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Dedifferentiation of Lineage-Committed Cells by a Small Molecule

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Among organisms that have regenerative capabilities, the urodele amphibians are unique in their use of a cellular dedifferentiation mechanism[1] at the damaged site to form a blastema which contains dedifferentiated progenitor cells. These cells can proliferate and redifferentiate under developmental control to regenerate a wide variety of tissues, such as limbs, tails, and lens. In mammals, epimorphic regeneration is largely limited by an irreversible differentiation process. As a consequence, stem cells,[2] in particular embryonic stem cells (ESCs) which can be expanded indefinitely and are pluripotent, have attracted considerable attention as a therapeutic approach to the damage caused by cardiovascular disease, neurodegenerative disease, diabetes and aging. However, the use of stem cells in cell replacement therapy remains problematic for a number of reasons, including the lack of robust methods for the propagation and efficient differentiation of stem cells, as well as host rejection of allogeneic cells. The ability to dedifferentiate or reverse lineage-committed cells to multipotent progenitor cells might overcome many of these obstacles. With an efficient dedifferentiation process, it is conceivable that healthy, abundant, and easily accessible adult cells could be used to generate different types of functional cells for repair of damaged tissues. Moreover, recent studies of the plasticity of murine myotubes[3,4] and other cells derived from adult tissues suggest that dedifferentiation may be possible in mammalian system.[5] However, in contrast to the differentiation process, methods for the control and study of dedifferentiation are lacking.

Cell-based phenotypic assays and, more recently, pathway screens of synthetic small molecules and natural products have historically provided very useful chemical probes of complex cellular processes.[6] The identification of small molecules which induce dedifferentiation of somatic cells would help to elucidate the molecular mechanism of this phenomenon and may ultimately allow us to regenerate tissues in vivo. Toward this end, we have begun to search for small molecules which can induce cellular dedifferentiation of lineage-restricted mammalian cells. Although little is known about the signaling pathways which might initiate such a process, protein kinases are likely to be involved. Therefore, libraries of heterocyclic compounds[7] designed around a large number of kinase-directed scaffolds were screened, including substituted purines, pyrimidines, quinazolines, pyrazines, pyrrolopyrimidine, pyrazolopyrimidine, phthalazines, pyridazines, and quinoxalines. Herein, we report the discovery of a small molecule from those libraries that induces a dedifferentiated phenotype in murine C2C12 myoblasts.

The murine C2C12 cell is a myogenic lineage committed myoblast. Upon withdrawal of serum, confluent C2C12 cells can differentiate and fuse into characteristic multinucleated myotubes. We have previously shown that a small molecule, myoseverine, has the ability to cleave multinucleated myotubes to generate myoblast-like cells,[8] which can be proliferated and redifferentiated into myotubes. However, myoseverine was found to bind microtubules, and its effects most likely stem from cytoskeleton remodeling rather than dedifferentiation. To identify molecules that induce true dedifferentiation, an alternative assay was devised which is based on the notion that lineage-reversed myoblasts should regain multipotency. Specifically, they should acquire the ability to differentiate into multiple nonpermitted cell lineages when exposed to conditions that typically induce differentiation of multipotent mesenchymal progenitor cells into adipocyptes, osteoblasts, or chondrocytes. Osteoblast formation was chosen for the primary screen since there are established osteogenic-inducing conditions and a high throughput assay for detecting the bone specific marker, alkaline phosphatase (ALP).[9]

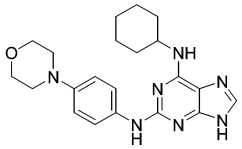
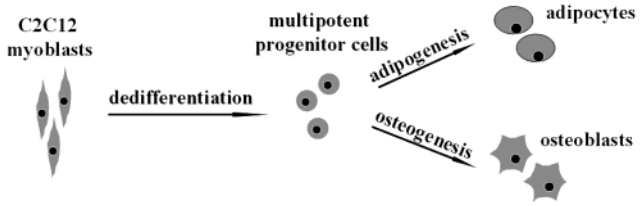


Figure 1. Reversine.

Scheme 1. Dedifferentiation Screening Scheme



A two-stage screening protocol (Scheme 1) was used in which C2C12 cells were initially treated with a library of 50 000 discrete small molecules for 4 days to induce dedifferentiation and then assayed for their ability to undergo osteogenesis upon addition of known osteogenic-inducing agents. To carry out the screen, C2C12 cells were plated in 384-well plates in growth medium (DMEM with 10% fetal bovine serum) and after overnight incubation (during which time cells attach to the bottom of the plate) 5 μM of compound was added. After 4 days, compound was removed, and the medium was changed to osteogenic-inducing medium[10] containing 50 μg/mL ascorbic acid-2-phosphate, 0.1 μM dexamethasone, and 10 mM β-glycerophosphate. The culture was maintained for an additional 7 days, and cells were lysed and then assayed for ALP activity using the fluorogenic substrate 2′-[2′-benzothiazoyl]-6′-hydroxybenzothiazole phosphate (BBTP). Because this screen could also find molecules which directly induce transdifferentiation of myogenic cells to osteogenic cells, compounds from the primary screen were tested to determine (1) whether they can induce osteogenesis in the absence of the osteogenesis-inducing cocktail and (2) whether cells treated with compounds can differentiate into adipocytes under conditions[11] that induce adipogenesis.

Among a series of 2,6-disubstituted purine analogues identified in the primary screen, a 2-(4-morpholinoanilino)-6-cyclohexylami-

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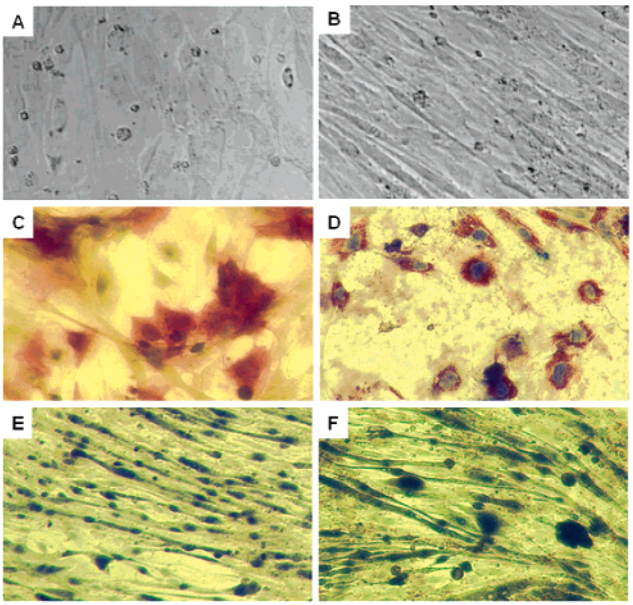

Figure 2. Morphological and histological characterization of cellular dedifferentiation induced by reversine. (A) Phase image of day 4 culture after reversine treatment; (B) phase image of day 4 culture after DMSO treatment; (C) ALP staining (red) of day 7 osteogenic differentiation culture after reversine treatment; (D) Oil red O staining (red) of day 7 adipogenic differentiation culture after reversine treatment; (E) ALP staining (red) of day 7 osteogenic differentiation culture after DMSO treatment; (F) oil red O staining (red) of day 7 adipogenic differentiation culture after DMSO treatment.

nopurine analogue (which we named reversine, Figure 1) was found to induce the highest level (7-fold) of ALP activity relative to the DMSO control treatment. On day 4 of compound treatment, striking differences were observed between the reversine-treated and untreated cells. In the control cells (treated only with DMSO), multinucleated myotubes were formed throughout the culture (Figure 2B). In contrast, in the presence of 5 μM reversine myotube formation was completely inhibited, and cells continued to grow to form a confluent culture of mononucleated cells (Figure 2A). In addition, myogenic-specific markers such as MyoD and myosin began to disappear. These results suggest that reversine is not simply acting as a selective toxin.[12]

After 4 days treatment, the compound was removed, and cells were then grown in osteogenic differentiation medium (ODM) or adipogenic differentiation medium (ADM). At the end of day 7, under ODM conditions, 35% of cells stained positive for ALP (Figure 2C); similarly when exposed to ADM condition, 40% of cells had the characteristic fat cell morphology, oil droplets inside the cytoplasmic membrane, and stained positive with oil red O (Figure 2D). Again, in the control culture, confluent C2C12 cells continue to form myotubes and were unaffected by the ODM and ADM conditions (Figure 2E,F). These results clearly demonstrate that reversine-treated lineage-committed C2C12 myoblasts cells regain multipotency. Consistent with this observation, in the absence of ODM reversine alone has no osteogenesis activity, further confirming that reversine induces dedifferentiation of C2C12 cells rather than trandifferentiation to osteogenic lineage.

A preliminary structure−activity relationship (SAR) analysis of the primary screen data revealed that both of the N9 hydrogen and the NH substitution at the C2 position of the purine ring are essential (removal of either completely abolishes activity). However, primary amines at the C6 position of the purine ring can be replaced with various heteroatoms, such as oxygen and sulfur without loss in activity, suggesting a H-bond donor at this position is not required. Only a limited group of aromatic substituents can be tolerated at the C2 position of the purine ring. We are continuing to explore the SAR in an effort to further enhance the potency and specificity of reversine, as well as to identify a linkage position on the molecule for attachment to a solid support for affinity experiments to elucidate its cellular target(s).

In summary, we have discovered a 2,6-disubstituted purine, reversine, which can induce myogenic lineage-committed cells to become multipotent mesenchymal progenitor cells which can proliferate and redifferentiate into bone and fat cells. This phenomenon recapitulates key aspects of epimorphic regeneration.

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Supporting Information Available: Detailed experimental procedures and spectra data of the compounds disclosed in this paper (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) At the effective concentration (1−10 μM), no significant cell death was observed during dedifferentiation stage. In addition, transdifferentiation of C2C12 myoblasts to osteoblasts or adipocytes was not observed under the conditions used to induce osteogenesis or adipogenesis. These observations suggest that reversine acts as a dedifferentiation-inducing agent rather than simply enriching certain type of progenitor cells by killing myoblasts.

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