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Wang, Heng; Simon, András

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Skeletal muscle dedifferentiation during salamander limb regeneration

by

Heng Wang and András Simon

Karolinska Institute, Department of Cell and Molecular Biology, Berzelius väg 35, 17177 Stockholm, Sweden

Correspondence to Andras.Simon@ki.se

Abstract

Salamanders can regenerate entire limbs throughout their life. A critical step during limb regeneration is formation of a blastema, which gives rise to the new extremity. Salamander limb regeneration has historically been tightly linked to the term dedifferentiation, however, with refined research tools it is important to revisit the definition of dedifferentiation in the context. To what extent do differentiated cells revert their differentiated phenotypes? To what extent do progeny from differentiated cells cross lineage boundaries during regeneration? How do cell cycle plasticity and lineage plasticity relate to each other? What is the relationship between dedifferentiation of specialized cells and tissue resident stem cells in terms of their contribution to the new limb? Here we highlight these problems through the case of skeletal muscle.

Tracking muscle cells in the salamander limb

Limb skeletal muscle fibers are formed by the fusion of somite-derived precursors. These multinucleate, elongated cells have a specialized cytoarchitecture built up by proteins, which make the fibers easily distinguishable from their precursor cells at the molecular level. A key feature of the myofibers in the context of the present review is the quiescent state of the myonuclei within the multinucleated syncytium, which is often referred to as the stable post-mitotic state [1,2].

Skeletal muscle has considerable regenerative capacity in all vertebrates, including mammals. However the myonuclei in mammals do not resume proliferation after an injury. Instead, a population of muscle stem cells, the so-called satellite cells, starts to proliferate and subsequently differentiates into muscle to replenish lost fibers [3–5]. Although satellite cells were first described in amphibians [6] *, their presence in adult salamanders [7–9] was unequivocally confirmed more than 40 years later by the isolation of single newt myofibers along with an attached population of cells expressing the canonical satellite cell marker, Pax7 [10]. This finding challenged the traditional view that solely the myofiber itself, rather than a quiescent stem cell population are the progenitor cells during salamander limb regeneration [11], and also highlighted the need to carry out cell type specific tracking experiments during limb regeneration.

Limb regeneration starts with a rapid wound healing followed by formation of a blastema from which the new limb develops [12,13]. Pioneering histological analyses

suggested more than half century ago that myofibers undergo fragmentation, and indicated the migration of mononucleate myofiber fragments into the salamander limb blastema [14*,15]. Furthermore, myofiber fragmentation temporally coincides with disorganization and histolysis of the stump tissues in general, and concomitant production of blastema cells [16]. Cell cycle reentry by myonuclei was also suggested but it is important to remember that the available tools at the time did not allow discrimination among myonuclei, satellite cell nuclei and the nuclei of other interstitial cells within muscle tissue [17]. The model of myofiber-dedifferentiation gained further support from several studies on myotubes, which are the *in vitro* model cell type for resident myofibers. Although myotubes lack striation, they do express a range of terminal differentiation markers, and their nuclei are stably quiescent. However, myotubes from the aquatic salamander, the newt, reenter the cell cycle and replicate their DNA upon appropriate stimulation, which is a distinctive feature of these cells compared to their mammalian counterparts [18*,19]. Furthermore, upon implantation of myotubes into the blastema, could give rise to mononucleate progeny in the blastema [20,21].

Although these studies collectively suggested a distinctive plasticity of differentiated salamander muscle cells, genetically integrated, heritable labeling of myonuclei was required to address whether and to what extent myofibers dedifferentiate during limb regeneration. These experiments were performed in the red spotted newt (*Notophthalmus Viridescens*) and the Mexican axolotl (*Ambystoma Mexicanum*), and revealed unexpected differences between these two salamander species [22] **. First, myofibers in newts gave rise to proliferating blastema progeny, but no such cells were found in the axolotl limb blastema. Second, in sharp contrast to the axolotl, the fraction of myofibers carrying the tracer was similar in pre-existing and regenerated muscle in the new limb in newt. Third, the newt blastema was largely devoid of $PAX7^+$ cells, except for a few cells appearing during the first few days of limb regeneration [10,23]. The axolotl limb blastema on the other hand contained a large number of $PAX7^+$ cells. To what extent these differences at the molecular level reflect differences in the cellular contribution of satellite cell progeny to the regenerating limb will be discussed further down. Importantly, the dissimilarities between the two species were independent of the developmental stages of the animals, since myofiber-progeny did not contribute to the new limb in axolotls that were experimentally induced to undergo metamorphosis, and $PAX7^+$ cells were also

lacking in the blastemas of larval newts. On the other hand, a recent analysis in the Japanase fire-bellied newt (*Cynops pyrrhogaster*) indicated that skeletal muscle dedifferentiation only occurs in metamorphosed animals [24]. Remarkably, that work also suggested that in larval stage the vast majority of blastema cells turn from being $PAX7^-$ into $PAX7^+$ between day12 to day15 after amputation. The possibility that proliferating $PAX7^+$ cells in the axolotl blastema are derived from myofibers, whose nuclei upregulate *Pax7* after amputation was raised [25], but the cell tracking experiments do not provide support for such a process.

Satellite cell progeny vs dedifferentiated cells in the blastema

Does the lack of $PAX7^+$ cells in the newt blastema mean that satellite cells do not significantly contribute to muscle (or to other tissues for that matter) in the regenerating limb? At a first glance this appears as a logical conclusion, especially in light of the contrasting observations in the axolotl [26]. However, it is important to keep in mind that the tracing experiments in newts specifically targeted myofibers, but not the satellite cells. Currently, it is perfectly possible that satellite cell progeny contribute to the limb blastema also in newts but these progeny downregulate expression of the *Pax7* gene within the blastema. If this were the case, a major difference between the newt and axolotl in terms of satellite cell contribution to the blastema would be at the level of gene regulation rather than in the cell source *per se* (Figure 1). In order to unequivocally determine the fate of satellite cells and to relate the contribution from satellite cells to myofiber dedifferentiation, one would need to trace satellite cell progeny during newt limb regeneration. So far this has not been feasible due to lack of suitable cell type specific promoter constructs.

As a surrogate approach to *bona fide in vivo* tracing, satellite cells were previously isolated and, following *in vitro* expansion, re-injected into to regenerating newt limb [10,23]. Although *in vitro* expansion could lead to such epigenetic changes in the cultured cells that naturally are not occurring, these experiments suggested that satellite cell progeny have the capacity to contribute to the regenerate. In addition, the experiments indicated that satellite cell progeny could not only give rise to muscle but also to other cell types in newts – a plasticity, which might be reflected by downregulation of *Pax7* in the satellite cell progeny [23]. This scenario would represent yet another difference between axolotls and newts. While axolotl muscle tissue, and presumably the satellite cells within, were shown only to form muscle

during limb regeneration [27] **, satellite cells may cross lineage boundaries in the newt. Again, the distinctive difference in the newt compared to the axolotl in that case would be the plasticity rather than the lack of contribution by satellite cells and their progeny.

Cell cycle plasticity and lineage plasticity

The results of the myofiber tracing studies in newts refined our understanding of myofiber plasticity from at least two aspects.

First, they showed that cell cycle reentry is a post-fragmentation event occurring in mononucleate myofiber progeny rather than in the myonuclei within the syncytium before breaking up of the myofiber. This is in line with earlier experiments showing that myotubes that were blocked to re-enter the cell cycle still could give rise to mononucleate (obviously non proliferating) progeny upon implantation into the blastema [21]. However they contrast other conclusions that some myonuclei did enter S-phase in the syncytium during limb regeneration [28]. Further experiments are required to resolve the discrepancy between the two studies. The mechanistic separation of cell fragmentation from cell cycle reentry is also consistent with the observations showing that, although without detectable proliferation, also axolotl limb and tail blastemas harbored mononucleate myofiber-derived progeny [22,29]. This indicates that fragmentation of myofibers may represent an alternative fate direction of the muscle fiber - a question that we will discuss further.

Second, they provided no evidence for the myofiber progeny to cross lineage boundaries, as the label introduced to intact muscle prior to limb removal was only found in muscle fibers and not elsewhere in the new limb. How the muscle identity of the myofiber progeny is maintained is not clear but myofiber derived mononucleate progeny that had lost expression of terminal muscle differentiation marker myosin heavy chain, still expressed the early myogenic factor Myf5 in the blastema [22]. It will be important to determine whether Myf5 expression is a prerequisite for retaining the myogenic commitment of myofiber progeny. Yet another open question is whether myofiber progeny acquire muscle stem cell properties, which also requires further investigations. So far we can conclude that dedifferentiated myofiber-derived cells neither do acquire Pax7-expression nor are they found in satellite cell position in the regenerated muscle within the new limb, suggesting that they act as lineage committed progenitors during regeneration.

Mechanisms of myogenic dedifferentiation

Three key features thus define dedifferentiation of skeletal muscle fibers during limb regeneration: (1) Fragmentation of the syncytium into mononucleate cells, (2) loss of terminally differentiated markers, but retention of at least one early myogenic determinant and (3) proliferation of the fiber-derived mononucleate cells. As outlined above, myofiber fragmentation does not depend on cycle reentry by the myonuclei, and conversely, fragmentation of the muscle syncytium does not predestine the derived mononucleate cells to proliferate. The underlying mechanisms of these two processes should thus be possible to disentangle from each other.

Means to force myotubes of both salamander and mammalian origin to reenter the cell cycle has been extensively explored. Key gate-keepers that prevent myonuclei reentering the cell cycle or initiate myogenic dedifferentiation have been identified, such as the retinoblastoma (Rb) protein [18], MSX1 [30], p21 [31], p19ARF [32], and thoroughly discussed in an excellent recent review [26]. Here we focus myogenic dedifferentiation cues specifically studied in the context of salamander limb regeneration.

A series of experiments involving both culture based assays and cell tracking approaches during limb regeneration showed that fragmenting muscle cells displayed hallmarks of a programmed cell death (PCD) process, such as activation of caspase-3, and that inhibition of caspase activity counteracted the derivation of mononucleate cells from both cultured myotubes as well as myofibers in the limb [33] *. Importantly, inducing a programmed cell death response by myotubes was sufficient to cause cellularization of cultured myotubes but only a fraction of the derived mononucleate cells could be rescued from dying by apoptosis inhibitors and induced to proliferate. Although still not proven, the emerging model suggests that limb amputation evokes myofibers to embark on a programmed cell death program, which is manifested by fragmentation of the syncytium. However, the derived mononucleate cells must be rescued from the full execution of the cell death program in order to gain ability for resuming proliferation within the blastema. This idea is consistent with the observations that axolotl myofibers also fragment into mononucleate cells during appendage regeneration [22,29], but these cells cannot be traced further during axolotl regeneration and presumably die.

At present it is unclear how the molecular components of the programmed cell death program cause myofiber disassembly. An experimentally approachable hypothesis is that caspases are involved in the disintegration of structural elements, which are required for maintaining the integrity of syncytium. Noteworthy in the context are the experiments showing that caspase activity is required for spermatid individualization during sperm maturation in drosophila – a process during which each spermatid becomes encapsulated by an independent plasma membrane [34]. Caspases might also expel obstacles of subsequent proliferation that reside in the chromatin structure.

What could be the reasons why, in contrast to the newt, myofiber derived mononucleate cells do not contribute to the regenerate in the axolotl (formally only proven in the limb)? Differences both in intrinsic cell properties as well as in extrinsic cues that cells encounter in the limb might provide explanations but no such differences have yet been identified. Assays on cultured newt myotubes indicated that inhibition of p53 activity is necessary for cell cycle reentry [35*,36] and p53 knockdown was also required to render mammalian myotube-derived mononucleate cells ability to resume proliferation [33]. However, p53 activity decreases also during axolotl blastema formation, and p53 stabilization led to impairment of limb regeneration [35]. Similarly, with a creative screening strategy using newt myotubes the Tanaka lab recently identified a MARCKS (Myristoylated alanine-rich C-kinase substrate)-like protein (MLP), which on one hand promotes proliferation of myofiber derived mononucleate cells in newts, and on the other hand initiates regeneration of both limbs and tails in the axolotl [37] **.

Future perspectives

Our understanding of how and to what extent skeletal muscle contributes to limb regeneration has significantly increased during the past years. In this review we also highlighted outstanding questions that still have not been addressed experimentally. One such issue is to determine the relative contribution from dedifferentiating myofibers and from satellite cells to the regenerating newt limb. Even if we have gained more insight to myofiber dedifferentiation at the cellular level, we are still short of insights into the underlying molecular mechanisms. One way forward is to combine cell tracking approaches with genome wide expression analyses and molecular manipulations using contemporary methods such as single cell sequencing

238 and genome editing technologies.

Figure legends

Figure 1. *Contribution of skeletal muscle cells to the blastema formation during newt limb regeneration.* Myofiber dedifferentiation results in proliferating, $Myf5^{+}/PAX7^{-}$ mononuclear cells (black) in the blastema that give rise to the skeletal muscle in the new limb. Lack of $PAX7^{+}$ cells in the newt blastema indicates either a minimal contribution of satellite cells (green) to the blastema formation or a down-regulation of *pax7* gene expression in the progeny of satellite cells.

Figure 2. *Model of myofiber dedifferentiation during newt limb regeneration.* Injury evokes myofibers to activate caspases, which are involved in the disassembly of the syncytium. The resulting fragments apoptotic fragments will either die or survive and proliferate. The identity of the pro-survival and proliferation cues is largely unknown. Although not proven in newts, downregulation of p53 activity is likely to play a role in cell survival. The MLP promotes proliferation of myofiber progeny during newt limb regeneration.

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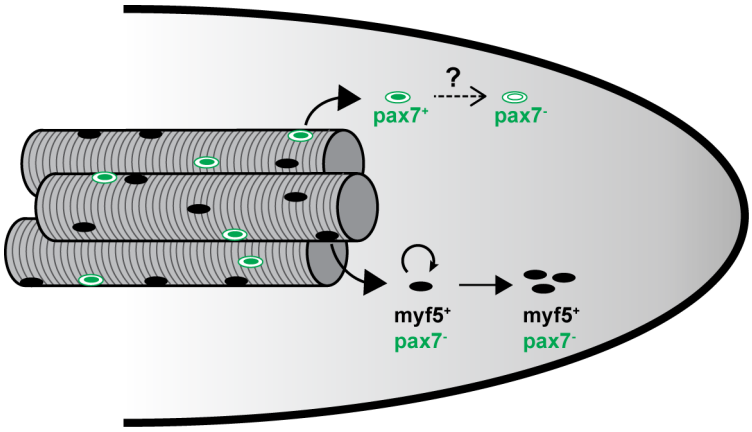
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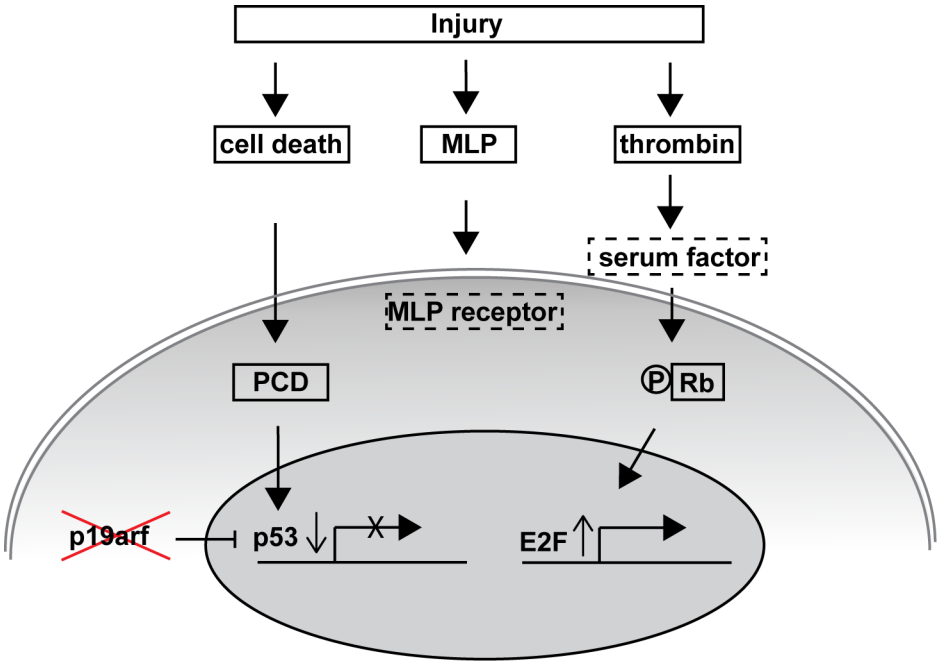
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