Artificial Organs
30(10):743–755, Blackwell Publishing, Inc.

Reversibility of the Differentiated State: Regeneration in Amphibians

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Abstract: In contrast to mammals, some fish and amphibians have retained the ability to regenerate complex body structures or organs, such as the limb, tail, eye lens, or even parts of the heart. One major difference in the response to injury is the appearance of a mesenchymal growth zone or blastema in these regenerative species instead of the scarring seen in mammals. This blastema is thought to largely derive from the dedifferentiation of various functional cell types, such as skeletal muscle, dermis, and cartilage. In the case of multinucleated skeletal muscle fibers, cell cycle reentry into S-phase as well as fragmentation into mononucleated progenitors is observed both in vitro and in vivo. **Key Words:** Regeneration—Urodele amphibians—Muscle dedifferentiation—Blastema—Cell cycle reentry—Cellularization.

THE PLASTICITY OF DIFFERENTIATION DURING REGENERATION IN SALAMANDERS

Regeneration in urodele amphibians

The unique ability of adult urodele amphibians such as the newt and axolotl to replace lost parts of the body and regenerate after wounding has fascinated researchers for over 100 years. In response to tissue damage or injury, an adult newt is capable of regenerating complex structures such as a limb, tail, spinal cord, heart ventricle, retina, lens, and jaws that are composed of a variety of tissues including muscle, skin, bone, cartilage, and nerves (Fig. 1). This remarkable regenerative capacity is thought to depend at least partly on dedifferentiation of cells at the site of injury, a process that is lacking in mammals and other vertebrates, where regeneration is limited to a small spectrum of organs (1–4).

The regeneration of an amputated urodele limb proceeds through a typical series of morphological and histological stages (5–7). Following amputation of the limb, epithelial cells begin to crawl over the amputation site to form a wound epithelium. Then, in response to undefined signals in the early regenerate, internal stump cells start to lose their specialized character in a process referred to as dedifferentiation. These dedifferentiated cells then proliferate to form a mesenchymal growth zone, known as the blastema, which harbors the cells that will later redifferentiate to form the regenerated limb (Figs. 2 and 3). Moreover, blastemal cells not only differentiate back into the cell type which they derived from, but also transdifferentiate into other cell types (10). The formation of the blastema in response to wounding represents a key difference compared to other vertebrates including mammals, which are not able to regenerate to this extent.

The source of cells for regeneration

The mature tissue source that gives rise to the regeneration blastema was controversial in early limb regeneration studies. Thornton's work on limb regeneration in larval axolotl (*Ambystoma punctatum*) and newt (*Triturus viridescens*) revealed transformation of muscle, cartilage, and other inner tissues of the limb into mesenchymal-like cells (11,12). An alternative hypothesis at this time was that the dedifferentiating epidermis is a major contributor to the arising blastema (13). This was contradicted by

Presented in part at the 43rd Tutzing Symposium "Regenerative Medicine—Membranes & Scaffolds" held November 20–23, 2005, in Tutzing, Germany.
Received April 2006; revised July 2006.
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FIG. 1. The red-spotted newt (*Notophthalmus viridescens*). Courtesy of Henk Wallays (Henk.Wallays@pandora.be).

observations of Chalkley, who showed that the earliest cell divisions begin in the inner limb tissues, in a relatively large zone away from the amputation plane, and that epidermis may not be required (14). Further confirmation of these results was obtained

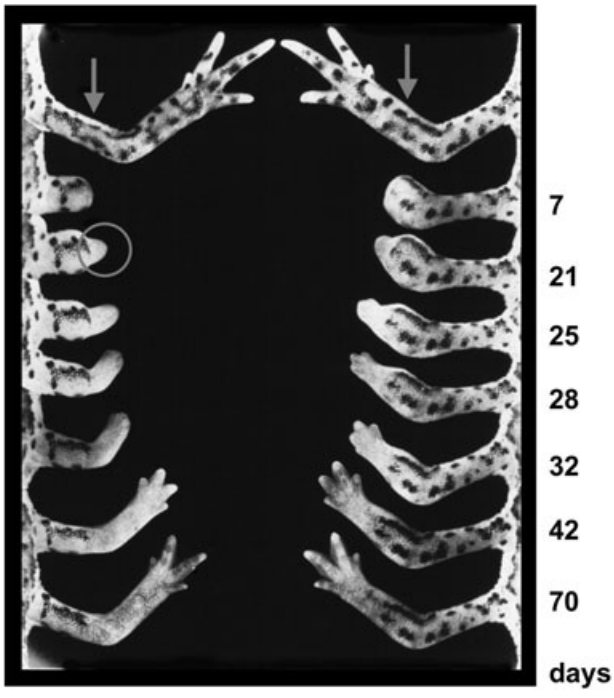

FIG. 2. Time course of limb regeneration. Forelimbs of adult newts (*Triturus viridescens*) were amputated at the level of lower arm (right) and upper arm (left) and photographs taken at 7, 21, 25, 28, 32, 42, and 70 days after amputation. During the third week, the blastema appears as a bud at the tip of the regenerating limb (circle). Note that only structures distal to the amputation plane are replaced, indicating the blastema contains a positional identity (reprinted from [8], p. 142, with permission from Elsevier).

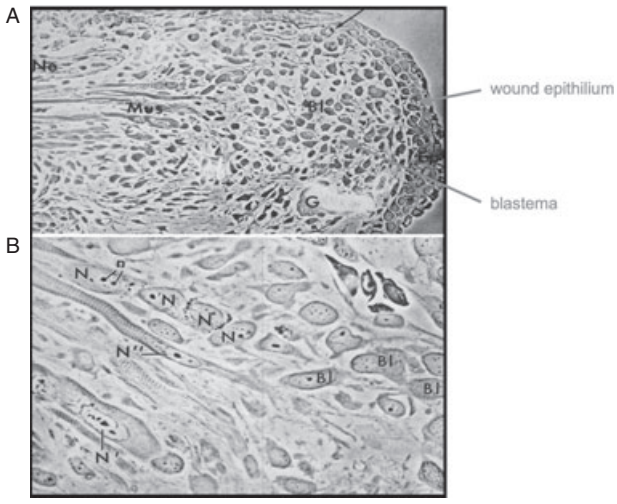

FIG. 3. Phase contrast photomicrograph of longitudinal section of a regenerating larval axolotl (*Ambystoma punctatum*) limb 5 days after amputation. (A) After 5 days, the regenerating limb is covered by at least a five-layer-thick apical wound epidermis (EP) underneath which the blastema has already appeared (Bl). Most of the blastema seems to have arisen from dedifferentiating muscle fibers (Mus), but Schwann cells from cut nerves (Ne) and connective tissue also contribute. (B) Magnification of the dedifferentiating muscle fibers from (A): During the process of muscle fiber dedifferentiation, the normally elongated nucleus (N″) enlarges (N′) and becomes rounded in shape (N). These changes indicate the initiation of DNA synthesis. Furthermore, dedifferentiation leads to a breakdown of myofibrils and a fragmentation of the multinucleated muscle cell into mononucleated cells (N), which contribute to the blastema (Bl) (reprinted from [9], p. 558, with permission from Elsevier).

with autoradiographic studies using tritiated thymidine. By tracing labeled cells, Hay and Fischman could provide the first direct evidence, that blastema cells in regenerating limbs of the newt indeed originate from the dedifferentiating internal tissues and not from the apical limb epidermis (15,16).

To date, a contribution of reserve stem cells to the blastema has never been ruled out, but there was no direct evidence for such a mechanism (7). Very recently, Morrison et al. identified a Pax7-positive satellite cell population, separated by a basement membrane adjacent to newt myofibers (17). These Pax7-positive cells were quiescent in an uninjured limb but became mitotic after amputation. Furthermore, Pax7-positive cells were found in the early blastema, suggesting that these satellite cells contribute to the regenerating limb. Nevertheless, dedifferentiation of cells at the wound site appears to be the crucial cellular response to injury that initiates blastema formation and here, we will focus only on the process of dedifferentiation.

A key question is how does injury initiate the regeneration response and what are the molecular

events occurring inside and outside the cells? Although experiments following labeled cells or grafts in animals after injury have provided insight about tissue behavior at the amputation plane, very little is yet known about the process of regeneration at the molecular and cellular levels, and no factor responsible for the initiation of dedifferentiation has been identified so far.

Reversal of the differentiated state

The process of cellular dedifferentiation during amphibian regeneration is thought to require two events in a cell: the loss of a differentiated phenotype and the reacquisition of proliferative capacity. Here, we will discuss three examples where a mature cell fulfills at least one these criteria during regeneration. The two definitive examples of cellular dedifferentiation are the transdifferentiation of pigmented epithelial cells of the iris or retina into lens and the dedifferentiation of muscle during limb and tail regeneration (3,18). In both cases, cells lose their differentiated phenotype to produce proliferating cells that contribute to the regenerated structure. In a third case, newt cardiomyocytes are able to proliferate in response to injury of the heart, but seem to retain other characteristics of a differentiated cell.

Cardiac muscle regeneration

In contrast to mammals, newts are able to functionally replace 30–50% of the heart ventricle after excision. Removal of ventricular tissue strongly increased DNA synthesis and mitosis in cells adjacent to the wound (19,20). Autoradiography using tritiated thymidine labeling along with electron microscopy failed to discover a reserve of undifferentiated satellite cells, but clearly identified differentiated cardiomyocytes as contributors to the regenerating heart ventricle (21–23).

Using primary cultures of adult newt cardiac muscle, a recent study revealed the heterogeneity of these cardiomyocytes with respect to proliferation. Interestingly, as with muscle and also pigmented epithelial cells, they are stimulated to reenter cell cycle by fetal bovine serum (FBS) in a dose-dependent manner. Although the majority can enter S-phase, only one-third of the cells traverse mitosis to then undergo additional rounds of cell division. Furthermore, cell cycle reentry is associated with phosphorylation of the retinoblastoma protein and injection of a plasmid encoding human cyclin-dependent kinase (CDK) inhibitor $p16^{INK4}$ into cultured cardiac myocytes decreased S-phase reentry approximately 13-fold (24). However, the identity of the serum factor that stimulates cardiomyocytes is not yet known.

Regeneration of the retina and lens

Amazingly, when the lens is removed from a larval or adult urodele (lentectomy), it can be regenerated by adjacent tissue. Amphibians differ greatly in their ability to regenerate a lens and in the source of cells that form the new lens. While salamanders of the family *Ambystomidae* are able to grow a new lens from a fragment of the old lens, some members of the family *Salamandridae* have the remarkable ability of growing new lens from the dorsal iris (1).

Regeneration of the lens from the dorsal iris (Wolffian regeneration) has been examined more extensively than any other type of lens regeneration. After removal of a newt lens, a defined population of dorsal iris cells loses their pigmentation, and then begins to divide to form a bulge in the epithelium. Over the next weeks, these cells continue to divide until they form a sphere of cells that eventually expresses the characteristic crystalline lens (18). These transitions have been reproduced in vitro by Eguchi and his colleagues, who have demonstrated that the depigmenting drug, phenylthiourea, and also the basic fibroblast growth factor (FGF) are required to induce retinal pigmented epithelial cells of the chick embryo to form lens in vitro (25). Furthermore, it was recently shown that active thrombin and a yet unknown thrombin-generated activity in serum are involved in this transition as these pigmented epithelial cells, like newt A1 myotubes, respond in culture to a thrombin-activated serum factor and reenter S-phase (26).

Regeneration of skeletal muscle

Studies of regenerating limbs or tails have revealed enormous tissue reorganization near the wound site. These histological changes affect all tissues in the regenerating stump; however, the most experimentally accessible example is the multinucleated skeletal muscle cell. Therefore, muscle has become the most intensively studied cell type for understanding regeneration at the cellular and molecular levels.

The first proposal that dedifferentiation of multinucleated muscle fibers at the wound site might contribute to the developing blastema came from Thornton and was confirmed by Elizabeth Hay's elegant electron microscopy observations already 40 years ago (9,11). In longitudinal sections of a regenerating axolotl limb, Hay interpreted changes of the nuclear shape in myofibers, from a normally elongated to an enlarged and rounded nucleus, as the beginning of DNA synthesis (Fig. 3). In addition, myofibrils disappeared and the syncytial fibers broke up into individual cells during dedifferentiation (9). However, this conclusion was derived from static

pictures and one could argue as well that the reverse process was occurring, that of cells fusing to form a muscle fiber.

Direct experimental evidence for the dedifferentiation theory of muscle cells was provided by implantation of labeled myotubes into a regenerating newt limb (27). Cultured newt limb myotubes were selectively microinjected with the lineage tracer rhodamine-dextran and introduced into regenerating limbs. One week after implantation, accumulation of rhodamine-dextran-labeled mononucleated cells was seen, strongly suggesting that the labeled myotubes had fragmented. Furthermore, these mononucleated cells were found to proliferate, as they were double-labeled with the cytoplasmic lineage tracer and ^{3}H-Thymidine that had been incorporated into the nucleus. In contrast, dedifferentiation of newt myotubes, left in cell culture, was never observed, suggesting a specific environment for reversal of the differentiated state in the regenerating limb. In later work, these experiments were more elegantly repeated using retrovirus-labeled implanted myotubes (28).

Although the experiments by Lo and Kumar et al. strongly supported the reversal of differentiation during the course of regeneration, it remained unclear whether endogenous muscle fibers close to a wound can undergo dedifferentiation and contribute to the mass of proliferating blastema cells. This was demonstrated by labeling of single muscle fibers in axolotl tail (29). Specifically, a single fiber was labeled by pressure injection of rhodamine-dextran, followed by distal amputation of the tail close to the labeled fiber. Dedifferentiation of mature muscle fibers occurred between 3 and 5 days after an amputation by the synchronous fragmentation of the multinucleate muscle fiber into mononucleated cells followed by rapid proliferation of these cells. Remarkably, in addition to amputation or severe tissue damage, a direct clipping of the muscle fiber was required to initiate this process. Based on these experiments, it was calculated that about 17% of the blastema cell mass derives from muscle dedifferentiation alone (30). In parallel, similar observations were made using isolated myofibers from axolotl limb and dissociated in culture. When striated myofibers were labeled with a cell tracker dye and then implanted back into the environment of a limb blastema, many examples of labeled mononucleated cells were observed in the regenerating limb 2–4 days later (31).

Aside from fragmentation of multinucleated cells, the second characteristic of dedifferentiating muscle cells is the reentry from a postmitotic state into a cell cycle accompanied by DNA replication. Studies with tritiated thymidine both in vivo (15) and with implanted myotubes (28) indicated that DNA synthesis occurs in the multinucleated myotube before fragmentation. Using injection of tritiated thymidine into regenerating limbs, followed by fixation of these tissues, Hay and Fishman found, in sections of the regenerating tissue, clear evidence for thymidine incorporation in polynucleated muscle fibers as early as the fourth day after amputation. However, between 10 and 20 days, incorporation was seen more commonly in rounded nuclei of mononucleated muscle fragments derived from the syncytial muscle fiber.

Similar results were obtained by implanting retrovirus-labeled cultured myotubes into the environment of a regenerating limb using incorporation of bromodeoxyuridine (BrdU) as a marker for DNA synthesis. After injection of BrdU into regenerates 9 days after implantation followed 24 h later by fixation and analysis, all the nuclei in several retrovirally labeled myotubes were found to be BrdU-positive, indicating S-phase reentry of these myotubes (28).

Neither of these studies addressed the issue of whether cell cycle reentry and budding are independent from each other or if both linked in one pathway. The first support for autonomous mechanisms came from implantation of cultured newt myotubes, where S-phase reentry was irreversibly blocked. When myotubes, inhibited from progressing through the cell cycle by X-irradiation or transfection with the cell cycle inhibitor $p16^{INK4}$, were implanted into the regenerating newt blastema, fragmentation still occurred (32).

Additional evidence for the independence of S-phase reentry and fragmentation was obtained from isolated and dissociated myofibers of axolotl. When dissociated, many of these myofibers underwent breakage into viable multinucleated fragments or even mononucleated cells (cellularization). However, labeling with tritiated thymidine did not reveal any S-phase reentry of myofibers up to 48 h after isolation, even in myofibers, showing clear signs of fragmentation and cellularization (31). Therefore, cell cycle progression and fragmentation of myofibers are likely independent events controlled by at least two distinct signaling pathways during regeneration.

MOLECULAR STUDIES OF DEDIFFERENTIATION IN SKELETAL MUSCLE CELLS

With the studies described earlier, the phenomenon of dedifferentiation was established, and the cellular events that occur during dedifferentiation

TABLE 1. *Summary of growth factor, serum, and blastema extract sensitivity of mammalian (mouse C2C12) and newt (A1) cells*

		Proliferate response		Proliferation + Fragmentation
Cell type	Cell form	Serum*	PDGF†	Blastema extract
Mouse C2C12	Mononucleates	+	+	na
	Myotubes	–	–	+
Newt A1	Mononucleates	+	+	na
	Myotubes	+	–	+

Myotubes and mononucleates were assayed for BrdU incorporation in response to growth factors such as PDGF, serum from various species, or blastema extract from a regenerating newt limb (35,36).

*Other tested sera were from fetal bovine, adult bovine, sheep, porcine, chicken, and human.

†Other tested growth factors were bFGF, EGF, IGF-1, and keratinocyte growth factor.

na, not applicable.

were defined. A key aspect of understanding regenerative ability will be to integrate this cellular understanding of the dedifferentiation process with a molecular understanding. What extracellular molecules are generated at the amputation site that trigger dedifferentiation to occur? How are they induced by injury? To study dedifferentiation on a molecular level, we and others have examined molecular factors that can induce the newt myotubes to dedifferentiate in vitro.

Newt A1 cells

In order to study muscle dedifferentiation of urodeles in vitro on the cellular level, a myogenic cell line derived from the red-spotted newt (*Notophthalmus viridescens*) limb has been commonly employed. These A1 cells were originally isolated from cultures of normal limb tissue and can be propagated without any sign of senescence for over 1 year in culture (33). Moreover, upon serum reduction (0.5% fetal calf serum) A1 mononucleated cells exit the cell cycle, fuse to form a polynucleated syncytium and start to express late markers of muscle differentiation, such as myosin-heavy chain, troponin T, myogenin, and myoD. Functionally, these differentiated myotubes become contractile in response to mechanical stimulation but, on the other hand, fail to display other features typically found in mature myofibers such as striation and peripheral alignment of nuclei. In contrast to plating at a high density, when few cells are cultured on substrates such as fibronectin, vitronectin, or laminin, newt A1 myotubes usually spread and form large myosacs containing one or more clusters of 5–10 nuclei.

Serum induces cell cycle reentry in newt A1 myotubes

To address what molecules trigger cell cycle reentry and proliferation in newt A1 myotubes, we have screened through a large number of known growth factors for their activity on newt myotubes. When newt A1 myotubes were exposed to common mitogens such as bFGF, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), or epidermal growth factor (EGF), they behaved similarly to myotubes from other animal species. They remained withdrawn from cell cycle while their mononucleate precursors were responsive to these factors. In contrast, treatment of the newt myotubes with elevated serum concentrations induced the cells to undergo cell cycle reentry and complete S-phase (34) while mammalian cells remained refractory (Table 1).

As demonstrated in a number of experiments described further, this DNA synthesis is not an artifact but leads to accumulation of myotube nuclei with 4 N DNA without any signs of apoptosis. Two days after serum addition, DNA synthesis in myotubes starts asynchronously with a peak of BrdU incorporation around day 4. A window of serum stimulation for as little as 8 h is sufficient to generate this response. When the magnitude of BrdU uptake into myotube nuclei was judged against normal proliferating mononucleated cells, both incorporation rates were comparable, and therefore activation of DNA repair machinery seems unlikely. Moreover, pulse chase experiments using tritiated thymidine and BrdU revealed that S-phase lasts about 48–72 h in the myotube nuclei, which is similar to the length of S-phase in mononucleated cells of newt limb (37,38). Finally, quantification of DNA content in nuclei of stimulated and nonstimulated myotubes suggests the traversal of a complete S-phase with an exact doubling of DNA content in each nucleus.

In order to develop an assay for the identification of the serum factor, we investigated the linearity of the myotube response to serum. Increasing concentrations of FBS displayed a dose-dependent response with a linear range from 5 to 20% positive myotubes using a BrdU pulse of 8 h. Concentrations higher than 20% serum led to a loss of linearity, and finally,

to a saturation of the myotube response at 25–30% BrdU-positive myotubes (34). Cumulative BrdU labeling resulted in 80% of the myotubes reentering S-phase.

Interestingly, the S-phase reentry of myotubes is sensitive to contact inhibition. When purified myotubes were plated within high- and low-density areas of mononucleated cells on the same dish, serum stimulation and BrdU labeling resulted in a high myotube response in the low-density environment compared with the high-density side (39). This indicates that, in addition to stimulation of soluble factors in serum, a loss of cell–cell contact may required for the cell cycle response of myotubes. It is also consistent with the in vivo finding, that myofibers require direct clipping for fragmentation (30).

Intracellular pathway of cell cycle reentry

One striking consequence of stimulation with serum is the phosphorylation of an intracellular cell cycle regulator, the retinoblastoma protein (pRb) that regulates progression through the G1 to S transition by complexing with the E2F family of transcription factors. The phosphorylation of pRb, by the action of the CDK 4 or CDK 6, releases members of the E2F family from this inhibitory pRb complex, allowing them to control the expression of several genes required for entry into S-phase (40,41).

In order to determine whether phosphorylation is required for cell cycle reentry, newt myotubes were injected with plasmids encoding the cell cycle inhibitor p16 or a pRb mutant. When human p16, which specifically inhibits the CDK 4 or CDK 6, was expressed in newt myotubes, no DNA synthesis was observed compared to control injections. More direct evidence for the central role of pRB phosphorylation was derived from injection with the pRb mutant Δ34 Rb, in which all eight CDK consensus phosphorylation sites had been mutated. Overexpression of Δ34 Rb competes with wild-type pRB for binding to E2F members as well as CDK 4/6 and therefore prevents all actions that require phosphorylation of endogenous pRB. Injection of Δ34 Rb into the newt myotubes led to an increased inhibition of DNA synthesis in myotubes compared to control injections, suggesting that phosphorylation of pRb is a critical step in cell cycle reentry of these cells (34).

Thrombin promotes cell cycle reentry during dedifferentiation

We have been pursuing the identity of the serum factor that initiates cell cycle reentry in newt myotubes, as it could provide the first molecular insight for understanding dedifferentiation. Serum represents the soluble fraction of blood after coagulation, which involves a proteolytic cascade terminating with thrombin cleaving fibrinogen to form the fibrin clot. Considering the primary role of thrombin in blood clotting, we examined the relationship between thrombin proteolytic activity and the myotube cell cycle-inducing activity. Indeed, thrombin was already known to function not only in fibrinogen cleavage, but also in activating platelets and fibroblasts by cleaving a specific G-protein-coupled receptor (42).

To determine if thrombin could directly induce myotube cell cycle reentry, pure thrombin was assayed on myotubes in differentiation media containing low levels of serum (1.5%) and in serum-free media (1% bovine serum albumin). Strikingly, only myotubes in 1.5% FBS reentered the cell cycle, indicating that thrombin-mediated reentry requires subthreshold concentrations of serum. This result suggested two possibilities: either thrombin acts directly through cleavage of a thrombin receptor (43,44) but additionally requires a second serum-derived growth factor, or thrombin acts indirectly by cleaving molecules in serum that then, in turn, act on myotubes. To distinguish between these possibilities, we determined if the cells needed to be exposed to active thrombin, or whether preincubation of the 1.5% serum with thrombin was sufficient to induce a cellular response (35). A medium containing 1.5% serum was incubated with pure thrombin. After 24 h of incubation with thrombin, the sample was then treated with D-Phe-Pro-Arg-chloromethylketone (PPACK) to completely inhibit thrombin protease activity before addition to cells. As a control, thrombin was inhibited with PPACK before incubation with 1.5% serum. In these experiments, the incubation of serum with thrombin prior to addition to cells was sufficient to induce cell cycle reentry. This indicates that thrombin proteolysis generates a downstream factor that induces cell cycle reentry in the newt myotubes (Fig. 4A). A similar (30) result was obtained when serum was incubated with the serum protease plasmin using, in this case, α2-antiplasmin for inhibition. Other proteases that were tested in similar assays were found to be negative, including trypsin, Factor Xa, Protein Ca, Factor IX, and Factor XII (35).

This characterization of thrombin activity on the newt myotubes allowed us to integrate the in vivo observations that thrombin is present and important for initiating regeneration, with a mechanistic insight that it induces cell cycle reentry in differentiated cells. Indeed, the exposure of newt iris pigmented epithelial cells to the thrombin-activated serum factor in culture also induces cell cycle reentry into these cells, again linking the in vivo observations with

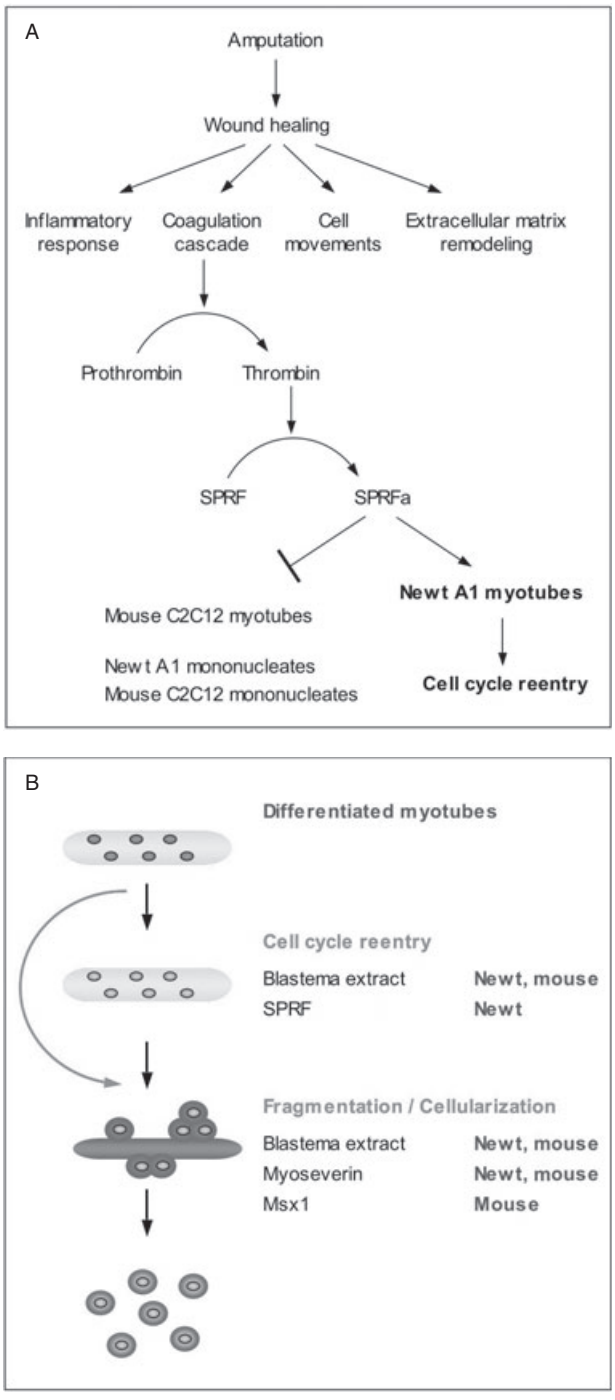

FIG. 4. Schematic diagrams of the S-phase reentry model and summary of cellular dedifferentiation effects observed in vitro on newt and mouse myotubes. (A) Schematic diagram of the activation of S-phase reentry by newt A1 myotubes in the context of the wound healing responses that leads to regeneration: Amputation of the limb triggers multiple responses such as inflammation, remodeling of the extracellular matrix, and cell migration. A major aspect of wound healing is activation of the conversion of prothrombin to thrombin. In mammals, this induces the conversion of fibrinogen to fibrin polymers and formation of a clot. In newt, thrombin activation, in addition, leads to cell cycle reentry from the differentiated state. This involves the conversion of a latent activity (SPRF to SPRFa) within blood (or serum), which can selectively stimulate newt A1 myotubes, but not their mononucleate precursors to undergo S-phase. Although the SPRFa activity was found in all animal sera tested so far and hence is likely to be a general product of thrombin activation, mouse C2C12 myotubes were refractory to this activity (adapted from 35). (B) Schematic diagram of muscle dedifferentiation in vitro—comparison of newt A1 and mouse C2C12 myotubes: in vitro an extracellular proteinaceous factor found both in serum and newt limb blastema extract is capable of pulling newt A1 myotubes out of G0 and allowing them to progress through S-phase, where they become arrested in a 4 N state. The G1-S transition is mediated through the phosphorylation of the pRB. In contrast to newt myotubes, mouse C2C12 myotubes can be stimulated to reenter cell cycle by newt limb blastema extract but not by the serum factor. Aside from cell cycle reentry, a second characteristic for dedifferentiation of muscle cells is the fragmentation of a multinucleated cell into mononucleated derivatives referred as to cellularization. Newt limb blastema extract and the microtubule-destabilizing drug myoseverin are capable of inducing fragmentation as well as cellularization in both newt A1 and mouse C2C12 myotubes. Furthermore, it was shown that overexpression of Msx1, a homeobox-containing transcriptional repressor, in mouse C2C12 myotubes causes fragmentation and even cellularization. Interestingly, isolated and dissociated myofibers from axolotl limb can undergo cellularization concomitant with the expression of Msx1, but without any signs of S-phase reentry. The curved arrow indicates that cell cycle reentry and fragmentation are thought to be independent events, as cell cycle reentry is not a requirement for fragmentation and the formation of viable mononucleated cells. Factors responsible for inducing myotubes to undergo DNA-synthesis, fragment, divide, and eventually redifferentiate are still unknown (30).

in vitro activities (26). Furthermore, these results indicate that the thrombin-activated serum factor is required to initiate regeneration in multiple contexts.

In vivo evidence that selective activation of thrombin is a critical event for limb and lens regeneration in vertebrates

In vivo evidence that thrombin activation is involved in initiating dedifferentiation came from localizing active thrombin in histological sections of regenerating tissue. This was performed by overlaying sections with membranes infused with a fluorogenic thrombin substrate. Thrombin proteolytic activity was observed exclusively in the blastema at the tip of the 8-day regenerating limb (35). By this time point, the cells of the tissue close to the amputation site had dedifferentiated and contributed to the forming blastema. Moreover, this signal was completely inhibited by inclusion of PPACK, an irreversible inhibitor of thrombin.

Studies of lens regeneration have provided further compelling in vivo evidence for the role of thrombin in initiating regenerative events. In adult newts, lens regeneration only takes place at the pupillary margin of the dorsal iris, where pigmented epithelial cells reenter the cell cycle and transdifferentiate into the lens (45). However, in cell culture, pigmented

epithelial cells both from dorsal and ventral iris possess the capacity to transdifferentiate, suggesting a missing stimulus in the ventral part of the iris after lens removal (46). Recently, Imokawa and Brockes demonstrated the transient and selective activation of thrombin at the dorsal margin in the newt eye after lentectomy is absolutely required for the activation of pigmented epithelial cells to undergo these regenerative events (47). Membrane overlay assays showed that after lentectomy, active thrombin is selectively localized to the dorsal pupillary margin. Furthermore, injection of the irreversible thrombin inhibitor, PPACK, into the eye strongly delayed lens regeneration. These results indicate that the selective activation of thrombin is a fundamental signal linking tissue injury to the initiation of regeneration in vertebrates.

The role of thrombin in this aspect of dedifferentiation of cells provides an interesting link between injury, the initiation of blood coagulation, and the local activation of factors for regeneration of missing tissue in urodeles (Fig. 4A) (48,49). It is also in agreement with experimental evidence, that injury alone elicits dedifferentiation and cell cycle reentry of cells (50,51).

Purification of a serum factor that triggers cell cycle reentry in newt A1 myotubes

A major goal in our laboratory has been to molecularly identify the thrombin-activated serum factor through classical biochemical approaches using the in vitro newt myotube cell cycle reentry assay. In an initial characterization of the activity in bovine serum, neither delipidation nor dialysis against a membrane with a molecular weight cut off between 6000 and 8000 Da abolished the activity. Gel filtration on Superose-12 suggested that the native molecular weight of the factor in serum is 150 000–300 000 Da (34). Furthermore, the activity appeared rather robust, as it was resistant to denaturation by sodium dodecyl-sulfate (SDS). The activity found in serum is always referred to as cell cycle reentry factor or S-phase reentry factor (SPRF).

S-phase reentry activity in crude bovine thrombin

In order to find a suitable starting material for the characterization and purification of the cell cycle reentry factor, several crude fractions derived from plasma or serum were tested. While, for example, platelet lysate, a potent source of growth factor activities, did not contain any detectable amount of activity, another plasma derivative enriched in thrombin contained significant amounts of SPRF activity. This commercially available crude bovine thrombin preparation was prepared from plasma and in addition to high concentrations of active thrombin, also contained at least 20-fold more cell cycle reentry activity per unit protein compared with bovine serum (35).

TABLE 2. *Characterization of S-phase reentry activity from serum and crude bovine thrombin, fractionated on Q-sepharose, on newt A1 cells*

	A1 Mononucleates	A1 Myotubes	A1 Myotubes +PPACK
FBS	+	+	+
CB-Thrombin	+	+	+
Q-FT	+	–	na
Q-100	–	+	–
Q-400	–	+	+

Starting material (crude bovine thrombin, CB-Thrombin) and fractions of the HiTrap Q column (flow through, Q-FT; 100 mM eluate, Q-100; and 400 mM eluate, Q-400) were added to mononucleated A1 cells and myotubes. Starting material and flow through both stimulated mononucleates, whereas with myotubes, stimulation was found for all fractions except Q-FT. However, when HiTrap Q fractions were treated with PPACK for inhibition of thrombin, S-phase reentry activity of myotubes was abolished in the Q-100 fraction. FBS was used as a control (39).

na, not applicable.

When crude bovine thrombin was applied to a HiTrap Q anion exchange column and bound proteins eluted with a linear gradient of NaCl, the activity fractionated as two distinct peaks at 100 mM (Q-100) and 400 mM NaCl (Q-400), respectively. Additional chromatographic fractionation of the Q-100 fraction on a HiTrap SP cation exchange column displayed one major peak, containing mostly α, β and γ forms of thrombin, eluting at 500 mM NaCl. Furthermore, the treatment of fractions with PPACK, a potent irreversible inhibitor of thrombin protease activity (52), completely abolished cell cycle reentry activity in the Q-100, but not in the Q-400 fraction when assayed in the medium containing 0.5% serum (Table 2). This data demonstrated that the activities in these two fractions are distinct and that the activity in Q-100 is linked to active thrombin, while the Q-400 fraction is linked to the downstream SPRF activity.

Purification of SPRF from crude bovine thrombin

In initial tests of the activity present in the crude bovine thrombin preparation, the SPRF activity was characterized as a thermolabile, proteinase K-sensitive glycosylated high-molecular-weight protein (53). It is resistant to denaturing conditions such as SDS, 8 M urea, high pH, and organic solvents such as ethanol or acetonitrile. Interestingly, under denaturing conditions, the serum factor behaves as a

low-molecular-weight protein hat displays charge heterogeneity on isoelectric focusing. After five column chromatography steps—cation exchange, hydrophobic interaction, heparin affinity, as well as size exclusion and anion exchange chromatography under denaturing conditions—we have achieved a 2600-fold purification starting from a commercially available crude bovine thrombin preparation (unpublished data). This represents about 50 000- to 100 000-fold purification over bovine serum. Silver-staining gels of the most purified fractions revealed 10 major protein bands. Based on the quantification of these gels and the amount of fractions assayed on newt A1 myotubes, the serum factor is thought to be a very potent growth factor activity, which acts at 1 ng/mL or even lower. We are currently pursuing a quantitative mass spectrometry approach in order to finally identify the S-phase reentry activity. Having this molecule in hand would be a first key for understanding the cellular and molecular differences between urodele amphibians and mammalians in response to injury and the regeneration of a missing body part.

OTHER FACTORS INVOLVED IN DEDIFFERENTIATION

A blastema protein extract induces cell cycle reentry and fragmentation of cultured newt myotubes

While newt myotubes clearly complete S-phase, neither mitotic figures nor budding were observed in culture, even up to 10 days after serum application, suggesting that the nuclei arrest in the G2-phase of the cell cycle. This also supports the theory of a second pathway in these myotubes, whereby other soluble factors provided by nerves and/or the wound epithelium or special requirements of the environment, such as signals from the extracellular matrix, are necessary for the full dedifferentiation of muscle cells and the formation of proliferating mononucleated blastema cells (51,54).

The budding activity has been observed in three situations, exposure of myotubes to blastema extracts, to pharmacological agents that disrupt the microtubule network, or by overexpression of the transcription factor, Msx1. In characterizing the blastema extract, Odelberg et al. found budding of myotubes into smaller myotubes or even proliferating mononucleates (36). Almost no evidence for cellular dedifferentiation was found when myotubes were treated with extracts from nonregenerating limbs, suggesting an activation of a factor or a set of factors after limb amputation, capable of inducing both DNA synthesis and cellularization (55). It is not yet understood whether the activities in serum and blastema extract act through similar mechanisms on myotubes. Compared to serum, blastema extract is a mixture of proteins and other biomolecules from several tissues and blood. Therefore, it seems likely that the extract might contain two factors, one essential for fragmentation and another for S-phase reentry.

Myoseverin, a microtubule destabilizing agent, causes fragmentation of cultured myotubes

The microtubule-binding molecule myoseverin was identified from a library of purine derivatives in a morphological differentiation screen using a mouse C2C12 muscle cell line, where it induced reversible fission of multinucleated myotubes into mononucleated cells. Myoseverin was also shown to act on newt A1 myotubes with similar cellularization effects (56). These results suggest that local depolymerization of microtubules could be a significant event in the pathway leading to cellularization of multinucleated myofibers.

Recently, another line of evidence for the importance of microtubule disassembly during fragmentation derived also from studies where cultured striated myofibers from axolotl limbs were treated with taxol. Typically, after isolation and dissociation, most untreated myofibers (80%) displayed morphological signs of fragmentation or even cellularization without S-phase reentry in the culture. However, when these cultures were exposed to the microtubule-stabilizing agent taxol, this number decreased to 16% (31).

Interestingly, myoseverin also induces changes in myogenic regulatory factor expression. Imokawa and his colleagues found an up-regulation of Myf5 associated with A1 myotube formation in culture as well as with myofibers in the newt. Conversely, stimulation with serum or treatment with myoseverin led to the down-regulation of Myf5 in cultured newt myotubes (56).

Msx1 and dedifferentiation of newt A1 muscle cells

Over the last few years, one molecule (Msx1) has emerged as the focus of several investigations into the mechanism of muscle dedifferentiation. This protein, a homeobox-containing transcriptional repressor, is expressed in rapidly proliferating mesenchymal cells during normal limb development. In studies of digit tip regeneration in fetal and newborn mice, Reginelli and colleagues demonstrated a restriction of the regenerative ability of mouse digit tips to an area where Msx1 is expressed (57). This led to the hypothesis that expression of Msx1 (and Msx2)

might be crucial for digit cells to take part in a regenerative response. The first direct evidence for an involvement of Msx1 in dedifferentiation came from a study of the temporal expression pattern in regenerating newt limbs. Msx1 was found to be strongly up-regulated during the initiation of regeneration, remained expressed throughout regeneration, but was not detectable in the fully regenerated limb, suggesting a correlation between the undifferentiated state and the expression of Msx1 (58).

Recently, functional evidence for the importance of Msx1 was provided using isolated muscle fibers from larval axolotls. Dissociated single fibers underwent fragmentation or even cellularization, and this coincided with the selective appearance of Msx1 mRNA and protein. Furthermore, the uptake of Msx1 antisense morpholinos from the culture medium caused a significant decrease in the expression of Msx1 protein and a marked inhibition of fragmentation (31). Taken together, these results suggest a key role for Msx1 during regeneration. However, no study has yet addressed whether extracellular signals, such as the factors present in serum or the blastema extract, lead to elevated levels of Msx1 expression in newt A1 or mouse C2C12 myotubes. A summary of the factors acting on newt and mouse myotubes that lead to cell cycle reentry and also the generation of mononucleated cells is presented in Fig. 4B.

CAN MAMMALIAN MYOTUBES DEDIFFERENTIATE?

Mouse C2C12 cells

From a tissue engineering perspective, it is important to establish whether mammalian cells have the potential to dedifferentiate or if this capacity has been irreversibly lost. For comparison of mammalian to newt muscle cells in the context of regeneration, a mouse myogenic cell line, C2C12, has been traditionally used. Mouse C2C12 muscle cells are a diploid, continuous cell line originally isolated by Yaffe from the thigh muscle of a 2-month-old C3H mouse (59). These cells were later subcloned and referred then as C2C12 (60). C2C12 cells are maintained as undifferentiated myoblasts in medium containing 20% fetal calf serum. To induce myoblast fusion and the formation of multinucleates cells, the culture medium is changed to 2% horse serum. This is associated with the expression of muscle markers such as myosin-heavy chain, troponin T, myogenin, and myoD (36). Within 2–3 days after the onset of cell fusion, muscle fibers exhibit spontaneous contraction (59). Very recently, it was reported that C2C12 cells are even able to form highly differentiated, contractile myotubes with peripheral nuclei and adult fast myosin expression. C2C12 coculture on a fibroblast monolayer resulted in a well-defined mature sarcomeric structure and a response to electrical stimulation comparable to mature myotubes (61). This suggests an important influence of the extracellular environment on differentiation and likely on dedifferentiation.

Cell cycle reentry in mouse C2C12 myotubes

In comparison to newt A1 myotubes, mouse myotubes are not stimulated to undergo S-phase reentry by elevated serum. When mouse C2C12 myotubes were generated and purified in a similar manner, no BrdU incorporation was found up to 72 h after serum stimulation (34). This significant difference in response to the serum factor might be one basis for the inability of mammals to achieve the regenerative potential of salamanders. In contrast, mononucleated mouse cells responded normally to serum. Table 1 summarizes the comparison of myogenic mouse C2C12 and newt A1 cells. It is unclear as yet, whether the lack of response derives from a missing or unresponsive receptor after muscle differentiation on the cell surface, an intracellular block of the S-phase pathway or an obstruction within the nucleus at the chromatin level.

Nevertheless, terminally differentiated C2C12 myotubes are not totally unresponsive to extracellular, proliferative signals. Incubation with serum led to an up-regulation of immediate-early genes such as c-*fos*, c-*jun*, c-*myc*, and *Id*-1, indicating that these cells are not confined to G0 but can partially traverse G1 (62). Moreover, the capability of C2C12 myotubes to undergo DNA synthesis was demonstrated by transfection with viral proteins such as SV40 large T antigen. Expression of this oncogene, which binds to the unphosphorylated form of the retinoblastoma protein, forced myotubes to reenter the cell cycle. Moreover, the pRb became rephosphorylated upon stimulation with serum in myotubes expressing SV40 large T antigen but not in nontransfected C2C12 myotubes (63). This suggests a link between the ability for cell cycle reentry and phosphorylation of pRb. However, like most retroviral oncogenes, the SV40 T antigen has no known cellular counterpart. The critical role of pRB was verified using cultured skeletal muscle cells derived from an Rb –/– mouse. In contrast to their wild-type counterparts, these Rb –/– myotubes reentered the cell cycle and synthesized DNA (64).

Very recently, Camarda and colleagues reported that terminal proliferation arrest is maintained in skeletal muscle cells by a pRb-independent

mechanism. In contrast to the earlier work, they used conditional knockout myotubes. In this case, excision of pRb after complete differentiation of myotubes caused reexpression of cell cycle regulators and down-regulation of muscle-specific genes, but did not trigger DNA synthesis. Further investigation revealed the presence of a second pocket-protein independent block in myotubes, which could be relieved by cyclin D1/CDK 4 coexpression (65).

Finally, evidence for the potential of mouse myotube nuclei to undergo DNA-replication in response to serum was obtained from the formation of interspecies hybrid myotubes by fusing mouse C2C12 and newt A1 myogenic cells (66). As expected under these conditions, C2C12 homokaryons remained arrested. In contrast, C2C12 nuclei in hybrids reentered the cell cycle upon serum stimulation, indicating that a pathway activated in newt cytoplasm can overcome the postmitotic arrest. Further experimental evidence supporting this idea came from treatment of mouse myotubes with a blastema extract of a regenerating limb. This latter result will be discussed below in more detail.

Fragmentation and cellularization of mouse myotubes

As their salamander counterparts, mouse C2C12 myotubes were found to be responsive to a newt blastema protein extract. Upon incubation with blastema extract, about 24% reentered cell cycle and underwent DNA replication. Furthermore, the treatment resulted in reduced expression of muscle differentiation proteins such as MyoD, myogenin, and troponin T. Most importantly, about 10% of the polynucleated murine myotubes undergo fission into proliferating cells.

As mentioned previously, the potential of mouse C2C12 cells to undergo fragmentation and cellularization was also demonstrated using a novel microtubule-depolymerizing drug (67). Myoseverin, a 2,6,9-trisubstituted purine, forced differentiated C2C12 myotubes to break into monoculeated cells. Immunoblot analysis after myoseverin treatment with subsequent addition of growth medium identified down-regulation of the differentiation markers Myf5, MyoD, and myosin heavy chain with a concomitant up-regulation of cell cycle proteins, cyclin A, and CDK2, indicating a reversal from the differentiated into a proliferative state. Moreover, this fragmentation occurred in the absence of S-phase reentry (68). This implies once more that cellularization is independent of S-phase traversal and points to a crucial function of myotube microtubule organization for generation of mononucleated progeny.

The fate of the mononucleated progeny after myoseverin treatment or other microtubule-depolymerizing agents has been controversial. Rosania et al. and Perez et al. reported an increase in colony-forming units from myoseverin-treated cultures (67,68). In contrast, Duckmanton et al., who lineage-traced individual myotubes through time-lapse microscopy, reported no proliferation in mononucleate cells derived from myoseverin-treated murine pmi28 myotubes (69). Whether the differences in conclusions derive from differences in cell proliferation assays or differences in cell lines is yet unknown.

Msx1 and mouse C2C12 muscle cells

Another sign for a regulative role of Msx1 in the dedifferentiation of muscle cells was obtained by the ectopic expression of the Msx1 gene in mouse C2C12 myotubes. Overexpression of Msx1 reduced the expression levels of myogenic factors such as MyoD, myogenin, and MRF4. Moreover, expression of Msx1 induced myotube fragmentation with subsequent proliferation and even transdifferentiation into cells expressing chondrogenic, adipogenic, or osteogenic markers upon stimulation (70).

Perspective

Dedifferentiation is a cellular response that occurs in multiple contexts of urodele regeneration, including lens, heart, limb, and tail regeneration. It is therefore a key cellular signature of regenerative ability. In the case of cardiac regeneration, the response is limited to cell cycle reentry with cells maintaining other features of their differentiated state, whereas dorsal iris epithelium and skeletal muscle dedifferentiation involve both cell cycle reentry and loss of differentiated character. In the latter cases, multiple signaling pathways are required to achieve the dedifferentiated state. Of the dedifferentiation activities observed, the serum activity that drives differentiated cells back into S-phase has been the most extensively characterized. The identification of the SPRF would provide an inroad into understanding regenerative ability on the cellular and molecular levels as mammalian skeletal muscle does not respond to the factor. Does the difference in response derive from a missing receptor on mouse myotubes or is there an intracellular block of the signaling cascade? Would ectopic expression of the SPRF receptor in C2C12 mouse myotubes lead to stimulation and subsequent S-phase reentry? What are the targets of the signaling pathway in the newt myotube? Is the identified serum activity a necessary signal for initiating the

regenerative response after wounding in the living newt or axolotl?

Little is known concerning the other factors that must be required for the fragmentation of myotubes into mononucleated cells and their traversal through mitosis. In particular, the interaction between muscle cells and extracellular matrix for dedifferentiation is of particular interest, as mechanical load or partial detachment seems to be necessary for fragmentation and cellularization of myotubes (29,31,50). On the contrary, possible sources for the mitogenic growth factor might be extracellular matrix (71) or nerves (7,72,73). In the latter case, it has long been recognized that denervation prior to or simultaneously with amputation of newt or axolotl limbs prevents proliferation and outgrowth of the blastema, but not dedifferentiation and DNA synthesis of cells. While several candidates for this neurotrophic or mitogenic factor have been proposed, including transferrin, fibroblast growth factors, substance P, glial growth factor, neuregulin, and insulin-like growth factors (7,54,74), only transferrin has been shown to fulfill this function in vitro. The characterization of dedifferentiation factors could serve as a first footstep to solve the mystery of replacing complete body parts after loss and greatly facilitate the understanding of the process of cellular dedifferentiation.

Acknowledgments: We would like to thank David N. Drechsel for helpful discussions and comments on the manuscript.

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