seminars in **CELL & DEVELOPMENTAL BIOLOGY**, Vol. 13, 2002: pp. 353–360
doi:10.1016/S1084–9521(02)00091-5, available online at http://www.idealibrary.com on IDEAL®

Mechanisms of muscle dedifferentiation during regeneration

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For many years people have known that amphibians have an amazing ability to regenerate lost body parts. In contrast humans have limited regeneration capacity and even simple wound healing results in scarring. Despite more than a century of scientific inquiry, this remarkable phenomenon remains poorly understood. Recent research has begun to provide insight into how this unique process that is now fully accepted to occur via the reversal of cell differentiation is executed at the molecular level. As more and more is known about regeneration and dedifferentiation we can begin to address the question: if given the right signals could mammals also regenerate body structures?

Key words: amphibians / dedifferentiation / blastema / myotubes

Introduction

Where do the cells in the regenerating structure come from?

In urodele amphibians regeneration occurs by formation of a wound epidermis in response to injury, followed by rapid proliferation of undifferentiated cells which form a structure referred to as a blastema. The undifferentiated cells making up the blastema eventually differentiate to replace all lost structures. For many years one of the major unanswered questions has been, from where do the cells in the blastema originate? Two major models have been considered. The first proposes that reserve 'stem' cells reside in adult tissues and these cells proliferate in response to local wounding in order to replace the lost tissue. A second, controversial hypothesis suggests that differentiated cells such as mature muscle fibers dedifferentiate to form mononucleate cells that then go on to populate the blastema.[1,2] Considering that muscle tissue in higher vertebrates harbors a population of reserve, mononucleate cells called satellite cells that lie adjacent to muscle fibers and are activated to divide upon muscle injury, and that potential reserve cells have been described for muscle in some urodele species,[3] the theory of dedifferentiation seemed unlikely. Remarkably recent studies show that muscle fiber dedifferentiation plays a key role in generating the blastema.[4–6] Here we review the current understanding of how muscle dedifferentiation occurs on both a cellular and a molecular level.

Muscle fibers dedifferentiate during regeneration

Histological evidence for muscle dedifferentiation

The proposal of dedifferentiation, whereby mature differentiated muscle fibers revert back to a 'stem cell' like state during regeneration was first described in the 1930s.[7] These studies on limb regeneration suggested that there was widespread dedifferentiation and cell plasticity during the formation of the blastema. Detailed electron microscopic observations of muscle fibers at the plane of amputation suggested that muscle fibers lost their myofibrillar structure, their nuclei became enlarged, and then mononucleate cells were budded off into the blastema.[1,8] Tritiated thymidine studies also revealed that DNA synthesis occurred at the same time as the nuclear enlargement and budding.[8] This result suggested that cell cycle re-entry was initiated prior to formation of the mononucleate cells, which would then proliferate to populate the blastema. In these studies, however, the process of muscle dedifferentiation was inferred

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from static images and so the causal relationship between the changes in muscle cells and blastema cell formation remained unproven and controversial. It was argued by others that these static images actually represented the fusion of myoblasts into newly forming muscle fibers instead of the budding off of mononucleate cells.[9]

Experimental evidence for dedifferentiation

The development of methods to follow the fate of muscle cells during regeneration was required to prove that muscle dedifferentiation indeed occurs during regeneration. Lo *et al.*[4] showed the first experimental evidence to support this 'budding off' theory of progenitor cell production. They implanted size-selected multinucleated myotubes, which had been formed in culture and injected with rhodamine dextran, into a regenerating newt limb. After 1 week, the lineage label was found in mononucleate cells. The average number of mononucleate cells increased over time, suggesting that cell division had occurred. Recently these implantation experiments were repeated using an integrated retroviral marker, reducing the possibility that mononucleate cells were derived from cytoplasmic transfer of the lineage tracer, and similar results were found.[5] Using BrdU injections, Kumar *et al.* also showed that some nuclei of the retrovirally labeled implanted myotubes re-entered S-phase, although it was unknown if these same nuclei after replicating their DNA would then 'bud off' and populate the regeneration blastema. Although Lo and Kumar's experiments strongly support the theory of dedifferentiation, the question still remained whether endogenous muscle fibers dedifferentiated to produce a significant number of cells of the blastema.

This issue has recently been addressed using *in vivo* labeling of muscle fibers in the axolotl tail to analyze muscle dedifferentiation and its contribution to the blastema.[6] By following the fate of individual muscle cells live during tail regeneration, these experiments showed for the first time that during regeneration, endogenous muscle fibers lying next to the plane of amputation dedifferentiate and form mononucleate cells (Figure 1). Moreover based on the frequency that dedifferentiation occurred it was estimated that nearly 17% of the blastema cells derive from muscle dedifferentiation alone.

Interestingly, specific conditions were required to trigger this process. Releasing the distal end of the

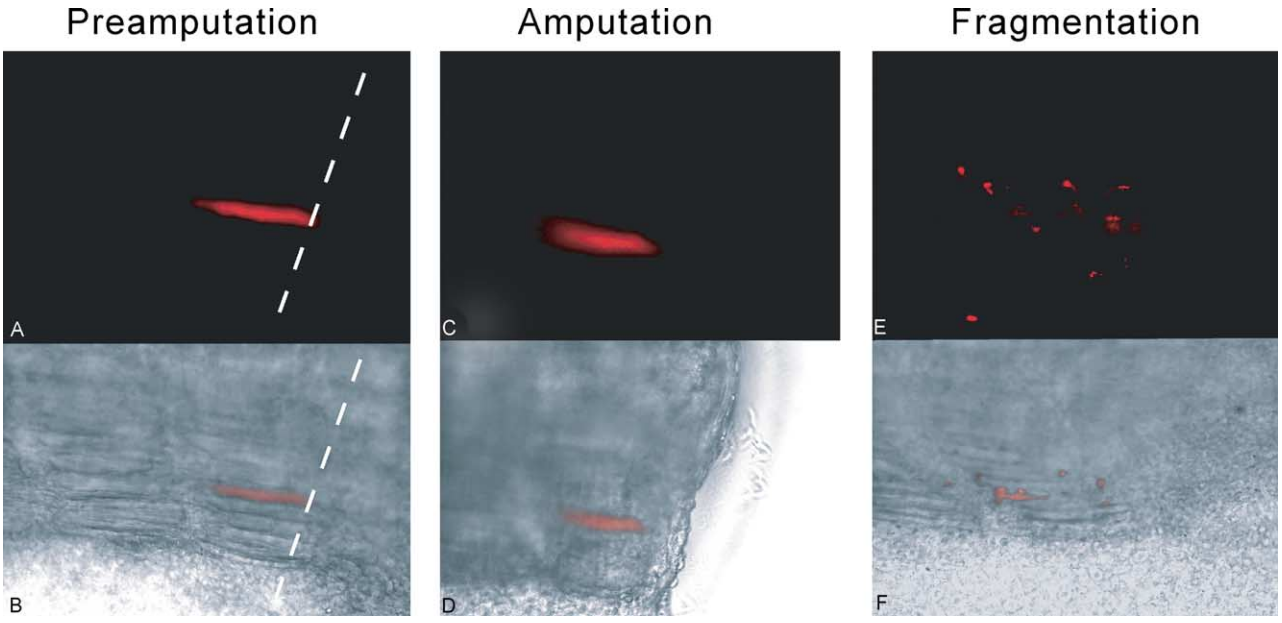

Figure 1. Tail amputation accompanied by clipping of the fiber results in dedifferentiation of a mature muscle fiber. (A) A single fiber was labeled with rhodamine dextran. (B) The matching DIC image with the overlay of the labeled fiber. The tail was amputated along the dotted line (A, B). (C) The retraction of the fiber in response to amputation. The overlay in (D) illustrates the position of the injected fiber in relation to the amputation plane. (E) At 5 days post-amputation the labeled fiber fragmented, giving rise to multiple mononucleate cells. (F) The position of the mononucleate cells can be seen clearly in the overlay.

myofiber from contact with neighboring cells was necessary but not sufficient to induce dedifferentiation. A second signal produced in response to tail amputation or severe tissue damage was also necessary. These two signals together induced the myofiber to 'fragment' and form mononucleate cells that then underwent rapid cell division. The identity of the extracellular cues and how they trigger dedifferentiation in the fiber is at present not known.

Cellular mechanisms underlying dedifferentiation: the relationship between cell cycle progression and formation of mononucleate cells

At least two distinguishable events, cell cycle re-entry and fragmentation into mononucleate cells, are required to create blastema cells from a muscle fiber *in vivo*. When do these events occur during dedifferentiation, and are they mutually dependent? Hay and Kumar's DNA-labeling experiments indicated that DNA synthesis occurs in the multinucleated myotube before fragmentation.[5,8] These findings raised the possibility that the nuclei proceed through to mitosis and bud off the fiber at cytokinesis as 2N nuclei. Alternatively, the nuclei may bud prior to mitosis as 4N nuclei and subsequently undergo cell proliferation. To examine the relationship between cell cycle progression and the fragmentation process Velloso *et al.* blocked S-phase re-entry either by X-irradiation or by transfection of the CDK4/6 inhibitor p16 in cultured myotubes that were then implanted into the regenerating newt limb blastemas.[10] These arrested myotubes could still form mononucleate cells even though the cells could not subsequently proliferate. This data showed that the fragmentation process is independent of cell cycle progression and thus strongly suggested that mononucleate cells were not formed through mitosis. This means that two parallel pathways are likely involved in dedifferentiation: a pathway to form mononucleate cells, and a separate pathway to re-enter the cell cycle with rapid proliferation occurring after fragmentation (Figure 2).

In the future, it will be fascinating to determine the relationship between the extracellular signals and the intracellular pathways. For example, is one extracellular signal dedicated to triggering cell cycle re-entry, and a separate extracellular signal for forming mononucleate cells, or is there overlap in the signaling pathways? Work described below suggests that the regenerating tissue may contain multiple overlapping cues to initiate the process of dedifferentiation.

Toward a molecular understanding of dedifferentiation

Cell cycle re-entry and dedifferentiation

Progress toward unraveling the signaling mechanisms for dedifferentiation has come from the development of functional assays that reproduce aspects of muscle dedifferentiation *in vitro*. Mouse myotubes have served as a paradigm for a cell type that becomes stably withdrawn from the cell cycle and refractory to growth factors upon differentiation. In contrast, newt myotubes in culture have been shown to re-enter S-phase in response to serum stimulation.[11] Using this assay two primary lines of experimentation have been directed at identifying the intracellular pathways that mediate cell cycle re-entry, and the extracellular cues that trigger these pathways. The first line of work was initially stimulated by the report that mammalian myotubes derived from mice lacking the retinoblastoma (Rb) gene re-enter S-phase in response to serum stimulation.[12] This suggested that in newts the cell cycle response pathway may regulate Rb, a known key regulator of the G1–S transition that is inactivated to allow transcription of S-phase re-entry genes by the E2F transcription factors. Tanaka *et al.* showed that serum stimulation of newt myotubes leads to the inactivation of the Rb protein via phosphorylation.[11] In contrast, Rb in wild-type mouse myotubes remains unphosphorylated, and the cells do not re-enter S-phase. The serum dependent phosphorylation of Rb in newt myotubes was the first molecular, intracellular difference to be identified between newt and mammalian myotubes related to a regeneration phenotype.

The question, however, remains: what is the intracellular block to S-phase entry that exists in mouse myotubes and is somehow relieved by the serum factor in newt myotubes? Is it a direct regulator of the Rb pathway or does it block an earlier step in cell cycle re-entry such as the G0–G1 transition? Again, work forcing cell cycle re-entry in mouse myotubes may provide a clue to this question. Tiainen *et al.* demonstrated that stimulation of mouse myotubes with serum induces the expression of immediate early response genes such as c-*fos* and c-*myc*, indicating that the mouse myotubes were transiting from G0 to G1, even though the myotubes never entered S-phase.[13] This suggests that the G0 to G1 transition is not the major block to cell cycle re-entry in mouse myotubes. Consistent with this finding, Latella *et al.* demonstrated in mouse myotubes that forced expression of cyclinD and cdk4, a kinase

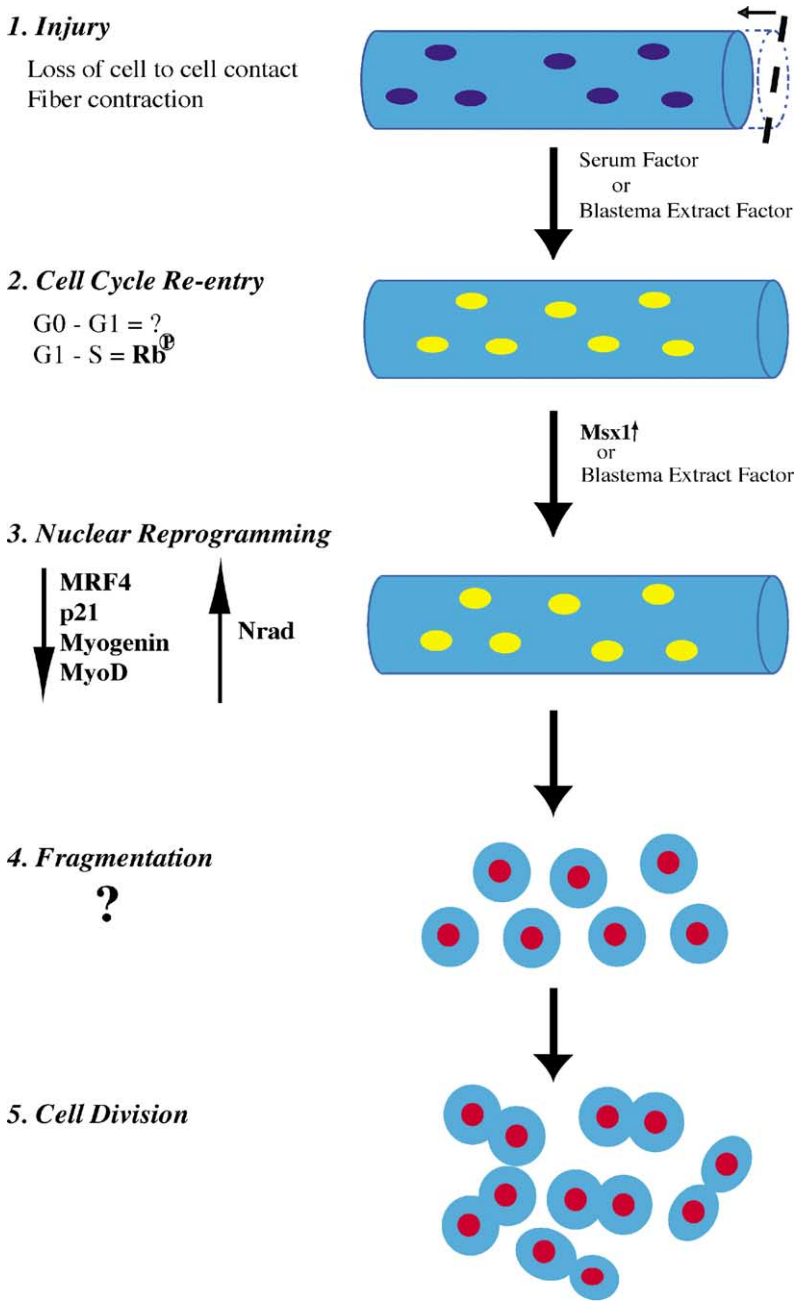

Figure 2. Schematic diagram of muscle fiber dedifferentiation. (1) *In vivo* muscle fibers retract in response to injury. Around the fiber, a blood clot is formed and the wound heals over. The retracted muscle fiber then re-elongates. (2) *In vitro* an extracellular factor found in both serum and newt limb blastema extract is capable of pulling the cells out of G0 and allowing them to progress to S-phase, where they become arrested in a 4N state. The G1–S transition is mediated via the phosphorylation of the Rb protein in newt myotubes. (3) Ectopic expression of *Msx-1* or the presence of newt blastema extract causes mammalian myotubes to down regulate markers of terminally differentiated muscle. Newt myotubes upregulate Nrad in the nucleus of muscle fibers at the plane of amputation. Steps 2 and 3 are likely to occur concomitantly. (4 and 5) *In vivo*, axolotl tail muscle fibers fragment and form mononucleate cells in response to clipping the end of a muscle fiber in combination with a signal released in response to severe tissue damage. *In vitro* a newt blastema extract can stimulate a response in both newt and mammalian myotubes, causing 9% of the myotubes in culture to fragment and form mononucleate cells. The factors responsible for inducing the cells to fragment, divide and eventually re-differentiate are still unknown.

that phosphorylates Rb at the G1 to S, was sufficient to induce S-phase re-entry in mouse myotubes cultured in high serum conditions.[14] These results raise the possibility that inhibition of the cyclinD/cdk4 kinase at G1 to S may be the critical block to S-phase re-entry in mouse myotubes. It will be interesting to determine whether this kinase pathway is the critical point of regulation during newt myotube cell cycle re-entry.

Extracellular signals leading to cell cycle re-entry

A second line of work to understanding cell cycle re-entry has involved analyzing the extracellular signals that trigger the process. Although cultured myotubes enter S-phase in response to a factor in serum, normal serum growth factors like PDGF or EGF are incapable of inducing the response.[11] Furthermore newt myoblasts that proliferate in response to PDGF and EGF are unresponsive to the partially purified extracellular factor, suggesting that sensitivity is acquired during the myogenic differentiation process.[15] Additional characterization of the factor revealed that it is activated by thrombin proteolysis to generate a ligand, which acts directly on newt myotubes.[16] An attractive aspect of this factor is that during wound healing, the first phase of regeneration, thrombin activity has been shown to be upregulated at the end of the limb stump.[16]

Contact inhibition of dedifferentiation

Another interesting aspect of this work is that serum stimulation of the cultured myotubes is inhibited by contact between the myotubes and mononucleate cells.[11] This *in vitro* characterization suggests that cell cycle re-entry requires at least two extracellular conditions: loss of cell–cell contact and a soluble extracellular factor derived from clotted blood (Figure 2). This phenomenon is reminiscent of that observed *in vivo* whereby release of cell–cell contact and tissue injury is necessary to induce dedifferentiation during axolotl tail regeneration.[6]

So far this extracellular factor has been found in all animal sera but only newt myotubes are responsive to it. Tanaka *et al.*[16] suggest that mammalian myotubes have lost the receptor to respond to the factor. Interestingly both nuclei of mouse/newt hybrid myotubes re-enter S-phase upon stimulation with serum, suggesting that the ability to respond is somehow conferred from the newt cytoplasm to the mouse nucleus.[17]

Can mammalian myotubes respond to dedifferentiation factors?

Based on the work described above it seems that mammalian myotubes may have either lost the receptors for responding to dedifferentiation signals or their downstream signaling pathways. However work by McGann *et al.* provides evidence to the contrary. A protein extract made from the newt limb blastema could stimulate 25% of mouse myotubes to re-enter S-phase and 9% of the myotubes to subsequently form mononucleate cells.[18] Newt myotubes displayed a similar response to the blastema extract. The mouse myotubes also exhibited a reduced expression of muscle differentiation markers like MyoD and myogenin. The authors speculate that the factors found in their extract are soluble extracellular proteins, which are capable of acting as ligands to induce receptors that can carry dedifferentiation signals to receptive cells. It is unclear why only a proportion of the myotubes formed mononucleate cells in response to the extract. Are there sub-populations of receptive myotubes, or is some feature of cell culture such as adhesion to the substrate simply blocking most myotubes from fragmenting?

Nonetheless the McGann *et al.* results imply that mammalian cells may not have lost the receptor, but simply that they never receive the correct signals in the mouse. So how can this data be reconciled with the previous results from Tanaka *et al.* with the serum factor? There are several interesting possibilities. The blastema factor and the serum factor could be completely different molecules that act through distinct pathways. Alternatively, the blastema extract may contain the serum factor plus an additional factor. For example, the blastema extract may represent a more 'complete' protein complex that is more potent than the serum factor. Although it may be appealing to think of one extracellular factor being responsible for inducing dedifferentiation, in reality perhaps several pathways remain to be elucidated for us to really understand how this unique phenomenon is induced and to allow us to try mimicking it in non-regenerative systems.

Is *Msx1* a master transcriptional regulator of dedifferentiation?

Msx genes are expressed in areas of cell proliferation during vertebrate development and regeneration

Little is known about the intracellular signaling pathway of dedifferentiation but one component appears

to be the *Msx-1* gene. *Msx* genes are a family of homeobox-containing transcription factors that are known to be expressed during embryogenesis in areas of epithelial to mesenchymal transitions. Interestingly, their expression is also associated with areas containing proliferating progenitor cells. During development, *Msx-1* is expressed in the growing end of the limb bud, where cells are maintained in an undifferentiated state. In fetal and neonatal mouse, Reginelli *et al.* found that the zone of *Msx-1* expression correlates with a zone in the finger-tip that retains regeneration ability.[19]

During urodele limb regeneration, the msx family members, *Msx-1* and *Msx-2* are expressed in the blastema.[20,21] Data from Carlson *et al.* suggests that *Msx-2* is one of the first known genes to be expressed during regeneration and in wound healing, and therefore it may be involved in a pathway common to both processes.[20] During limb regeneration *Msx1* is found to be upregulated later at mid-bud, when all cells in the blastema are already believed to be in an undifferentiated state.[21,22]

Functional studies of the *Msx-1* gene have shown that it can keep cells in an undifferentiated state. For example, expression of *Msx-1* in cultured mouse myoblasts prevented formation of myotubes in response to normal differentiation signals.[23] On a molecular level, expression of *Msx-1* results in down regulation of the myogenic regulator factor, MyoD, consistent with the cells transforming into a less differentiated state.[24]

The role of Msx-1 in dedifferentiation

In addition to its role in maintaining cells in an undifferentiated state, *Msx-1* may also be involved in causing differentiated cells to dedifferentiate. Odelberg *et al.* used ectopic expression of *Msx-1* to force dedifferentiation of mouse myotubes.[25] Early after *Msx-1* induction, the levels of genes associated with muscle differentiation, MRF4, p21 and myogenin, were reduced in the myotubes, followed by a down regulation in MyoD levels. Approximately 9% of the myotubes produced smaller myotubes or proliferative mononucleate cells, which were capable of being forced to redifferentiate into several lineages including osteoblasts, chondrocytes and adipocytes.

Since ectopic expression of *Msx-1* acts directly in the nucleus, it bypassed the need for a receptor to respond to an external signal so the upstream activators of the process that are normally used during regeneration still remain to be elucidated. One potential candidate molecule found by Shimizu-Nishikawa *et al.* that is upregulated during newt limb regeneration is Nrad, a ras protein associated with diabetes that is thought to act as a switching molecule with GTPase activity.[26] It is the first factor found to be upregulated, 4 h post-amputation specifically in the nuclei of muscle fibers at the plane of amputation. This upregulation is found in different types of regenerating structures such as the limb, the tail and heart muscle in response to amputation. The question arises, is rad part of the upstream pathway which eventually turns on *Msx-1* or might it be involved in the cell cycle re-entry pathway?

Open questions about the role of Msx-1 in dedifferentiation

The *Msx-1* data gives us an important clue to the intracellular pathway of dedifferentiation but a number of key questions remain. It is not known whether the *Msx-1* expressing myotubes enter S-phase before dedifferentiating. Furthermore it is not clear if ectopic expression of *Msx-1* alone is sufficient to cause dedifferentiation, or if a combination of *Msx-1* overexpression with the serum that was present in the medium is required to cause mouse myotubes to form mononucleate cells. Therefore, it is also not yet clear if *Msx-1* is involved in both myotube fragmentation and cell cycle re-entry or just one of these pathways.

How do Odelberg's molecular results in cultured mouse myotubes relate to *in vivo* dedifferentiation during urodele regeneration? In their experiments, Odelberg *et al.* found that *Msx-1* had dedifferentiating activity but not *Msx-2*. Although ectopic expression of *Msx-1* appears to lead to dedifferentiation *in vitro*, *Msx-1* upregulation has not been observed in myotubes next to the plane of amputation by *in situ* hybridization *in vivo*. In the regenerating limb *Msx-2* is expressed during the dedifferentiation phase while *Msx-1* is upregulated at later stages of regeneration, after dedifferentiation is finished.[21] The significance behind the differences between the *in vivo* gene-expression patterns and the *in vitro* dedifferentiation activities is not clear. In their experiments Odelberg *et al.* used the mouse *msx* genes, which may have different activities, compared with the amphibian homologues. Alternatively, other genes that have similar activities to *Msx-1* may be the true inducers of dedifferentiation *in vivo*. Clearly it will be important to identify other genes in the dedifferentiation pathway.

It will also be necessary to test the role of *Msx-1* *in vivo*. For example, one test would be to ectopically express *Msx-1* in a subset of fluorescently labeled myofibers *in vivo* in the mouse and to determine if they

would then dedifferentiate in response to wounding. It would also be interesting to inactivate *Msx-1* function in dedifferentiating muscle during regeneration.

In the *in vitro* cell assays, the mononucleate cells derived from *Msx-1* expressing myotubes could differentiate into other mesenchymal lineages such as adipocytes and chondrocytes. It will be interesting to determine whether the dedifferentiated muscle cells found *in vivo* in a urodele limb or tail blastema display the same range of potential as those created *in vitro*.

The molecular pathway of muscle dedifferentiation

Figure 2 indicates what is currently known about the mechanism of muscle dedifferentiation. This figure represents a consolidation of observations from *in vivo* and *in vitro* experiments, as well as experiments in urodele and mouse cells. Therefore, while the cellular events of muscle dedifferentiation are reasonably well defined, the relationships between various molecular events are speculative. After injury, the muscle fiber loses contact with its neighbors and contracts before re-elongating. Soluble factors in serum and/or the blastema induce the muscle cell nuclei to undergo S-phase. Concomitantly extracellular blastema extract and/or intracellular *Msx-1* expression causes the gene transcriptional program of the muscle cell to change with down-regulation of myogenic genes and cell cycle regulators such as p21. *In vivo* data indicates that the Nrad gene would be upregulated in these early stages. The formation of mononucleate cells from the syncytium appears to occur prior to mitotic division.

There are many open questions about the relationship between the currently known players in a pathway. *Msx-1* appears to play a key role in establishing and maintaining cells in an undifferentiated state. Yet it is still unclear exactly at which step in the dedifferentiation pathway it acts. Furthermore, what is its relationship to the signals to initiate dedifferentiation? For example, does the blastema extract induce dedifferentiation through Nrad and the *Msx-1* gene?

Future perspectives

By focusing on muscle cell dedifferentiation as a unique cell process that distinguishes urodeles from other non-regenerating species, the fascinating but complex problem of regeneration has just begun to be understood on the cellular and molecular level. The technical advances in following dedifferentiating cells in regenerating tissue, as well as *in vitro* functional assays have been a crucial starting point for delineating the underlying regulatory pathways.

Clearly many more molecular players must be identified to understand how dedifferentiation occurs in urodele amphibians, and why it does not occur in mammals. Importantly, although the extracellular activities have been characterized, their molecular identity is still unknown. A key goal is to identify the serum factor and the blastema extract factor.

Further down the line, the identity of cell surface receptors and the intracellular pathways that these factors activate will play a crucial role in our understanding of dedifferentiation. Toward this end two important steps currently being pursued are the generation of a broad spectrum of molecular markers and the identification of gene candidates from regenerating urodele tissue. Combining these approaches with the development of better functional assays where dedifferentiation processes such as mononucleate cell formation can be assayed easily, these candidate genes can then be tested in the mammalian system. With such tools in hand, we may finally understand, why can amphibians regenerate but mammals not?

Acknowledgements

We would like to thank Stephano DeRenzis, Martin Srayko, Werner Straube and David Drechsel for helpful discussion and input on the manuscript.

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