PLASTICITY AND REPROGRAMMING OF DIFFERENTIATED CELLS IN AMPHIBIAN REGENERATION

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Adult urodele amphibians, such as the newt, can regenerate their limbs and various other structures. This is the result of the plasticity and reprogramming of residual differentiated cells, rather than the existence of a 'reserve-cell' mechanism. The recent demonstrations of plasticity in mouse myotubes should facilitate comparative studies of the pathways that underlie the regenerative response, as well as proposing new approaches to promote mammalian regeneration.

URODELE
An order of the class Amphibia, which comprises newts and salamanders, which have elongated bodies, short limbs and a tail.

METAZOAN
Refers to the kingdom Animalia (animals), which comprises ~35 phyla of multicellular organisms.

PHYLOGENY
Evolutionary history that is sometimes represented by the hypothesized ancestor–descendant relationship of a group of organisms.

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The only adult vertebrates that can regenerate their limbs are the URODELE amphibians, of the order CAUDATA. Regeneration of the salamander limb, together with several other examples of urodele regeneration, was first reported by Spallanzani in 1768. The ability to regenerate large sections of the body plan is widespread in METAZOAN PHYLOGENY, and the discovery of this ability was an important aspect of the emergence of experimental biology in the eighteenth century[1]. It provoked intense public discussion on the nature of generation and the basis of individual identity, which are issues that continue to be explored in present-day debates about reproductive cloning. As a problem in molecular cell biology, urodele regeneration provides crucial information about the reversal and plasticity of the differentiated state[2,3]. In addition, limb regeneration is a key system in which to study how positional identity in cells is established[4–7]. Although much work on development and evolution serves to underline the similarities and continuity between different phylogenetic contexts[8,9], it is an all-embracing concern of research on regeneration to understand the relevant distinctions between species that regenerate and those that do not[3,10]. In the case of urodeles and mammals, this takes on a biomedical imperative in view of the current interest in regenerative medicine.

From our mammalian perspective, the ability of an adult newt to regenerate its limbs (FIG. 1a, b) might seem exceptional or even exotic, but it is unlikely that regeneration arose independently in different phylogenetic contexts[3,10,11]. In only six phyla are there no examples of adult regeneration, yet in all contexts in which it is found, there seem to be examples of closely related species that have marked differences in regenerative ability (BOX 1). The hypothesis from phylogeny is that regeneration is a primordial attribute of metazoans that has been lost subsequently for reasons that are not yet understood. In analysing the cellular and molecular mechanisms that underlie the regenerative responses in urodeles, it has been informative to compare them with the mammalian case. This helps to pinpoint the crucial differences between urodeles and mammals, and will shed light ultimately on the evolutionary basis of regeneration.

Regenerative responses in urodeles

An adult newt can regenerate its jaws[12], lens[13], retina[14] and large sections of the heart[15], as well as its limbs and tail, in response to molecular events that signal tissue damage or removal. Although the processes of tissue restoration unfold in a different way in the heart, limbs and tail of an adult newt, in all cases, the outcome seems to depend on the plasticity of differentiated cells that remain after tissue removal.

Plasticity in the heart. After removal of the apical region of the newt ventricle, the heart seals by contraction

around the clot (FIG. 2a). The adult CARDIOMYOCYTES re-enter the cell cycle and divide in a zone that surrounds the clot[15,16]. If the animal is injected with tritiated thymidine — to identify those cells that are in S PHASE — ~10% of the cardiomyocytes in this region are labelled in a one-day period. In comparable experiments with the adult mammalian heart, very few cells label after injury[17]. Thymidine labelling carried out in conjunction with ultrastructural studies has identified cardiac MYOFIBRILS as a marker of cell identity.

Plasticity in the iris. After removal of the newt lens (lentectomy), the reactive population comprises pigmented epithelial cells, which are invariably located at the dorsal pupillary margin of the iris (FIG. 2b). These cells re-enter the cell cycle, lose their pigment granules and convert into lens cells, a process that is referred to as transdifferentiation[18–20] (BOX 2). Elegant clonal cultures of newt pigment-epithelial cells have established conclusively that these cells transdifferentiate into lens[20,21].

Plasticity in the limb. After amputation of the limb at any point along its proximodistal axis (shoulder to fingertip), the wound surface is covered rapidly by epithelial cells, which form the wound epidermis at the end of the stump. In contrast to lens regeneration, which depends on the plasticity of epithelial cells of the iris, in limb regeneration, it is the mesenchymal cells in a zone underlying the wound epidermis that re-enter the cell cycle. The cartilage, connective tissue and muscle cells lose their differentiated characteristics, and become blastemal cells — the progenitor cells of the regenerate. These cells divide to form the blastema, a mesenchymal growth zone that undergoes proliferation, differentiation and morphogenesis to regenerate the limb[22,23] (FIG. 1b).

Extensive evidence indicates that multinucleate newt MYOTUBES and MYOFIBRES re-enter the cell cycle and undergo conversion to mononucleate cells. Such evidence comes from the results of experiments on muscle cells in culture[24–26], cells implanted from culture into a limb blastema[27–29], and observations of live myofibres in a regenerating tail[30], which are all discussed later. In addition, earlier observations of implantation of labelled cartilage also provide strong evidence for reversal of this cell type[31].

In each of these three cases of regeneration, the initial mobilization of differentiated cells occurs within a zone of ~100 μm from the site of tissue removal. It is obviously crucial that such changes do not propagate into the main body of the tissues that are concerned, and the signalling events that link tissue removal with plasticity clearly accomplish this spatial restriction. Although these three cases involve re-entry into the cell cycle, the cardiomyocyte is the only example to retain differentiated function, a feature that is also observed in culture. Although in one sense cell division is necessary to generate the extra cells that are needed for the regenerate, it is less clear how important it is for the events of plasticity and reversal, and this issue needs to be addressed experimentally in each context.

The urodele strategy for regeneration of most structures is therefore the respecification of differentiated cells to a local progenitor cell, rather than a PLURIPOTENT CELL. So, if iris epithelial cells are transplanted to the limb blastema, they give rise to a lens[32,33], and limb blastemas always give rise to a limb after transplantation, even after relocation to the anterior chamber of the eye[34]. This is in contrast to recent findings of the extensive plasticity of stem cells after transplantation in mammals[35] (BOX 2). At present, there is no evidence that adult stem cells can contribute to urodele limb regeneration, although it is not possible to exclude this possibility completely. One advantage of the urodele mechanism might be that it allows the progenitor cells to derive local cues from their differentiated parental cells.

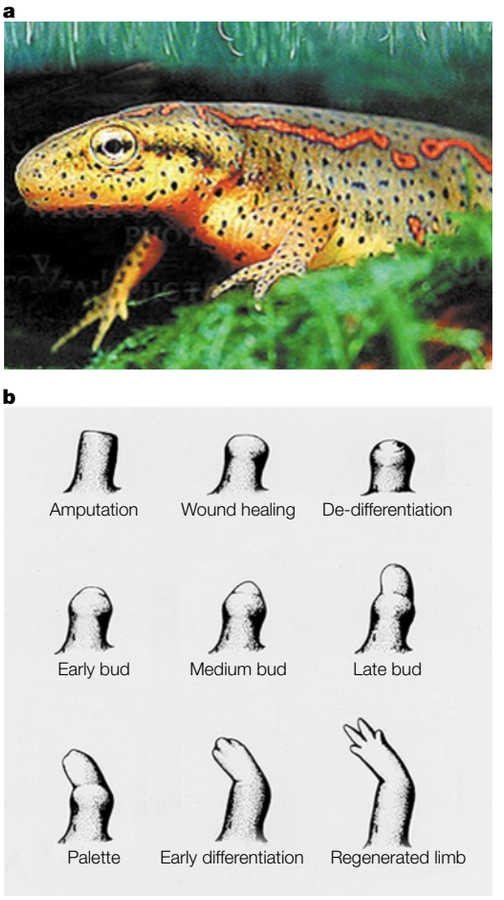


Figure 1 | **Urodele limb regeneration. a** | The North American red spotted newt, *Notophthalmus viridescens*. **b** | Stages of limb regeneration in an adult newt[66]. Reproduced with permission from REF. 66 © 1973 Springer-Verlag.

Plasticity of differentiated cells in mammals?

Plasticity of cellular differentiation provides a convenient cellular assay to compare a differentiated urodele cell with its mammalian counterpart. It is important to recognize that there are examples of regeneration in mammals that do involve plasticity. For example, liver regeneration seems comparable to cardiac regeneration in newts in that the HEPATOCYTES divide without loss of differentiated function[36]. The regeneration of myelinated peripheral nerves requires that SCHWANN CELLS divide and lose

CARDIOMYOCYTE
A muscle cell of the heart.

S (SYNTHESIS) PHASE
The phase of the eukaryotic cell cycle in which DNA is synthesized.

MYOFIBRIL
The structural unit of striated muscle fibres. Several myofibrils make up each fibre.

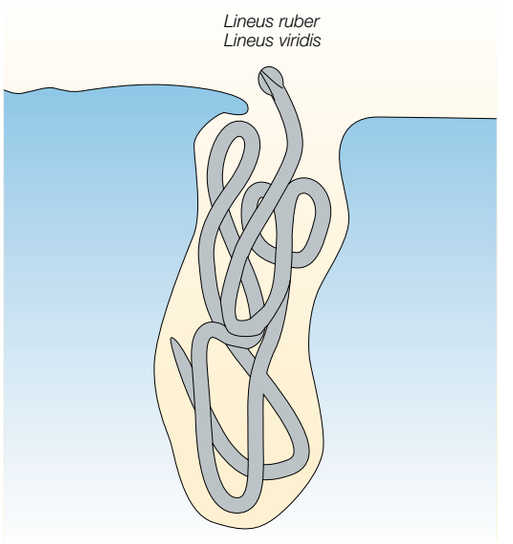
MYOTUBE
The multinucleate structure that is formed by the fusion of proliferating myoblasts and is characterized by the presence of certain muscle-specific marker proteins.

MYOFIBRE
A skeletal-muscle fibre that consists of one long multinucleate cell.

PLURIPOTENT CELL
A stem cell that can give rise to more than one differentiated cell type.

HEPATOCYTES
The parenchymal cells of the liver that are responsible for the synthesis, degradation and storage of a wide range of substances.

Box 1 | Phylogeny and regeneration



There are several phyla, such as ROTIFERS and nematodes, that show cell constancy in the adult, and are not capable of regeneration[61]. Furthermore, the phyla that include examples of regeneration often have marked interspecies variation. *Lineus ruber* and *Lineus viridis* are two species of nemertine worm, which are so similar that it is said to require a specialist to distinguish them. However, *L. ruber* has excellent bidirectional regeneration after transecting the primary body axis, whereas *L. viridis* has no discernible regenerative abilility[62].

It has often been noted that in some invertebrates, the process of asexual reproduction is closely related to regeneration[10,61]. Some animals undergo a process of self-induced fission, after which they regenerate from the fragments. This might be achieved by tearing forces that are generated from opposite movements of parts in some starfish or sea anemones, or even by twisting into several pieces[61]. In TURBELLARIANS and ANNELIDS, fission involves distinct division planes, and in some species, the new organs form before separation — a process that is known as paratomy.

So, what is the most promising system for future studies of regeneration? Although it is important to continue to study several models, two compelling candidates are fin regeneration in zebrafish[63], which has allowed the isolation of several conditional mutants, and PLANARIAN regeneration, in which powerful new approaches have been developed.

ROTIFERA
A small phylum of microscopic multicellular organisms. They have a wheel-like ciliated organ (from which they derive their name) that they use for swimming and feeding.

TURBELLARIANS
A class of platyhelminthes that comprises mostly aquatic and free-living organisms. They have a ciliated epidermis for locomotion and a simple gut.

ANNELID
A segmented worm.

PLANARIAN
Describes free-living members of the invertebrate phylum platyhelminthes.

SCHWANN CELL
A cell that produces myelin and ensheathes axons in the peripheral nervous system.

MYELIN
Proteins that are produced by Schwann cells or oligodendrocytes that cause adjacent plasma membranes to stack tightly together.

MESENCHYME
Immature connective tissue that consists of cells that are embedded in extracellular matrix.

GREEN FLUORESCENT PROTEIN
An autofluorescent protein that was originally isolated from the jellyfish *Aequorea victoria*. It can be genetically conjugated with proteins to make them fluorescent. The most widely used mutant, EGFP, has an emission maximum at 510 nm.

5′ BROMODEOXYURIDINE
(BrdU). A base analogue of thymidine, which is often used experimentally to label dividing cells.

expression of MYELIN before they redifferentiate in conjunction with the regenerating axons[37,38]. It therefore seems to be more closely related than liver regeneration to the general case of the urodele mechanism.

It seems unlikely that the regulation of the differentiated state is fundamentally different in urodeles and mammals. To study this further, we have compared urodele multinucleate skeletal muscle cells with their mouse counterparts. To what extent are the two intrinsically different in terms of regulation of the differentiated state or responsiveness to injury-related signals, or are there distinctive signals in the urodele context that mouse cells never encounter?

Reversal of muscle differentiation

Multinucleate skeletal myotubes are formed by the fusion of mononucleate precursor cells. The myotube enters a state of post-mitotic arrest in which it is entirely refractory to the growth factors that stimulate division of its precursors[39]. The change in cytology — from mononucleate to multinucleate — together with the post-mitotic arrest provides two indices for the reversal of the myogenic phenotype (de-differentiation). Newt A1 cells, which were originally derived from limb MESENCHYME[40], fuse in culture to form multinucleate myotubes, which express markers of late muscle differentiation, such as myosin heavy chain, and are also refractory to protein growth factors[25,40].

Assaying for de-differentiation in the blastema. A1 cells can be stably infected with retroviruses that express genes for alkaline phosphatase or GREEN FLUORESCENT PROTEIN (GFP), so that after differentiation, labelled myotubes can be readily distinguished and purified from any labelled mononucleate cells[27]. Other methods of labelling involve microinjecting the myotubes with a lineage tracer, such as rhodamine dextran[28,29], or labelling them with a lipophilic cell tracker dye[27].

Implantation of labelled myotubes under the wound epidermis of an early limb blastema showed that many were converted to mononucleate cells, which divided and contributed to the regenerate — a process that is referred to here as cellularization[27–29] (FIG. 3a, b). If the myotubes were double labelled in their nuclei and cytoplasm before implantation, the mononucleate progeny derived both markers, which shows that both compartments of the multinucleate muscle cells were conserved after cellularization[27,28].

A second index for the reversal of differentiation is re-entry to S phase by the nuclei in a myotube. After implantation of myotubes into a newt blastema, and injection of the animals with 5′ BROMODEOXYURIDINE (BrdU) to label nuclei in S phase, residual myotubes had several positively stained nuclei. In some cases, all of the nuclei in a myotube were labelled[27]. It is not clear if such myotubes undergo subsequent cellularization after re-entry, but it is clear that the environment of the blastema leads to reversal of both aspects of myogenic differentiation.

Implantation is an extremely useful approach because it enables prior transfection, labelling and purification of the myotubes in culture (FIG. 3b). Importantly, both aspects of reversal that are discussed above have also been observed in endogenous multinucleate muscle fibres after amputation. First, the nuclei within muscle fibres at the site of limb amputation were labelled after BrdU injection into adult newts[27]. The identity of the labelled nuclei was verified after serial sectioning, which showed that they were present in muscle fibres. Second, the

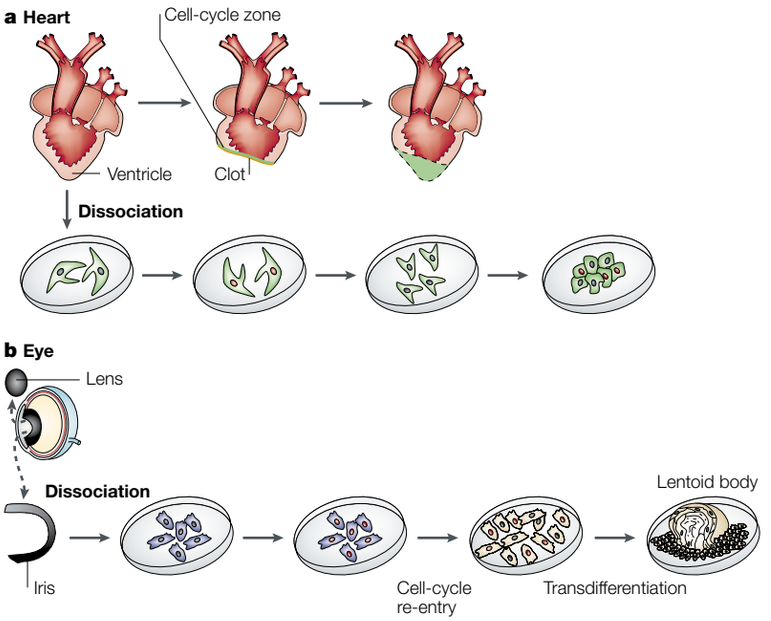


Figure 2 | **Plasticity of differentiated cells during regeneration of newt heart or lens.** **a** | After removal of the apex of the ventricle, the heart is sealed by a clot (red), and the adult cardiomyocytes return to the cell cycle in a zone (green) around the clot, proliferate and regenerate the ventricle. If cardiomyocytes are dissociated into a culture dish, they can divide and form groups of beating cells. It is striking that some cardiomyocytes complete the cell cycle and then resume beating. **b** | After removal of the lens, the pigmented epithelial cells of the dorsal margin of the iris give rise to the new lens. If the iris is dissociated, pigmented cells will re-enter the cell cycle, de-pigment and transdifferentiate into lentoid bodies (a small cluster of lens cells) that express lens markers.

regenerating tail of the larval axolotl was visualized under NOMARSKI OPTICS to observe the behaviour of single fibres after microinjection of a lineage tracer[30]. Such fibres fragmented synchronously into mononucleate cells and underwent rapid proliferation. The activation of this process apparently required both 'clipping' at the end of the fibre as well as tissue injury in the vicinity.

These experiments have established that the response of myotubes that are implanted into the limb blastema is similar to that of resident myofibres, and underline the importance of a more detailed analysis of both re-entry to the cell cycle and cellularization. Furthermore, it is noteworthy that the muscle fibres make an important cellular contribution to the blastema[30]. The authors estimate that a 4-day old blastema contains ~900 cells, of which approximately 150 come from the cellularization of muscle fibres.

Cell-cycle re-entry in newt myotubes

Newt myotubes clearly differ from their vertebrate counterparts in that they enter and traverse S phase after serum stimulation in culture[24,25]. The nuclei in the myotube double their DNA content and are stably arrested in G2. The response to serum is not observed for other vertebrate myotubes, with the exception of mouse cells that lack both copies of the retinoblastoma gene *Rb*[41]. pRb has a familiar and essential role in regulating the transition from G1 to S phase. Several lines of evidence[42,43] indicate that it is important for maintenance of the differentiated state in vertebrate myotubes, not only for stable arrest from the cell cycle[41], but also for transcription from certain muscle promoters that depend on activation of members of the myocyte enhancer factor 2 (MEF2) family of transcription factors[44]. Newt myotubes express pRb, but serum stimulates a pathway that leads to its inactivation by phosphorylation, and hence triggers progression from G1 into S phase[24]. If the myotubes are injected with a plasmid that encodes mammalian $p16^{INK4}$ — a specific inhibitor of the cyclin-D–CDK4 protein kinase that inactivates Rb — they are effectively blocked from entering S phase after serum treatment[24]. These data provide the first clear evidence that differentiated urodele cells are intrinsically different from their mammalian counterparts. The newt myotube can activate a pathway that leads to phosphorylation of pRb and re-entry to the cell cycle, and we

Box 2 | **Transdifferentiation**

Transdifferentiation was originally defined by Okada[18] as the conversion of one differentiated cell type into another. The original, and still the most studied, example is the conversion of pigmented epithelial cells from the iris into lens cells, which occurs during lens regeneration in newts and in cultures of pigment cells from various species (FIG. 2b). This can still occur to some extent if cell division is blocked, although it normally occurs in conjunction with cell-cycle re-entry.

In urodele limb regeneration, transdifferentiation could occur in a switch between the chondrogenic–connective-tissue lineage and the myogenic lineage. If cartilage is selectively labelled and introduced into a limb blastema, the occurrence of labelled nuclei in muscle is negligible. However, if cultured myotubes are labelled by microinjection of rhodamine-dextran[28] or with an integrated retrovirus[27], small clones of labelled mononucleate cells are detected occasionally in the cartilage of the regenerate.

There are several cases of transdifferentiation in mammals[64], such as the conversion of pancreatic EXOCRINE CELLS to hepatocytes *in vivo* under conditions of copper deficiency, as well as *in vitro*. Other examples include the transition between smooth and skeletal muscle in the developing oesophagus, and the conversion of myoblasts to adipocytes.

There has been much recent interest in transdifferentiation of stem cells; for example, the ability of haematopoietic stem cells or mesenchymal stem cells to give rise to neural and other epithelial derivatives after transplantation. The interpretation of these results is controversial at present[65], in view of the possibility of heterogeneity in the starting stem-cell populations and the contribution of cell fusion between different stem cells. However, transdifferentiation remains an important area for understanding cell plasticity.

NOMARSKI OPTICS
Also known as differential interference contrast microscopy, this technique forms images of high contrast and resolution in unstained cells using birefringent prisms and polarized light.

EXOCRINE CELL
A cell that makes up part of an exocrine gland, which discharges its secretion through a duct.

G2
The phase of the cell cycle through which cells progress after S phase but before M phase.

HETEROKARYON
A cell that contains two nuclei in a common cytoplasm.

propose that the regulation of this pathway is pivotal to the initiation of regeneration.

Regulation of cell-cycle re-entry by thrombin. The activity of vertebrate serum on newt myotubes is not due to the presence of typical protein growth factors, such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF); these factors act on mononucleate A1 cells but not on the myotubes[24,25] (FIG. 4). The post-mitotic arrest as defined in mouse myotubes is therefore comparable to that in newt myotubes, and this is consistent with the pivotal role of pRb in both cases. The distinction is that the urodele myotubes are responsive to an activity in serum that has no effect on the mouse cells. Serum is the soluble fraction of clotted blood, and results from the activation of prothrombin to generate the serine protease thrombin. Thrombin activates the clotting cascade and various other events that mediate the response to injury. It is possible to pre-incubate sub-threshold concentrations of serum with thrombin, and then inactivate all of the residual protease activity. Such digests can generate considerable activity on the newt myotubes[25]. When crude prothrombin is activated *in vitro*, the resulting thrombin preparations contain a distinct activity that acts directly on newt myotubes in serum-free medium[25]. We hypothesize that this ligand is generated downstream of prothrombin activation both in regeneration and in culture, and it is this factor that acts on the myotubes and other differentiated cells to promote re-entry (FIG. 4).

The activation of vascular prothrombin after injury occurs in relation to a protease complex known as Tissue Factor, which is assembled on the cell surface. Thrombin formation is subject to strict spatial and temporal regulation, as it is essential that clot formation is restricted to the wound area and does not spread. It is therefore tempting to speculate that urodele regeneration — for example, in the heart, limb or eye — is linked to the acute events of injury or tissue removal by the local activity of thrombin. Thrombin activity is increased locally in the early mesenchymal blastema of the limb[25], but more striking are recent findings that prothrombin is selectively activated on the dorsal margin of the iris after lentectomy, and that inhibition of thrombin in this context blocks cell-cycle re-entry on the dorsal margin (Y. Imokawa and J. P. B., unpublished observations). We propose that activation of prothrombin and generation of an activity referred to as Fa (FIG. 4) are important for the initiation of urodele regeneration in several contexts. It will be important to determine whether Fa is active on various differentiated cell types.

Cell-cycle re-entry by mouse myonuclei. Although myotubes of the mouse C2 cell line are not normally responsive to serum and the thrombin-based manipulations[24], their nuclei can be induced to re-enter the cell cycle by this pathway under some experimental conditions. This involves fusing mononucleate newt A1 and mouse C2 cells to obtain viable hybrid interspecies myotubes (HETEROKARYONS)[26]. The mouse nuclei can be identified by a species-specific antibody to nuclear lamin B. These cells grow at 33 °C under conditions that support serum-induced cell-cycle re-entry of A1 myotubes, as well as the post-mitotic arrest. If the heterokaryons are stimulated with serum or thrombin, mouse nuclei frequently enter S phase — sometimes in conjunction with newt nuclei and sometimes alone[26]. So, mouse myotubes have apparently lost responsiveness to the thrombin-derived factor[24–26], but their nuclei remain responsive to the intracellular consequences of the pathway, such as the phosphorylation of pRb, and in this respect, are quite comparable to newt nuclei.

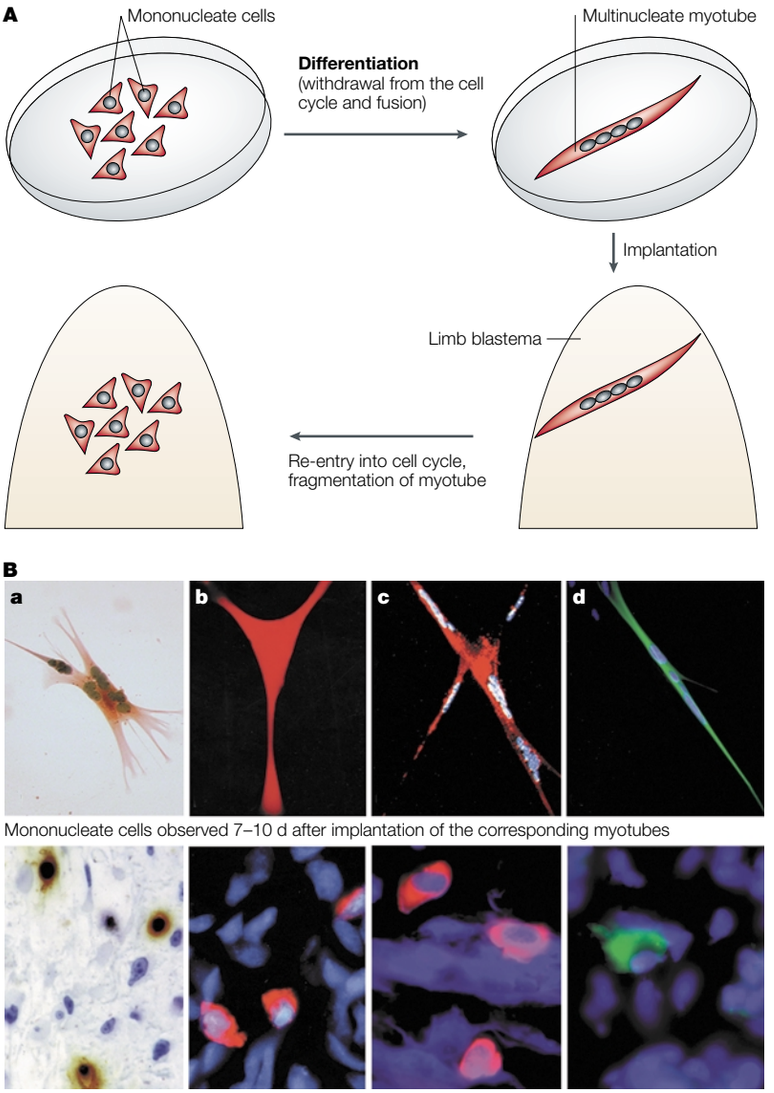


Figure 3 | **Plasticity of urodele myogenesis. A** | Mononucleate cells fuse to form multinucleate myotubes, but this process can be reversed. After implantation into a limb blastema, the nuclei within myotubes re-enter S phase and the myotubes are fragmented into viable mononucleate cells, which divide and contribute to the regenerate. **B** | Examples of implantation assays with newt myotubes that are labelled by different methods. Top panels show myotubes in culture labelled with an integrated provirus that expresses **a** | the marker enzyme human placental alkaline phosphatase, **b** | the lineage tracer rhodamine-dextran, **c** | the lipophilic cell tracker dye PKH-26 , or **d** | green fluorescent protein. Bottom panels show mononucleate cells observed 7–10 days after implantation of the corresponding myotubes. Nuclei in (**b**), (**c**) and (**d**) are stained with Hoechst dye, whereas those in (**a**) are stained with haematoxylin. Reproduced with permission from REF. 3 © 2001 Cambridge Univ. Press.

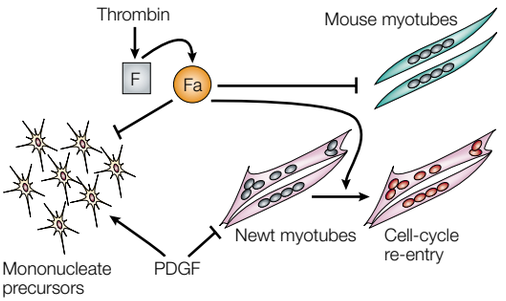


Figure 4 | **Thrombin activity counteracts post-mitotic arrest in newt myotubes.** Thrombin acts indirectly by cleaving a substrate (F) that is present in vertebrate serum or plasma[25] to induce cell-cycle re-entry. Interestingly, the resulting activity, referred to as Fa, does not affect mononucleate cells, whereas growth factors such as platelet-derived growth factor (PDGF) have the reverse specificity. Mouse myotubes are completely refractory to the thrombin-generated activity.

Cellularization

An important mechanistic issue is the relationship between the two aspects of plasticity — re-entry to the cell cycle and cellularization[27]. One possibility is that nuclei proceed into mitosis and the myotube is fragmented by CYTOKINESIS. Alternatively, myotube nuclei could bud off the main axis of the myotube in the presence or absence of cell-cycle re-entry. A1 newt myotubes can be blocked from cell-cycle re-entry either by X-irradiation or by microinjection of a plasmid that encodes mammalian $p16^{INK4}$. Such arrested myotubes, when implanted into a limb blastema together with a differentially labelled control population, were effectively converted to mononucleate cells[29] (FIG. 5). So, although re-entry and cellularization occur in parallel after implantation, they are not linked mechanistically. One other example of cellularization that is not dependent on mitosis, and which has some parallels with the process in newt myotubes, is that of the avian OSTEOCLAST-like multinucleated GIANT CELLS[45]. These cells are formed by fusion of mononuclear monocytes, and in culture they bud off mononuclear osteoclasts from the apical surface.

These findings on the independence of re-entry and cellularization have been reinforced by observations on striated muscle fibres, which can be dissociated from the limbs of larval salamanders and maintained in culture as adherent cells. These cells are apparently activated by the dissociation process to undergo several aspects of morphological plasticity, which can be analysed by time-lapse studies and by microinjection of a lineage tracer into the fibres. Some of them break into smaller fibres, or discharge viable multinucleate buds that subsequently adhere to the substrate. The most striking observation is that nuclei can aggregate in some fibres to form a lobulated, 'cauliflower' structure, which gives rise to a colony of dividing mononucleate cells. These events all occur in the absence of S-phase entry in the muscle-fibre nuclei (A. Kumar *et al.*, unpublished observations). This accessible system offers the potential for a more detailed exploration of the mechanisms of cellularization.

Cellularization of mammalian myotubes. An important impetus for the study of cellularization has come from the recent discovery of two methods that induce this process in mouse myotubes. In one approach, mononucleate C2 cells were stably transfected with the homeobox gene *Msx-1* linked to a conditional promoter[46]. Several studies have previously indicated that *Msx* genes promote cell proliferation, and that their expression is inversely correlated with differentiation[47–49]. After fusion of the stably transfected C2 cells, expression of *Msx-1* in myotubes was induced and this led to a decrease in the expression of myogenic regulatory genes. About 5% of the myotubes were induced to cleave into viable fragments — as described above for the larval myofibres — and another 5% fragmented into mononucleate cells, which proliferated. In some cases, the clonal progeny of a single myotube was isolated, propagated and shown to be capable of either CHONDROGENIC, ADIPOGENIC or myogenic differentiation, depending on the culture conditions[46]. It has been proposed[50] that Msx-1 is a master regulator of

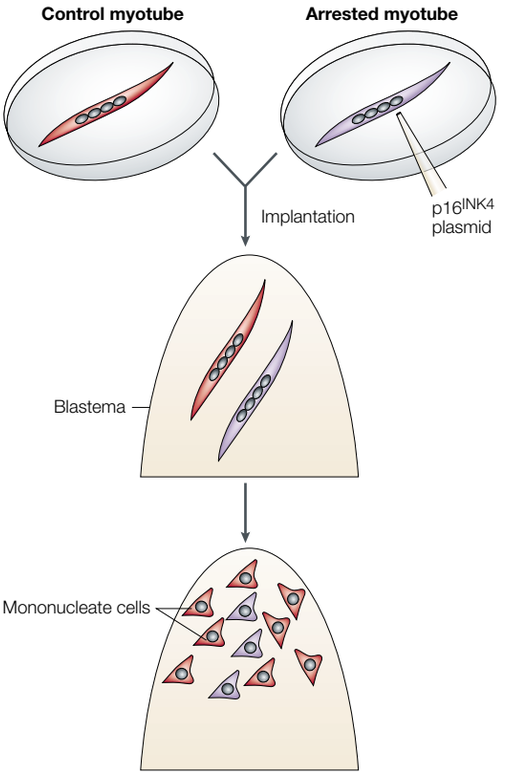


Figure 5 | **Arrested myotubes undergo cellularization after implantation into the newt blastema.** The figure shows a cultured newt myotube (purple) injected with a plasmid that expresses the cyclin-dependent-kinase inhibitor $p16^{INK4}$ — to arrest cell-cycle progression — along with a control myotube that is labelled with the lineage tracer rhodamine-dextran (red). After implantation, both give rise to mononucleate cells in the blastema (see REF 29).

CYTOKINESIS
The process of cytoplasmic division.

OSTEOCLAST
A mesenchymal cell that can differentiate into a bone-degrading cell.

GIANT CELLS
Large multinucleated cells that are thought to result from the fusion of macrophages.

CHONDROGENIC
Able to form cartilage.

ADIPOGENIC
Able to form fat or adipose tissue.

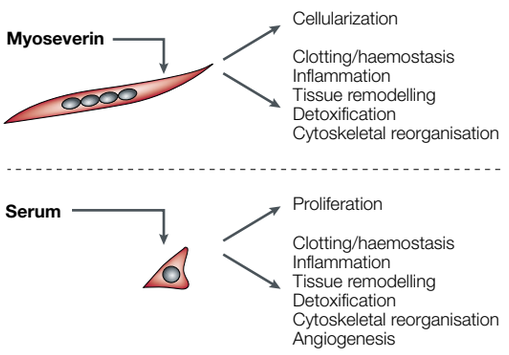


Figure 6 | **Categories of genes that are regulated after myoseverin treatment of myotubes or serum stimulation of fibroblasts.** In addition to the overall response of proliferation or cellularization, both myoseverin treatment (upper) and serum stimulation (lower) alter the expression of categories of genes that are implicated in tissue repair and remodelling, and wound healing[51,53]. The interpretation has been that fibroblasts encounter serum in the context of tissue injury, and that the changes in expression level of these genes orchestrate the behaviour and activities of this cell type. For myoseverin, it is plausible that as cellularization occurs during urodele regeneration, the programme of gene expression might also occur normally in this context.

the programme for cellularization that is expressed in urodele regeneration, and that it can also induce this programme in mammalian myotubes.

A second impetus to the analysis of these issues has come from the application of 'chemical genetics'. A large combinatorial library of trisubstituted purines was screened to identify a compound that would induce mammalian myotubes to undergo the regenerative response of newt myotubes[51]. One compound, with methoxybenzyl and isopropyl substituents, effectively fragments the myotubes within 24 hours to yield viable mononucleate cells that can divide and also fuse again to re-form myotubes. This compound — called myoseverin — seems to have two activities in mammalian myotubes. First, it depolymerizes MICROTUBULES. Second, it induces changes in the expression of a specific complement of genes that is involved in repair, wound healing and regeneration (FIG. 6a). Although there are other agents that can fragment microtubules, they tend not to yield viable mononucleate cells that can fuse into myotubes again[52]. Therefore, it is possible that both activities — that is, microtubule depolymerization and changes in gene expression — are important for the generation of viable mononucleate cells.

One possibility is that the compound that is responsible for fragmenting myotubes might also, by chance, regulate genes that are implicated in tissue remodelling and repair. The alternative and more attractive hypothesis, as proposed by Rosania *et al.*[51], is that myoseverin can activate the expression of a programme that mediates cellularization and other functions that are relevant to regeneration. DNA MICROARRAY analysis has identified ~90 genes so far, the expression of which was up or downregulated at least twofold by myoseverin[51]. Interestingly, many of these genes belong to categories that are regulated in fibroblasts in response to serum[53] (FIG. 6b). A crucial question, which is now open to investigation, is whether the mechanism of myoseverin action overlaps with the endogenous programme of cellularization in urodeles, as well as with the action of Msx-1 that has been outlined above.

The results with myoseverin raise the question of the identity of the endogenous signal that activates this response in the urodele muscle fibres during the initial phase of regeneration. A recent paper[54] has provided evidence that ligands that are present in crude extracts of the early regenerating newt limb can induce both A1 newt myotubes and C2 mouse myotubes to undergo cell-cycle re-entry and cellularization. The cellularization event indicates the presence of myoseverin-like activity on both newt and mouse cells. However, the S-phase re-entry of mouse myotubes is somewhat surprising in view of the evidence discussed earlier that there is a clear difference in responsiveness to serum and thrombin between newt and mouse myotubes. Further analysis of the factors that are present in the crude extracts should provide more information.

Perspective

In view of the evolutionary and biomedical issues, it is interesting to compare the relevant examples of plasticity in myotubes and pigment epithelial cells from urodeles and mammals. Although mammalian myotubes are thought not to re-enter the cell cycle or undergo conversion to mononucleate cells under normal circumstances, the intracellular pathways that mediate these responses are apparently intact, although it remains to be determined if the myoseverin or Msx-1 programme(s) does overlap with the urodele programme. Recent results[54] have raised the possibility that there are one or more distinctive signals for regeneration in the blastema. Nevertheless, the thrombin-generated activity is apparently a ubiquitous signal to which mammalian cells have lost responsiveness[25]. Although newts are the only adult vertebrates that can regenerate their lens, pigmented epithelial cells from several vertebrates can be converted to lens cells in culture[55]. It will be necessary to identify the blastemal signals[25,54] that trigger these responses of differentiated cells, as well as the molecular basis of any differences between the differentiated cells of the two species. It is already clear that this approach to regeneration — that is, the investigation of the plasticity of differentiated cells — is a productive and informative one, particularly in the absence of a comprehensive genetic analysis (BOX 1).

In view of the overlap, or even correspondence, in pathways between urodeles and mammals, it could be predicted that regenerative responses would be subject to genetic variability. The most striking example at present is the MRL mouse, a strain that has a markedly enhanced ability to restore tissue after wounds such as an ear punch[56,57]. After CRYOGENIC INFARCTION, this mouse

MICROTUBULE
A hollow tube, 25 nm in diameter, that is formed by the lateral association of 13 protofilaments, which are themselves polymers of α- and β-tubulin subunits.

DNA MICROARRAYS
Devices that are used to analyse complex nucleic acid samples by hybridization. They make it possible to quantitate the amount of different nucleic acid molecules that are present in a sample of interest.

CRYOGENIC INFARCTION
Obstruction of the blood supply as a result of extremely low temperatures.

can restore significant cardiac function, and this response is associated with cell-cycle progression of cardiomyocytes in the vicinity of the lesion[58]. The analysis of the various loci that are associated with these aspects of the MRL phenotype should be informative[59].

At present, the main approach to mammalian regenerative medicine is the isolation of appropriate stem cells, followed by manipulations that are aimed at directing their differentiation towards the morphogenesis of complex structures[60]. Although this attracts considerable interest at present, many of the applications are sufficiently problematic to warrant consideration of alternative and complementary approaches. The urodele strategy — the limited respecification of residual differentiated cells — is so successful that it would be surprising if it were not eventually tried as a therapeutic approach in some contexts of mammalian regeneration. The example of myoseverin[51,52] shows that the responses that are discussed in this review are a potential target for therapeutics that are directed at regulating the stability of the differentiated state.

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