

COMMENTARY

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Lessons from the swamp: developing small molecules that confer salamander muscle cellularization in mammals

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Abstract:

The ability of salamanders, such as newts, to regenerate damaged tissues has been studied for centuries. A prominent example of this regenerative power is the ability to re-grow entire amputated limbs. One important step in this regeneration process is skeletal muscle cellularization, in which the muscle fibers break down into dedifferentiated, mononuclear cells that proliferate and form new muscle in the replacement limb. In contrast, mammalian skeletal muscle does not undergo cellularization after injury. A significant proportion of research about tissue regeneration in salamanders aims to characterize regulatory genes that may have mammalian homologs. A less mainstream approach is to develop small molecule compounds that induce regeneration-related mechanisms in mammals. In this commentary, we discuss progress in discovering small molecules that induce cellularization in mammalian muscle. New research findings using these compounds has also shed light on cellular processes that regulate cellularization, such as apoptotic signaling. Although formidable technical hurdles remain, this progress increases our understanding of tissue regeneration and provide opportunities for developing small molecules that may enhance tissue repair in humans.

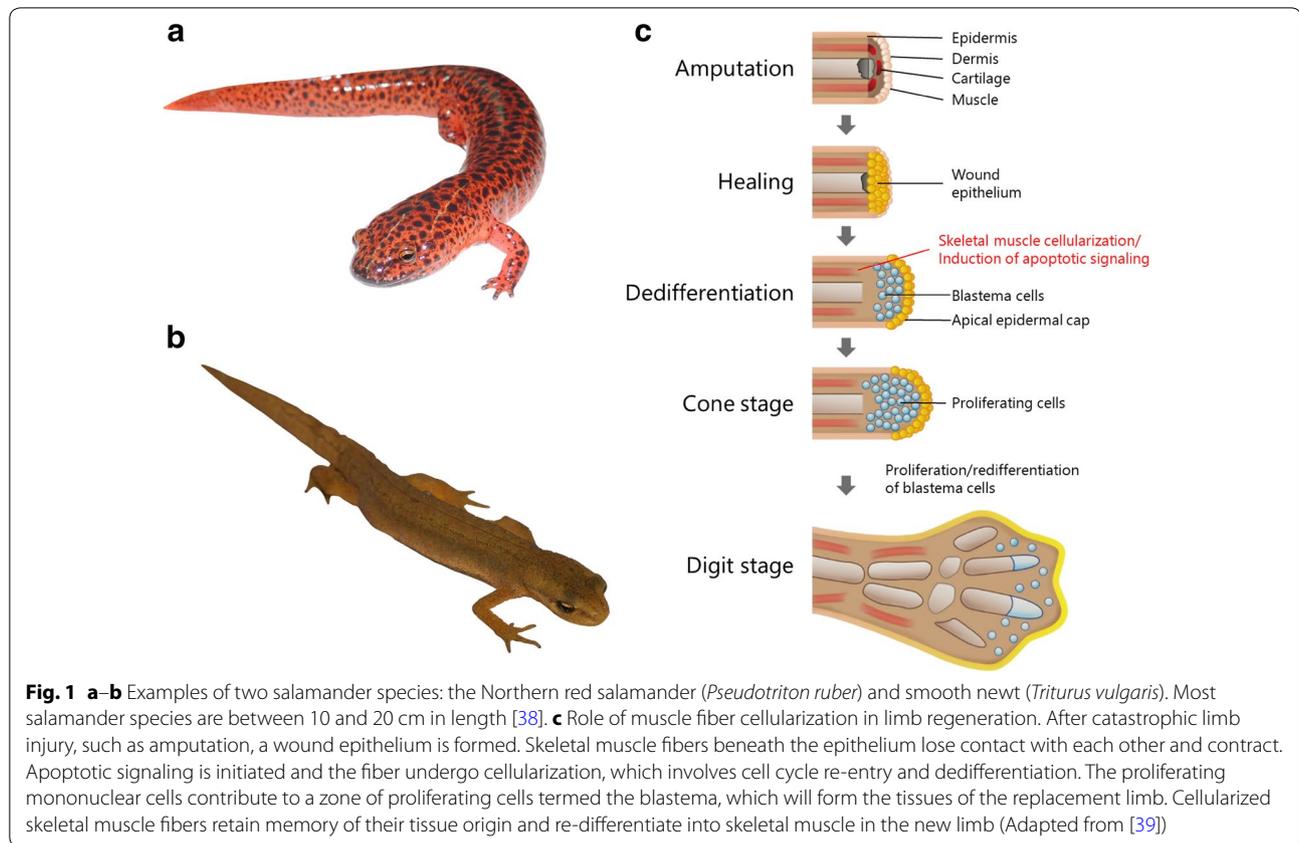
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For centuries, scientists have been both fascinated and beguiled by the regenerative capacity of animals such as flatworms, starfish and salamanders [1, 2]. Among these species, salamanders can be thought of as the ‘champions of regeneration’ because they are tetrapod vertebrates that can completely regenerate multiple tissues, such as the lens, ventricle and limb, and partially regenerate their intestine and spinal cord ([3–5], two examples of salamander species are shown in Fig. 1a–b). Characterizing the cellular and molecular mechanisms underlying limb regeneration responses have been the subject of high profile research (for example, [6–9]), with the aim of identifying genes or developing strategies to confer this ability to humans. Much progress has been made in characterizing the signals and cellular responses that initiate and guide these regeneration processes, although our

knowledge remains incomplete. However, it has become clear that there are fundamental differences in the initial response of mammals and salamanders to injuries such as limb amputation. One difference is the cellularization of skeletal muscle in salamanders, which contributes dedifferentiated cells to a zone of regenerative cells termed the blastema (described below). In the context of limb regeneration, this had led to the development of small molecules that can change the behavior of mammalian muscle tissue *in vitro* to resemble the injury response observed in salamanders. In this commentary, we summarize recent progress in the characterization of these small molecules and discuss their potential to be developed as therapeutic agents to enhance tissue regeneration in humans. Moreover, we also describe recent advances in the identification of new drug targets that could be used for the screening and development of compounds that enhance regeneration responses.

There are fundamental differences in the cellular response of salamanders and mammals to limb amputation (discussed in [10]). In mammals, there is simple

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closure of the wound, followed by healing and scarring. In salamanders, wound closure also occurs with the formation of a blood clot at the site of injury. However, within 6–12 h post-amputation, epidermal cells from the limb stump start to migrate and eventually cover the entire wound surface, forming a structure termed the wound epithelium (Fig. 1c). This epithelium proliferates to form an apical epidermal cap. The epidermis also synthesizes retinoic acid, which is produced as a gradient across the proximal–distal axis of the blastema to provide positional identity [11–13]. The cells in the tissues beneath the wound cap begin to dedifferentiate, including multinucleated skeletal muscle fibers, which break down into single, proliferating cells via the process of cellularization (Fig. 1c). Thus, the limb tissues beneath the amputation site revert to a mass of dedifferentiated cells termed the blastema [14]. A significant proportion of blastema cells originate from multinucleated skeletal muscle fibers in the limb stump [15, 16]. The blastema cells proliferate and re-differentiate over a period of weeks to produce the tissues of the regenerating limb [17, 18]. Blastema cells that originated from cellularized muscle fibers retain memory of their tissue of origin and only form musculature in the regenerating limb [19]. This dedifferentiation does not occur in mammalian skeletal

muscle after amputation, suggesting that cellularization is an important step in limb regeneration.

An interesting feature of muscle fiber cellularization is that it can be modelled *in vitro* using skeletal muscle myotubes [20]. Genetic manipulations or exogenous agents that induce myotube fragmentation can be observed using microscopy and provide a simple assay for candidate compounds that produce cellularization in mammalian muscles [21]. The development of combinatorial chemistry in the 1990s provided small molecule libraries based on known bioactive molecules (in the order of thousands or tens of thousands [22]) that could be screened for inducers of cellularization.

The first reported success using this approach was the discovery of myoseverin (Table 1), which produced cellularization in myotubes derived from the mouse C2C12 myoblast cell line [23]. Myoseverin was found to bind tubulin in the myotubes and destabilize the cytoskeletal network. However, in contrast to other known tubulin binding molecules, myoseverin also increased the expression of genes related to the wound healing response. Subsequent analysis showed that myoseverin down-regulated the myogenic transcription factor, Myf5, in salamander myotubes, which is also observed during the early stages of cellularization [24]. However, further

Table 1 Small molecules used to induce cellularization, a key step of salamander limb regeneration, in mammalian myotubes

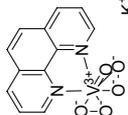
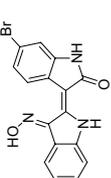
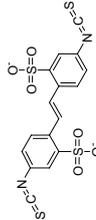
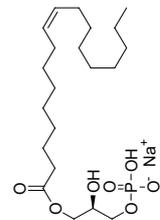
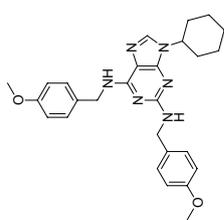
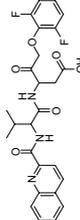
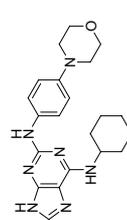
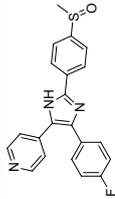
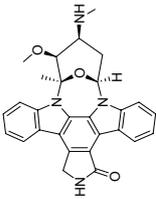
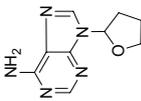
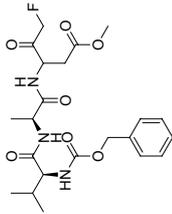
Structure	Name	Myotube type	Reference	Mechanism	Notes
	BpV [dipotassium bisperoxo (5-hydroxypyridine-2-carboxyl) oxovanadate (V)]	Primary mouse myotubes	[29]	Tyrosine phosphatase inhibitor	10 μM; used in combination with staurosporine and Q-VD
	BIO (6-bromoindirubin-3-oxime)	Mouse C2C12 myotubes and primary mouse myotubes	[33]	Glycogen synthase kinase-3β inhibitor	2.5 μM; used in combination with myoseverin and reversine
	DIDS (5-isothiocyanato-2-[2-(4-isothiocyano-2-sulphonyl)ethenyl]benzene-1-sulfonic acid)	Mouse C2C12 myotubes	[35]	Voltage-dependent anionic channel blocker	100 μM; used in combination with staurosporine and Q-VD/Z-VAD
	Lysophosphatidic acid	Mouse C2C12 myotubes	[33]	G-protein-coupled receptor activator	30 μM; used in combination with myoseverin and reversine
	Myoseverin [9-isopropyl-N2,N6-bis-(4-methoxybenzyl)-9H-purine-2,6-diamine]	Mouse C2C12 myotubes and primary mouse myotubes	[33, 35]	Microtubule disruption	20 μM; used in combination with BIO/lysophosphatidic acid/SB203580/SQ22536 and reversine
	Q-VD (N-(2-quinolyl)-L-valyl-L-aspartyl-(2,6-difluorophenoxy)methylketone)	Mouse C2C12 myotubes and primary mouse myotubes	[29, 35]	Pancaspase inhibitor	10 μM; used in combination with staurosporine and BpV
	Reversine [2-(4-morpholinoamino)-6-cyclohexylaminopurine]	Mouse C2C12 myotubes and primary mouse myotubes	[33]	Aurora kinase inhibitor	250 nM; used in combination with myoseverin and BIO/lysophosphatidic acid/SB203580/SQ22536

Table 1 continued

Structure	Name	Myotube type	Reference	Mechanism	Notes
	SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole]	Mouse C2C12 myotubes	[33]	p38 MAP kinase inhibitor	10 μ M; used in combination with myoseverin and reversine
	Staurosporine	Mouse A1/C2C12 myotubes and primary mouse myotubes	[35]	ATP-competitive kinase inhibitor	1 μ M; used in combination with DIDS/ and Q-VD/Z-VAD
	SQ22536 [9-(tetrahydro-2-furanyl)-9H-purin-6-amine]	Mouse C2C12 myotubes	[33]	Adenylyl cyclase inhibitor	300 μ M; used in combination with myoseverin and reversine
	Z-VAD [N-benzyloxycarbonyl=Val-Ala-Asp(O-Me)]	Mouse C2C12 myotubes	[35]	Pancaspase inhibitor	10 μ M; used in combination with staurosporine and DIS

studies of myoseverin using single cell analysis indicated that this compound may only produce myotube fragmentation [25]. The mononuclear cells did not proliferate and remained refractory. Work from our own laboratory showed that further manipulation was required to produce cellularization, such as down-regulation of the cell cycle inhibitor, p21^{Cip1} [26]. Therefore, myoseverin should be categorized alongside other tubulin-binding molecules that cause myotube fragmentation, such as taxol, colchicine and nocodazole. Nevertheless, myoseverin has been useful in the development of small molecule cocktails to induce cellularization (described below).

Numerous genetic manipulations have been shown to produce cellularization in mammalian myotubes, such as down-regulation of retinoblastoma protein (Rb) and Ink4a/alternative reading frame (ARF) [27], ectopic expression of Msx-1 [9], or overexpression of Twist [28]. This suggests that small molecules could be developed that modulate these targets in mammalian myotubes. The response of mammalian muscle to injuries such as amputation is fiber death rather than regeneration. Paliwal and Conboy [29] speculated that BpV, a tyrosine phosphatase inhibitor that delays myoblast differentiation and increases proliferation, could be used to produce myotube cellularization. However, cell cycle reentry in myotube nuclei has been linked with apoptosis [30]. Therefore, Paliwal and Conboy combined BpV treatment with the apoptosis inhibitor, Q-VD (Table 1). Using genetically labelled mouse myotubes that express yellow fluorescent protein, they observed that the combined chemical treatment induced cellularization, with approximately 10–15% of the myotube nuclei reverting to proliferating mononucleated cells. Marker gene analysis indicated that these cells resembled myoblasts, with increased expression of the myogenic factors Pax7 and MyoD. After transplantation into sites of skeletal muscle damage, the cells re-differentiated into muscle fibers, providing further evidence that combined treatment with BpV and Q-VD induced reversible cellularization in mammalian myotubes. This study provided the first evidence that compound treatment could induce a prominent step of salamander limb regeneration (cellularization) in mammalian muscle tissue.

A critical step in the cellularization process is the initiation of proliferation in the nuclei of the differentiated myotube. It can be hypothesized that small molecules which have been previously shown to induce proliferation in differentiated mammalian cells, such as cardiomyocytes, could be combined with myotube fragmentation compounds to achieve cellularization. In our laboratory, we tested the compound, BIO (Table 1), an inhibitor of glycogen synthetase kinase-3 β (GSK-3 β), which

was previously shown to increase proliferation in refractory mammalian cardiac muscle cells [31, 32]. Sequential treatment of mouse primary myotubes with myoseverin and BIO induced cellularization [33]. Step-wise treatment with the small molecule, reversine, an epigenetic regulator that produces dedifferentiation [34], induced pluripotent potential in the mononuclear cells as indicated by re-differentiation into the neuronal lineage. The cell cycle inhibitor, p21^{Cip1} is known as a 'gatekeeper' that maintains skeletal muscle differentiation. Using three different small molecules that have been shown to down-regulate p21 expression or stability; lysophosphatidic acid (activator of G-protein-coupled receptors), SQ22536 (adenylyl cyclase inhibitor) and SB203580 (p38 MAP kinase inhibitor) (Table 1) our laboratory demonstrated that these molecules can induce cellularization in mouse C2C12 myotubes when combined with myoseverin treatment [33]. This suggests that different signaling pathways can be manipulated to induce cellularization if they converge on a common target, such as p21^{Cip1}.

Recently, new insights about the role of chemically-induced apoptosis in cellularization were reported [35]. Mammalian myotube fragmentation using myoseverin was shown to induce apoptosis in the mononuclear cells. Using the well-known apoptosis-inducing compound, staurosporine (Table 1), it was observed that the induction of apoptosis is a critical event in myotube fragmentation. However, to prevent cell death and achieve cellularization, the apoptotic process should be 'intercepted,' which was achieved using the anti-apoptotic small molecules DIDS (inhibitor of voltage-dependent anionic channels) and Z-VAD or Q-VD (pancaspase inhibitors) (Table 1) [35]. Significantly, similar processes were revealed in regenerating salamander limb tissues. Tracing the activity of the apoptosis 'executioner' cleaved caspase-3 showed that blastema cells maintained caspase activity without undergoing apoptosis. Therefore, sequential treatment with staurosporine and DIDS/Q-VD produces cellularization in mammalian myotubes, which mimics the initiation and suppression of apoptosis pathways observed in the regenerating salamander limb.

Table 1 provides a list of the small molecules that have been used to induce cellularization in mammalian myotubes. Currently, these molecules are only effective in combination: there is no reported single molecule treatment for producing cellularization. An interesting feature is that, even though numerous genetic manipulations have been shown to achieve cellularization, such as overexpression of Msx-1 or down-regulation of Rb/ARF [9, 27], these molecules target different proteins, such as tubulin or GSK-3 β . Therefore, there is ample scope to develop new small molecules that achieve cellularization by targeting known genetic regulators.

In summary, significant progress has been achieved in the development of small molecules that produce a critical step of limb regeneration: cellularization in mammalian muscle tissue. Recent research indicates that apoptotic signaling without full progression to apoptosis is a significant stage of regeneration in salamanders. Therefore, focusing on small molecules that induce apoptosis in muscle, such as doxorubicin [36], could provide new candidate compounds for initiating cellularization. However, it is also apparent that major technical hurdles remain before there is any possibility of completely regenerating mammalian limb tissues. For example, the cellularization process has been recapitulated in mammalian myotubes, but there is no data to suggest that this can be reproduced in fully differentiated mature muscle fibers, which are thicker and striated with contractile proteins. Moreover, small molecule-induced cellularization has only been reported for mouse myotubes. There is no report about the effectiveness of this methodology in human muscle tissue. Finally, there is no established animal model for testing the potential for these small molecules to enhance tissue regeneration *in vivo*. The p21^{Cip1} knockout mouse shows appendage regeneration with the closure of ear punctures [37]. It could be envisaged that small molecules which induce myotube cellularization by targeting p21^{Cip1} could be tested in an ear punch model using genetically normal mice. Overall, numerous small molecules have been developed that induce a key step of salamander limb regeneration, cellularization, in mammalian muscle tissue. Unfortunately, research progress is stalled at *in vitro* analyses of myotubes derived from rodent tissues. Further studies of these molecules *in vivo* using animal models and confirmation of their effects in human tissues are required to assess their potential to regenerate tissues that have been lost to injury or disease.

Authors' contributions

JU researched the manuscript and made the figure and table. DWJ and DRW wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All material in this commentary is stored on the institutional computer of the corresponding author (DRW) with cloud based back-up.

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