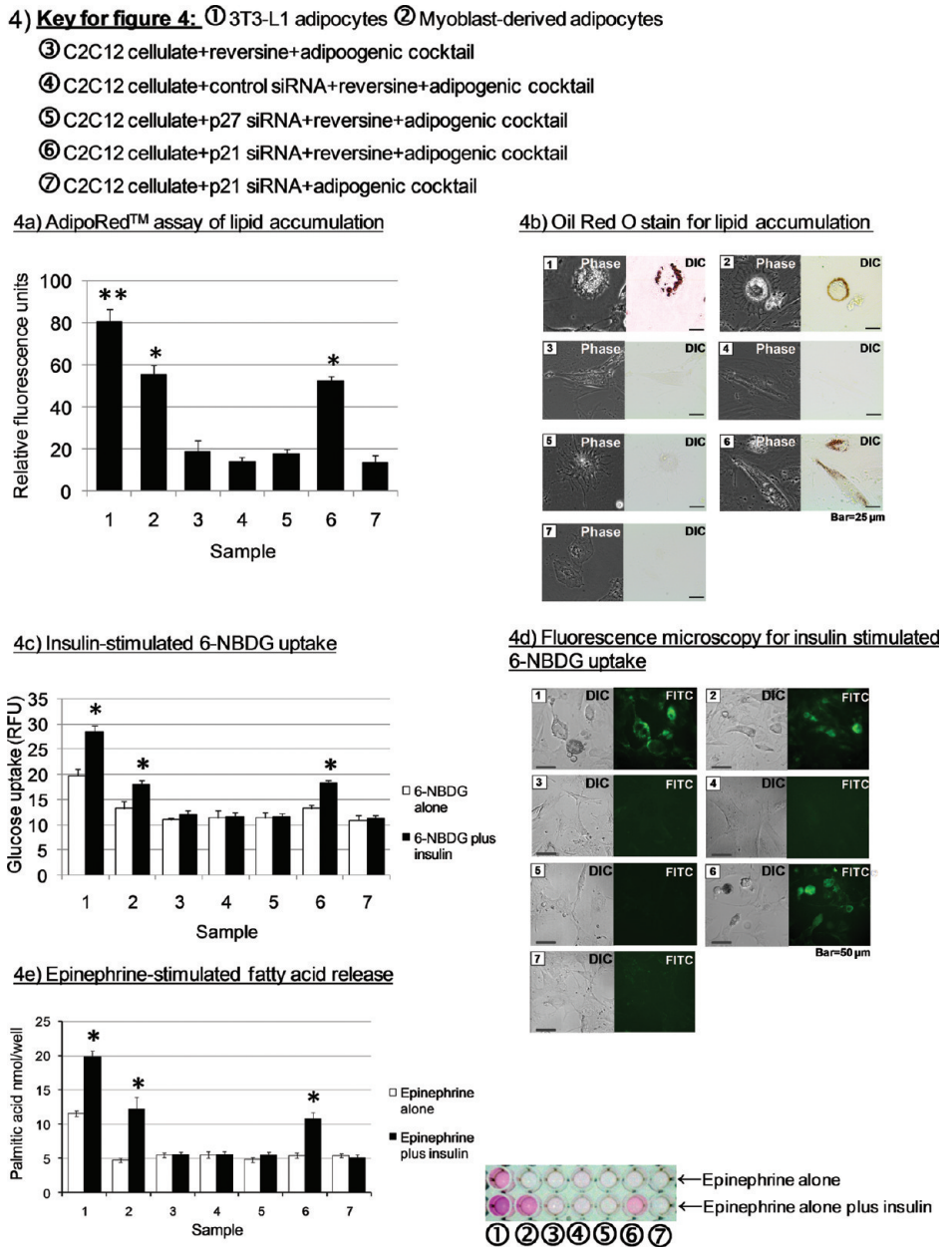


Figure 4. p21 down-regulation and reversine treatment together facilitates adipogenic conversion. (a) C2C12 cellulate treated with p21 knock-down, reversine and cultured in adipogenic media can accumulate lipid, as shown by AipoRed labeling. C2C12 myoblasts treated with reversine and cultured in adipogenic media also accumulated lipid, as previously described.[11] However, replacing p21 siRNA with p27 siRNA or control siRNA failed to produce lipid accumulation. In addition, p21 siRNA treatment plus adipogenic cocktail (without reversine) failed to increase lipid accumulation. Error = SD; $^{*}P < 0.05$ compared to C2C12 cellulate + reversine + adipogenic cocktail; $^{**}P < 0.05$ compared to myoblast-derived adipocytes. (b) Lipid accumulation in C2C12 cellulate treated with p21 knock-down, reversine, and adipogenic cocktail could also be visluaized by Oil Red O staining. (c) C2C12 cellulate treated with p21 knock-down, reversine, and adipogenic cocktail showed increased uptake of the fluorescent glucose analogue 6-NBDG after insulin stimulation (an adipocyte-specific function). The amount of 6-NBDG uptake was less than that observed in 3T3-L1 adipocytes after insulin stimulation. Replacement of p21 siRNA with control siRNA or p27 siRNA failed to produce cellulate capable of insulin-stimulated glucose uptake. In addition, p21 siRNA treatment plus adipogenic cocktail (without reversine) failed to produce cells capable of insulin-stimulated glucose uptake. Error = SD; $^{*}P < 0.05$ compared to 6-NBDG alone. Data is representative of three independent experiments. (d) Uptake of the fluorescent glucose analogue 6-NBDG after insulin stimulation could be visualized by fluorescent microscopy. 6-NBDG was found to accumulate in cells possessing characteristic adipocyte morphology caused by lipid accumulation in the cytoplasm. (e) C2C12 cellulate treated with p21 knock-down, reversine, and adipogenic cocktail showed insulin-sensitive free fatty acid release after epinephrine-stimulation (an adipocyte-specific function). Replacement of p21 siRNA with control siRNA or p27 siRNA failed to produce cells that possessed this adipocyte-specific function. Also, treatment with p21 siRNA and adipogenic cocktail (without reversine) failed to produce cells that possessed this adipocyte-specific function. C2C12 myoblasts treated with 500 nM reversine and adipogenic cocktail also showed insulin-sensitive free fatty acid release after epinephrine-stimulation. The amount of free fatty acid release and insulin sensitivity was less than that observed in 3T3-L1 adipocytes. Error = SD; $^{*}P < 0.05$ compared to epinephrine alone. Data is representative of three independent experiments. A photograph of the assay plate is included in the figure. Experimental groups are shown next to their corresponding wells on the microplate.

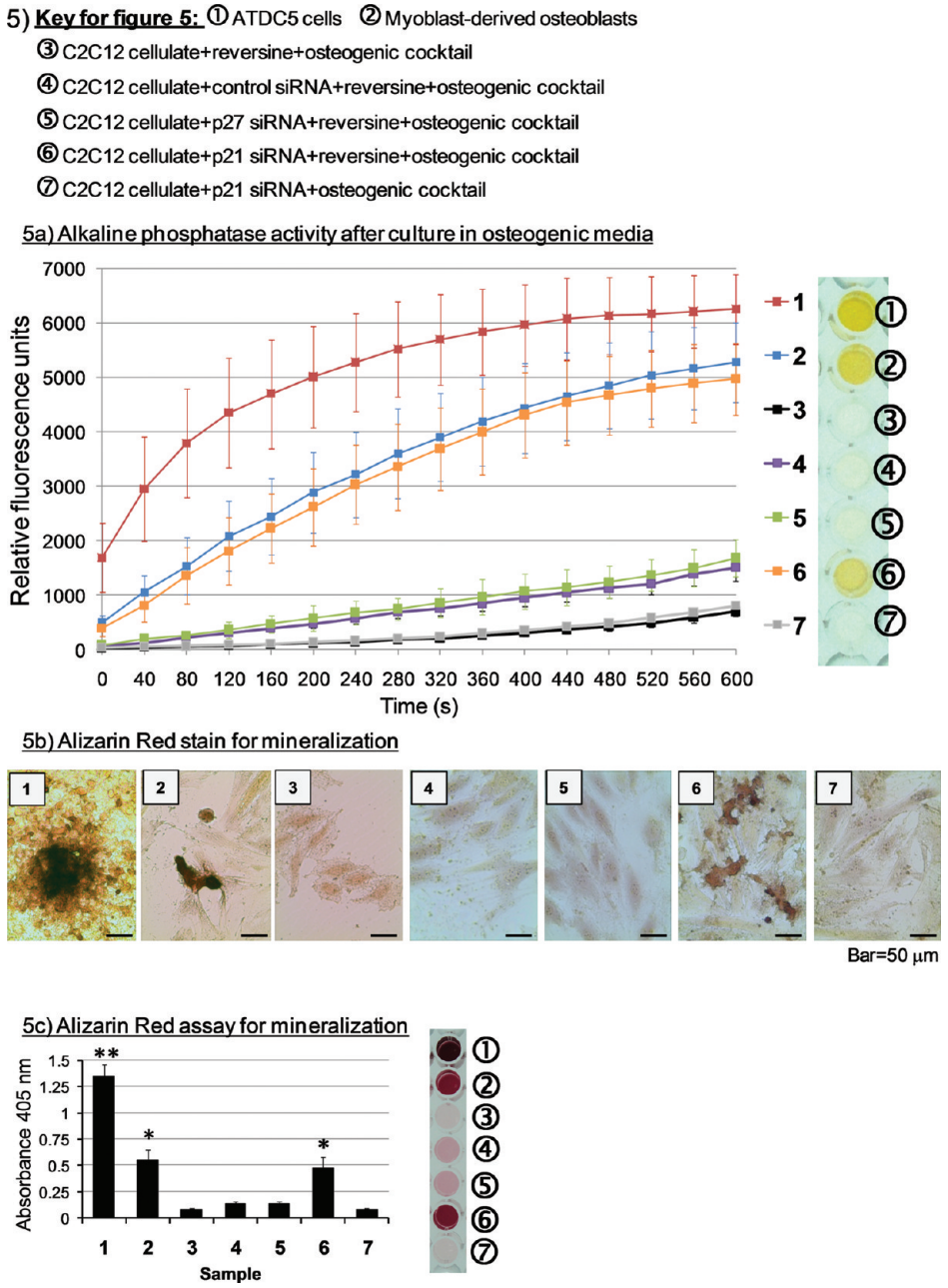


Figure 5. p21 down-regulation and reversine treatment together facilitates osteogenic conversion. (a) Alkaline phosphatase activity increased in C2C12 cellulate after treatment with reversine, p21 knock-down, and culture in osteogenic conditions. Replacement of p21 siRNA with control siRNA or p27 siRNA resulted in a marked reduction in alkaline phosphatase activity. Also, treatment with p21 siRNA and adipogenic cocktail (without reversine) reduced alkaline phosphatase activity. ATDC5 osteo-chondrogenic precursor cells cultured in osteogenic conditions were used as a positive control. Also included in the figure is a photograph of the AttoPhos substrate solution 300 s after addition of cell lysate. Experimental groups are shown next to their corresponding wells on the microplate. (b) Alizarin Red S staining to visualize mineralization in ATDC5 cells and C2C12 cellulate after 10 d of culture in osteogenic promoting media. Treatment of the C2C12 cellulate with p21 siRNA, 500 nM reversine, and osteogenic cocktail induced regions of mineralization, similar to C2C12 myoblasts treated with 500 nM reversine and osteogenic cocktail. Removal of reversine from the treatment condition or replacement of p21 siRNA with control siRNA or p27 siRNA reduced mineralization. (c) Semiquantification of mineralization by colorimetric assay. Treatment of C2C12 cellulate with 500 nM reversine and p21 siRNA induced mineralization after osteogenic cocktail. The degree of mineralization was similar to C2C12 myoblasts treated 500 nM reversine and osteogenic cocktail. However, mineralization in these cells was lower than that observed in ATDC5 osteo-chondrogenic precursors cultured in osteogenic cocktail. Error = SD; $^{*}P < 0.05$ compared to C2C12 cellulate + control siRNA + reversine + osteogenic cocktail. $^{**}P < 0.05$ compared to myoblast-derived osteoblasts. Data is the overall average of three independent experiments.

dedifferentiation following limb amputation.[4] Our comparison of different small molecules that induce skeletal muscle cellularization is useful because there are known differences in the effects of these chemicals in different tissues.[16,31,32] Using an optimized protocol for the differentiation of a myoblast cell line, it was found that the small molecule myoseverin was more efficient for deriving viable mononuclear cells from muscle syncytia (Figures 1a and b). The relatively low cytotoxicity of myoseverin

dx.doi.org/10.1021/cb2000154 |ACS Chem. Biol. 2011, 6, 553–562

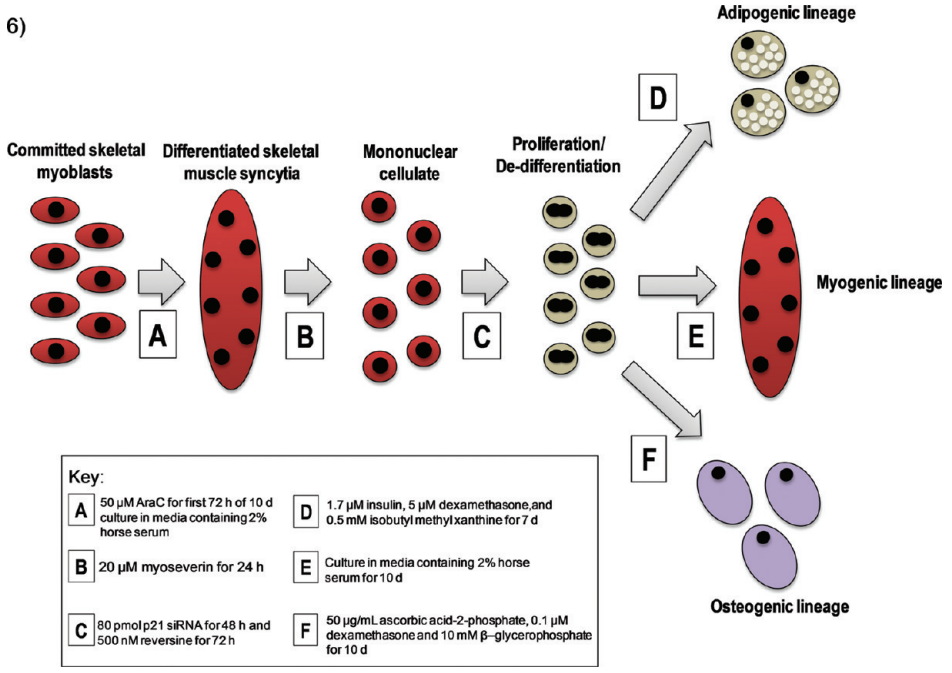


Figure 6. Schematic diagram of the chemical approach to obtain multipotent cells from terminally differentiated skeletal muscle tissue.

was noted in the original description of this compound,[10] although our study is the first analysis of myoseverin action in myotubes derived from myoblasts treated with the antimitotic agents AraC or 5-FU.

Cells derived from muscle syncytia treated with myoseverin were found to remain refractory (Figure 2b and c). This finding is in line with the report by Duckmanton *et al.*[14] and contrasts with the original reports of myoseverin action,[10,12] possibly emphasizing the need to obtain a high purity of myotubes before analyzing cellularization. Knock-down of the cyclin-dependent kinase inhibitor (CDKN) p21 induced a proliferative response in the cellularized myotubes (Figures 2b−d). Knock-down of the cyclin-dependent kinase inhibitors p27 or p57 or the tumor suppressor p53 (TP53/LFS1) failed to induce proliferation, indicating that p21 is a key regulator of cell cycle withdrawal in the muscle cellulate. In addition, the combination of myoseverin and p21 knock-down induced mammalian muscle to behave in a manner that grossly follows an important early step of appendage regeneration in urodele amphibians: muscle fiber cellularization and cell cycle re-entry.[15,33]

Recent studies of appendage regeneration in urodele amphibians suggest that the muscle cellulate forms a population of precursor cells that later redifferentiate into new muscle tissue, *i.e.*, there is no contribution to other tissue types in the regenerating limb (reviewed in 33). Our study has shown that the muscle cellulate obtained by treatment with small molecules can also redifferentiate into muscle myotubes. The inhibitory effect of reversine treatment on redifferentiation that we observed may be linked to the down-regulation of myogenic regulatory factor expression.[34] Thus, we believe that the action of p21 knock-down on myogenic regulatory factor expression is an interesting area of future investigation, because p21 knock-down reversed the inhibitory effect of reversine on redifferentiation (Figures 3d and e). This occurred despite the finding that reversine treatment or p21 knock-down reduced acetylcholine receptor expression in the muscle cellulate (Figure 3a and b). However, we found that both p21 knock-down and reversine treatment of the muscle cellulate are required to induce the formation of precursor cells that could adopt divergent fates (Figures 4 and 5). This may be due to the dependency of reversine action on cell cycle progression.[35] Knock-down of p21 in the muscle cellulate would, therefore, induce cell cycle progression and provide a cellular environment favorable for the action of reversine. Cell lineage switching also occurs in the urodele appendage regenerate, where dermis-derived fibroblasts have been shown to form precursors that contribute to cartilage, connective tissue, and tendons.[33] Thus, the chemical approach described in our study allows adult mammalian skeletal muscle to adopt a higher degree of multipotency that is observed in natural examples of appendage regeneration. A schematic diagram of our chemical approach is shown in Figure 6.

The ability of reversine-treated myoblasts to redifferentiate into adipogenic and osteogenic cells has been described previously.[11] However, our data suggests that this finding should be interpreted with a degree of caution. Previous studies of reversine assessed the expression of lineage-specific markers or phenotypic markers, such as lipid accumulation during adipogenesis. Our study attempted to monitor fat and bone cell formation using more rigorous, functional assays and comparison with known cell types. For example, we have shown that reversine treated muscle cells accumulate lipids. However, their functional "performance" compared to lineage-specific adipocytes is significantly lower (Figure 4c and e). It may be possible to further "push" adipogenesis in reversine-treated muscle cells by modifying the cocktail of adipogenic factors, such as treatment with thiazolidinediones that activate the key adipogenic factor peroxisome proliferator-activated receptor-γ.[36]

The new chemically defined method of producing multipotent cells from differentiated muscle, presented in this paper, offers a number of possible areas for further study. For example, it has recently been shown that myoblasts can form neurogenic cells after reversine treatment and culture in neuron differentiation

media.[37] A similar "jump" from the mesodermal lineage (including fat and bone cells) to the neuroectodermal lineage may be possible in cellularized muscle fibers. Alternatively, muscle biopsies could be subjected to this chemical-based protocol in an attempt to derive patient-specific multipotent cells. For example, vastus lateralis skeletal muscle strips can be obtained from patients under local anesthetic.[38] Skeletal muscle is a favorable tissue type for this approach, because it can regenerate lost tissue by activating resident muscle precursor cells.[39] Moreover, this chemical approach has similarities with the early events of urodele amphibian appendage regeneration, and it may be enlightening to attempt to transfer this method to mammalian models of wound healing, with the aim of deriving functional progenitor cells at the wound site before the onset of inflammation and wound closure. Therefore, the results of our study have implications for the development of methods to induce limb regeneration in mammals and suggest new approaches for the future development of induced pluripotent stem cells from refractory, terminally differentiated mammalian tissues.

METHODS

A description of the following methods can be found in the Supporting Information for this manuscript: a description of reagents, cell culture, flow cytometry, measurement of cell viability, Western blotting, cell counting, α-bungarotoxin labeling of acteylchloine receptors, quantification of myoblast differentiation, RT-PCR, quantification and visualization of lipid accumulation during adipogenesis, analysis of insulin-stimulated glucose uptake, free fatty acid release assay, alkaline phosphatase assay, Alizarin Red staining and quantification of mineralization using Alizarin Red.

Cellularization of Skeletal Muscle Myotubes. Myotube cultures were treated with either 20 μM myoseverin, 20 μM myoseverin B, 1 μM colchicine, or 0.5 μM nocodazole for 24 h (within the concentration range reported to induce myotube cellularization[12,14]). Cellularization was found to produce relatively large amounts of cellular debris, in addition to mononuclear cells. The cell monolayer was trypsinized and gently centrifuged at 750 rpm for 5 min. The cell pellet was gently resuspended in 10 mL of proliferation media and passed through a 70 μm mesh (BD Biosciences) to remove cellular debris and seeded in 6-well culture dishes at a density of 1.5×10^6 cells/well. Cellulate was used for experiments 24 h after seeding.

siRNA-Mediated Gene Silencing. Cellulate (C2C12 myotubes 24 h after treatment with 20 μM myoseverin) was seeded in 6-well plates at a cell density of 1.5×10^6 cells/well, after sieving through a 70 μm mesh (BD Biosciences). Twenty-four hours after seeding, cells were transfected with 80 pmol of siRNA, following the manufacturer's protocol (Santa Cruz Biotechnology). For flow cytometry analysis, triplicate wells were used for each siRNA to ensure that sufficient numbers of cells would be available. Cellulate was used for experiments 48 h post-transfection.

Dedifferentiation of Mononuclear Cells Derived from Cellularized Skeletal Muscle. The first report of reversine employed a dose of 5 μM for 4 days.[11] However, we found that this concentration produced widespread cytotoxicity in the mononuclear cells derived from myotubes (>30% of the cell monolayer was disrupted). Therefore, to reduce cytotoxicity, the mononuclear cells were treated with a lower concentration of reversine for a shorter time course (500 nM for 3 days). After 3 days of incubation, reversine was removed and the mononuclear cells were induced to undergo adipogenesis and osteogenesis (as described below).

Induction of Osteogenesis in Cellularized Skeletal Muscle Myotubes. The induction of osteogenesis in cellularized skeletal muscle myotubes was carried out as described previously for reversine-treated myoblasts.[11,40] Cells were cultured in DMEM + 10% FBS supplemented with 50 μg/mL ascorbic acid-2-phosphate, 0.1 μM dexamethasone, and 10 mM β-glycerophosphate for 10 days. The culture conditions were then switched to mimic endochondral bone formation in ATDC5 cells: culture in α-minimum essential medium containing 5% FBS, 10 μg/mL human transferrin, 3×10^{-8} M sodium selenite and 10 μg/mL bovine insulin, in 97% air/3% CO_2, for an additional 10 d. Culture media was changed every 48 h.

Induction of Adipogenesis in Cellularized Skeletal Muscle Myotubes. The induction of adipogenesis in cellularized skeletal muscle myotubes was carried out as described previously for reversine-treated myoblasts.[11] Cells were cultured in DMEM + 10% calf serum supplemented with 1.7 μM insulin, 5 μM dexamethasone, and 0.5 mM isobutyl methyl xanthine for 7 days.

Statistical Analysis of Data. The Mann−Whitney *U* test was used to determine significance. A *P* value (two-tailed) of less than 0.05 was considered to be significant.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +82-62-715-2509. Fax: +82-62-715-2484. E-mail: darren@gist.ac.kr.

ACKNOWLEDGMENT

This research was supported by two grants from the National Research Foundation funded by the Korean government (MEST basic science research program (NRF-2010-0006002) to D. Williams and MEST basic science research program (NRF-2009-0067894) to D. Jung). We also wish to thank the following members of the Gwangju Institute of Science and Technology: J. Kim, for flow cytometry, W. Kim, for technical assistance and J. Chun, for provision of ATDC5 cells. We also acknowledge the Bioimaging Research Center and Cell Dynamics Research Center, Gwangju Institute of Science and Technology, for provision of microscopy facilities.

REFERENCES

(1) Grafi, G. (2009) The complexity of cellular dedifferentiation: implications for regenerative medicine. *Trends Biotechnol. 27*, 329–332.

(2) Tsonis, P. A. (2000) Regeneration in vertebrates. *Dev. Biol. 221*, 273–284.

(3) Luc, B. (1998) Transdifferentiation versus stem cell hypothesis for the regeneration of islet beta-cells in the pancreas. *Microsc. Res. Tech. 43*, 332–336.

(4) Brockes, J. P., and Kumar, A. (2008) Comparative aspects of animal regeneration. *Annu. Rev. Cell Dev. Bio.l 24*, 525–549.

(5) Takebe, I. (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. *Naturwissenschaften 58*, 318–320.

(6) Martin, A. B., and Schultz, P. G. (1999) Opportunities at the interface of chemistry and biology. *Trends Cell Biol. 9*, M24–M28.

(7) Min, J., Kyung Kim, Y., Cipriani, P. G., Kang, M., Khersonsky, S. M., Walsh, D. P., Lee, J. Y., Niessen, S., Yates, J. R., 3rd, Gunsalus, K., Piano, F., and Chang, Y. T. (2007) Forward chemical genetic approach identifies new role for GAPDH in insulin signaling. *Nat. Chem. Biol. 3*, 55–59.

(8) Williams, D., Jung, D.-W., Khersonsky, S. M., Heidary, N., Chang, Y.-T., and Orlow, S. J. (2004) Identification of Compounds that Bind Mitochondrial F1F0 ATPase by Screening a Triazine Library for Correction of Albinism. *Chem. Biol. 11*, 1251–1259.

(9) Xu, Y., Shi, Y., and Ding, S. (2008) A chemical approach to stem-cell biology and regenerative medicine. *Nature 453*, 338–344.

(10) Rosania, G. R., Chang, Y. T., Perez, O., Sutherlin, D., Dong, H., Lockhart, D. J., and Schultz, P. G. (2000) Myoseverin, a microtubule-binding molecule with novel cellular effects. *Nat. Biotechnol. 18*, 304–308.

(11) Chen, S., Zhang, Q., Wu, X., Schultz, P. G., and Ding, S. (2003) Dedifferentiation of lineage-committed cells by a small molecule. *J. Am. Chem. Soc. 126*, 410–411.

(12) Perez, O. D., Chang, Y.-T., Rosania, G., Sutherlin, D., and Schultz, P. G. (2002) Inhibition and reversal of myogenic differentiation by purine-based microtubule assembly inhibitors. *Chem. Biol. 9*, 475–483.

(13) Brockes, J. P., and Kumar, A. (2002) Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nat. Rev. Mol. Cell. Biol. 3*, 566–574.

(14) Duckmanton, A., Kumar, A., Chang, Y.-T., and Brockes, J. P. (2005) A single-cell analysis of myogenic dedifferentiation induced by small molecules. *Chem. Biol. 12*, 1117–1126.

(15) Tanaka, E. M., Gann, A. A., Gates, P. B., and Brockes, J. P. (1997) Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. *J. Cell Biol. 136*, 155–165.

(16) Tiainen, M., Pajalunga, D., Ferrantelli, F., Soddu, S., Salvatori, G., Sacchi, A., and Crescenzi, M. (1996) Terminally differentiated skeletal myotubes are not confined to G0 but can enter G1 upon growth factor stimulation. *Cell Growth Differ. 7*, 1039–1050.

(17) Myers, T. K., Andreuzza, S. E., and Franklin, D. S. (2004) p18INK4c and p27KIP1 are required for cell cycle arrest of differentiated myotubes. *Exp. Cell Res. 300*, 365–378.

(18) Pajalunga, D., Mazzola, A., Salzano, A. M., Biferi, M. G., De Luca, G., and Crescenzi, M. (2007) Critical requirement for cell cycle inhibitors in sustaining nonproliferative states. *J. Cell Biol. 176*, 807–818.

(19) Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science 267*, 1018–1021.

(20) Thelen, M. H., Simonides, W. S., and van Hardeveld, C. (1997) Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca2+-ATPase gene. *Biochem. J. 321* (Pt 3), 845–848.

(21) Im, Y. S., Shin, H. K., Kim, H. R., Jeong, S. H., Kim, S. R., Kim, Y. M., Lee do, H., Jeon, S. H., Lee, H. W., and Choi, J. K. (2009) Enhanced cytotoxicity of 5-FU by bFGF through up-regulation of uridine phosphorylase 1. *Mol. Cells 28*, 119–124.

(22) Garcia de Herreros, A., and Birnbaum, M. J. (1989) The acquisition of increased insulin-responsive hexose transport in 3T3-L1 adipocytes correlates with expression of a novel transporter gene. *J. Biol. Chem. 264*, 19994–19999.

(23) Dimitriadis, G., Maratou, E., Boutati, E., Psarra, K., Papasteriades, C., and Raptis, S. A. (2005) Evaluation of glucose transport and its regulation by insulin in human monocytes using flow cytometry. *Cytometry, Part A 64A*, 27–33.

(24) Jung, D. W., Ha, H. H., Zheng, X., Chang, Y. T., and Williams, D. R. (2011) Novel use of fluorescent glucose analogues to identify a new class of triazine-based insulin mimetics possessing useful secondary effects. *Mol. Biosyst. 7*, 346–358.

(25) Nishide, M., Yoshikawa, Y., Yoshikawa, E. U., Matsumoto, K., Sakurai, H., and Kajiwara, N. M. (2008) Insulinomimetic Zn(II) complexes as evaluated by both glucose-uptake activity and inhibition of free fatty acids release in isolated rat adipocytes. *Chem. Pharm. Bul. (Tokyo) 56*, 1181–1183.

(26) Kawabe, K., Yoshikawa, Y., Adachi, Y., and Sakurai, H. (2006) Possible mode of action for insulinomimetic activity of vanadyl(IV) compounds in adipocytes. *Life Sci. 78*, 2860–2866.

(27) French, D. M., Kaul, R. J., D'Souza, A. L., Crowley, C. W., Bao, M., Frantz, G. D., Filvaroff, E. H., and Desnoyers, L. (2004) WISP-1 is an osteoblastic regulator expressed during skeletal development and fracture repair. *Am. J. Pathol. 165*, 855–867.

(28) Gregory, C. A., Gunn, W. G., Peister, A., and Prockop, D. J. (2004) An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal. Biochem. 329*, 77–84.

(29) Hochedlinger, K., and Plath, K. (2009) Epigenetic reprogramming and induced pluripotency. *Development 136*, 509–523.

(30) Marieb, E. K. H. (2007) *Human Anatomy & Physiology*, 7th ed., Pearson Benjamin Cummings, San Francisco.

(31) Huby, R. D. J., Weiss, A., and Ley, S. C. (1998) Nocodazole inhibits signal transduction by the T cell antigen receptor. *J. Biol. Chem. 273*, 12024–12031.

(32) Ai, H., Ralston, E., Lauritzen, H. P., Galbo, H., and Ploug, T. (2003) Disruption of microtubules in rat skeletal muscle does not inhibit insulin- or contraction-stimulated glucose transport. *Am. J. Physiol. Endocrinol. Metab. 285*, E836–844.

(33) Tweedell, K. S. (2010) The urodele limb regeneration blastema: the cell potential. *ScientificWorldJournal 10*, 954–971.

(34) Amabile, G., D'Alise, A. M., Iovino, M., Jones, P., Santaguida, S., Musacchio, A., Taylor, S., and Cortese, R. (2009) The Aurora B kinase activity is required for the maintenance of the differentiated state of murine myoblasts. *Cell Death Differ. 16*, 321–330.

(35) Chen, S., Takanashi, S., Zhang, Q., Xiong, W., Zhu, S., Peters, E. C., Ding, S., and Schultz, P. G. (2007) Reversine increases the plasticity of lineage-committed mammalian cells. *Proc. Natl. Acad. Sci. U.S.A. 104*, 10482–10487.

(36) Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. (1996) Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology 137*, 354–366.

(37) Lee, E. K., Bae, G. U., You, J. S., Lee, J. C., Jeon, Y. J., Park, J. W., Park, J. H., Ahn, S. H., Kim, Y. K., Choi, W. S., Kang, J. S., Han, G., and Han, J. W. (2009) Reversine increases the plasticity of lineage-committed cells toward neuroectodermal lineage. *J. Biol. Chem. 284*, 2891–2901.

(38) Zierath, J. R., Krook, A., and Wallberg-Henriksson, H. (2000) Insulin action and insulin resistance in human skeletal muscle. *Diabetologia 43*, 821–835.

(39) Morgan, J. E., and Partridge, T. A. (2003) Muscle satellite cells. *Int. J. Biochem. Cell Biol. 35*, 1151–1156.

(40) Sze Wan, S., Mei Kuen, T., Pak Ham, C., Miguel, M., Dong Qing, C., and Kenneth, K. H. L. (2007) Induction of growth arrest and polycomb gene expression by reversine allows C2C12 cells to be reprogrammed to various differentiated cell types. *Proteomics 7*, 4303–4316.