Appendage Regeneration in Adult Vertebrates and Implications for Regenerative Medicine

Jeremy P. Brockes* and Anoop Kumar

The regeneration of complex structures in adult salamanders depends on mechanisms that offer pointers for regenerative medicine. These include the plasticity of differentiated cells and the retention in regenerative cells of local cues such as positional identity. Limb regeneration proceeds by the local formation of a blastema, a growth zone of mesenchymal stem cells on the stump. The blastema can regenerate autonomously as a self-organizing system over variable linear dimensions. Here we consider the prospects for limb regeneration in mammals from this viewpoint.

The goal of regenerative medicine is to restore cells, tissues, and structures that are lost or damaged after disease, injury, or aging. The current approaches are influenced by our understanding of embryonic development, of tissue turnover and replacement in adult animals (*1*–*3*), and by tissue engineering and stem cell biology (*4*). The regeneration of organs and appendages after injury occurs in diverse animal groups and provides another important viewpoint, in addition to the demonstration that complex adult tissues can be rebuilt. The lessons of biological regeneration have not been extensively assimilated, in part because this attribute appears remote and exceptional from a mammalian perspective. This Review is concerned principally with lessons from regeneration in salamanders, the species of adult vertebrates that possesses the most extensive abilities (*5*, *6*). We identify three properties of regeneration in salamanders—autonomy, scaling, and plasticity—and discuss some of the cellular and molecular mechanisms underlying them. It may be desirable to implement these properties in the context of mammalian regeneration.

Regenerative medicine currently uses three approaches (Fig. 1) (*4*): the implantation of stem cells to build new structures, the implantation of cells pre-primed to develop in a given direction, and the stimulation of endogenous cells to replace missing structures. Each of the different aspects identified in the first two examples—the generation of an appropriate cohort of regenerative cells, their regulated division and differentiation, and the restoration of the appropriate part of the structure—must be evoked from endogenous cells in the third approach. These processes operate in adult animals that regenerate, and in addition, the regenerative response must be initiated by signals responsive to tissue injury or removal. One candidate signal in salamanders is the local activation of thrombin, a regulator of hemostasis and other aspects of the response to injury, as well as an activator of S phase (the phase of chromosome replication) reentry in differentiated cells (*7*–*9*).

A salamander can regenerate its limbs and tail, upper and lower jaws, ocular tissues such as the lens and retina, the intestine, and small sections of the heart (*10*–*13*). The various contexts for regeneration do not present an equivalent degree of difficulty. To restore the intricate and discontinuous pattern of the vertebrate limb is a different proposition from replacing a patch of cardiac tissue in the ventricle. Nonetheless, recent efforts at tissue engineering of heart muscle have underlined that even in the heart, it is quite challenging to achieve an appropriate vascular and electromechanical integration after implantation (*14*). The salamanders are unusual among adult vertebrates in their ability to regenerate an entire limb from a blastema, and this property is a particular focus here. Regeneration of the digit tip in fetal mammals does not proceed from a blastema but rather from progenitor cells in the nail bed (*15*). The limb blastema consists of a mound of mesenchymal stem cells at the end of the stump (Fig. 2A). The critical questions for research into limb regeneration are concerned with the blastema, and its properties offer a distinct perspective for regenerative medicine.

Autonomy of the Blastema

If a blastema is removed from its limb by transection at the amputation plane and is transplanted to an appropriate location, such as the anterior chamber of the eye or a tunnel bored in the connective tissue of the dorsal fin (Fig. 3A), then it forms a normal regenerate (Fig. 3B) (*16*, *17*). The blastemal cells derived after amputation at any level on the proximodistal (PD) axis give rise precisely to the distal structures—wrist-level cells regenerate a hand, shoulder cells regenerate an arm. This property is stably expressed by blastemas transplanted to the fin or eye and is called positional memory. The limb blastema, as illustrated in Fig. 2A, is a self-organizing system that is independent of any templating or inductive activities from the limb stump (*18*). The significance of this property can be illustrated by contrasting different strategies for the repair of a bone lesion resulting in a gap. The approach of tissue engineering depends on the implantation of a scaffold seeded with appropriate stem cells (Fig. 3C) (*19*, *20*). The salamander has no mechanism for local tissue regeneration of such a gap, but if the limb is amputated at an appropriate level, the blastema will reconstruct the distal skeletal elements (Fig. 3D). This outcome is independent of the presence of elements proximal to the amputation plane, as expected from blastemal autonomy (*21*). This property is a tantalizing one for attempts to regenerate complex structures in mammals, because it suggests that the isolation or engineering of a cell functionally

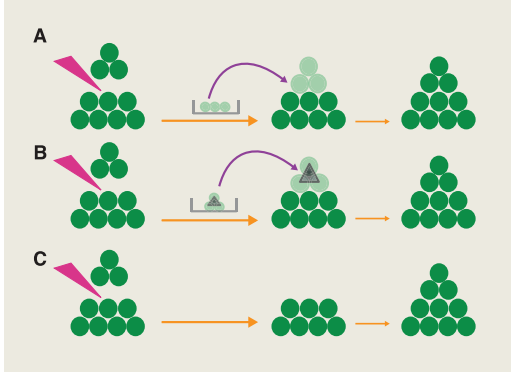

Fig. 1. Schematic of three approaches to regenerative medicine. (**A**) Implantation of stem cells (light green) from culture leads to the restoration of the structure. (**B**) Stem cells are provided with a scaffold (triangle) in order to guide restoration. (**C**) The residual cells of the structure are induced to make a regenerative response.

Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK.

*To whom correspondence should be addressed. E-mail: j.brockes@ucl.ac.uk

equivalent to a salamander blastemal cell could obviate the necessity for much further intervention. What are the mechanisms that endow the limb blastema and its cells with this ability?

Blastemal cells are derived by dedifferentiation from adult mesenchymal cells at the plane of amputation, and they derive critical cues about their identity and potentiality from their precursors. Such cues include limb identity, and indeed when regenerative cells are transplanted between different tissue contexts in the salamander, they retain their original identity (*18*, *22*). The regenerative territories for forelimb, hindlimb, and tail identity have been mapped by inserting a peripheral nerve branch into the vicinity of a superficial wound at different locations on the body and observing the identity of the resulting appendage (*23*). The retention of such specification in adult differentiated cells may be one major step for the loss of regenerative ability in other vertebrates. The most striking example of local cues in blastemal cells is the specification of transverse and PD axial identity in limb regeneration (*24*, *25*). Positional memory is a critical aspect for the autonomy of limb regeneration, because it specifies the initial population of blastemal cells in relation to the extent of the axis to be regenerated. An understanding of its molecular basis is generally important for our appreciation of how stem cells are specified to give rise to different structures, rather than to different cell types.

When blastemal cells from different PD levels are juxtaposed in experimental configurations, this leads to the activation of cell division, movement, and adhesion (*26*, *27*). This mechanism operates even when single cells or small groups of cells in a distal location are respecified to a more proximal identity and then relocated over the distances characteristic of an adult salamander limb (*28*, *29*). The view that limb morphogenesis is driven by local differences between cells (*30*) has led to the hypothesis that PD identity is encoded by a molecule or molecules at the cell surface, possibly as a graded level of expression along the PD axis. This is consistent with the ability of retinoic acid (RA) and precursor retinoids such as vitamin A to respecify distal blastemal cells to a more proximal identity. Such respecification from wrist to shoulder levels occurs continuously over a 2.5-fold range of retinoid concentration, suggesting that the differences in gene expression that underlie PD identity may be relatively small (*31*, *32*).

These considerations have led to the identification of *Prod 1*, a gene that is regulated by PD location and RA. *Prod 1* encodes a small protein that is linked to the cell surface by a glycosylphosphatidylinositol (GPI) glycolipid anchor (*33*). It is apparently the newt ortholog of mammalian CD59, as evidenced by the prediction of secondary structure. The difference in expression at mRNA and protein levels is shown for mid-humerus and mid-radius blastemas, as well as for the gradient of expression in the normal limb (Fig. 4, A to C). The CD59 protein in mammals is associated with the inhibition of the terminal phase of complement activation, and it is also able to mediate activation of intracellular nonreceptor tyrosine kinases (*34*–*36*). When proximal and distal blastemas are confronted in culture (Fig. 4, B and C), the proximal member reproducibly engulfs the distal, and engulfment is selectively blocked by two antibodies against the protein Prod 1 (*33*). Compelling evidence for its relevance to PD identity has come from electroporating a Prod 1 expression vector into distal cells of the limb blastema of the larval axolotl. Whereas labeled cells in control blastemas maintain their distal location and give rise to tissues in the regenerated hand, labeled cells in the contralateral blastema receiving the Prod 1 vector relocate and contribute to the upper arm (Fig. 4D) (*28*). Taken together, the evidence suggests that Prod 1 is a cue for local cell identity that is expressed in the normal limb and persists in blastemal cells. Questions remain as to which extracellular and surface ligands may interact with it and how it mediates cell interactions based on differences in expression between neighbors. We have suggested that neighbors may titrate the relative expression of Prod 1 by homophilic adhesion between cells, leaving spare Prod 1 molecules on the proximal cell to interact with ligand (*33*). This mechanism may dictate the extent of growth, movement, and adhesion during patterning and hence define the morphogenetic autonomy of the blastema.

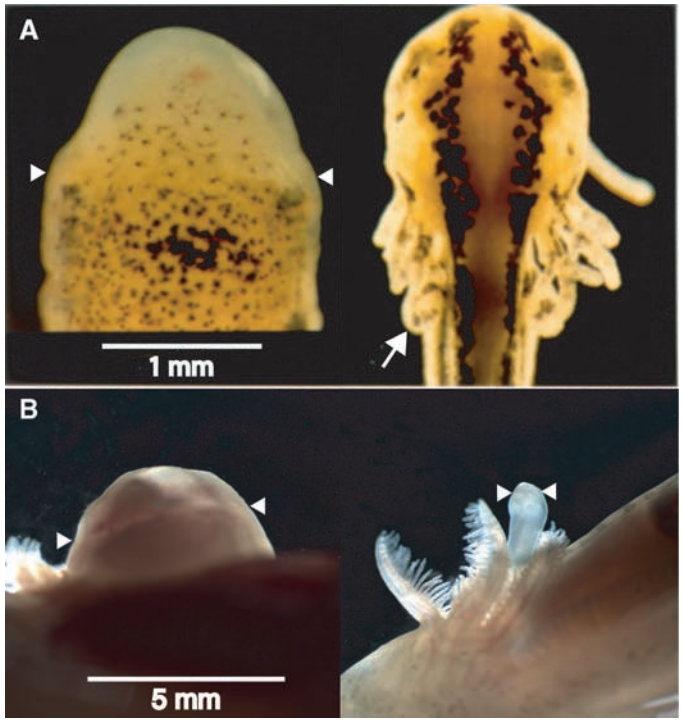

Fig. 2. Scaling differences in limb regeneration and development. (**A**) An adult newt (*Notophthalmus viridescens*) limb blastema (left) (arrowheads mark the original plane of amputation) next to a newt embryo (right) showing the developing limb bud (arrowed). The specialized epithelium surrounding the blastema is called the wound epidermis. (**B**) An adult axolotl limb blastema (left) (from an animal 16 cm in length) next to a 4-cm larval axolotl limb blastema (right) (arrowheads mark the amputation plane). The scale bars apply to the pair of (A) or (B) images, respectively.

Scale of Regeneration

There can be a major difference in the scale of limb development and adult limb regeneration (Fig. 2A), or of larval and adult limb regeneration (Fig. 2B). The difference in the time taken to generate the limb between members of each pair is only about twofold. In larger axolotls, there is a tendency for the cross-sectional area of the blastema to be smaller than the stump from which it arose (*37*); but nonetheless, regeneration can occur on a scale close to that of the limb bud or on the scale of an adult limb with a linear dimension that can be 10-fold greater. This property is important, because it would be inappropriate to regenerate a larval limb on an adult stump, but the mechanisms underlying it are not fully understood. One aspect of developmental mechanisms that is particularly hard to reconcile with scaling is the activity of morphogens, in particular, the principle that spatial localization can be derived from an extracellular diffusion gradient in conjunction with concentration thresholds (*38*, *39*). It seems difficult to implement this principle in the context of an adult limb, and historically, this has led to a substantial divergence in the mechanisms proposed for limb development and regeneration.

These differences between development and regeneration can be accommodated by recent findings in relation to the activity of RA on the PD axis and of sonic hedgehog (Shh) in digit specification on the anteroposterior (AP) axis. It has been proposed that an RA gradient operates in mouse limb development as a consequence of its synthesis near the midline of the animal and its degradation by the product of the *Cyp 26* gene, expressed at the distal end of the limb bud (*40*). If this mechanism

operates in salamander development, it apparently leads to a stable gradient of expression of *Prod 1*/*CD59* in the adult limb, as discussed above (Fig. 4A), so that regeneration may converge with development at a stage after the action of a putative extracellular gradient of RA. In the case of Shh, there is evidence that it is required in regeneration just as in development, because misexpression at the anterior margin of the axolotl blastema or treatment with the antagonist cyclopamine give the same phenotypes as the chick or mouse limb bud (*41*, *42*). Digit identity may depend on the time of exposure to Shh as cells move away from the source and their responsiveness is regulated, as opposed to a spatial gradient of Shh protein (*43*, *44*). These remarks are directed at the derivation of tissue pattern in the regenerate from extracellular concentration gradients, but not at the activity of diffusible ligands in general. The division of blastemal cells is dependent on signals provided initially by regenerating axons that ramify throughout the blastema (*45*–*47*) and later by the wound epidermis, a transient structure that surrounds the early regenerate (Fig. 2A) (*48*).

Plasticity of Differentiated Cells

One contribution to a mechanism that is able to operate at different scales is the founder population of blastemal cells, which is recruited from differentiated mesenchymal cell types across the amputation plane. The plasticity of differentiated cells is a notable feature in different contexts of non-neural regeneration in salamanders, but this term encompasses a range of phenomena (*49*). The regeneration of sections of the adult heart depends on the ability of cardiomyocytes to reenter the cell cycle in the vicinity of the lesion (*50*). Dissociated cardiomyocytes from the adult newt ventricle reenter S phase in culture, and about a third of the cells progress through mitosis and may enter successive cell divisions, in contrast with their mammalian counterparts. This is accomplished without major loss of differentiated properties, and cells promptly resume beating after cytokinesis (*51*). In lens regeneration, pigment epithelial cells at the dorsal margin of the iris reenter the cell cycle after removal of the lens, lose their pigmentation, and transdifferentiate into lens cells (*52*–*54*). In limb and tail regeneration, multinucleated myotubes or striated myofibers undergo cellularization to give rise to mononucleate progeny that resume division (*55*–*58*). In experiments where cultured myotubes are labeled by selective microinjection or by retroviral integration and then implanted into the limb blastema, transdifferentiation to labeled chondrocytes occurs only at a frequency of about 0.1% of mononucleate cells. The nuclei in multinucleate muscle cells may also reenter S phase, although this is apparently not required for cellularization to occur (*59*, *60*). The range of responses shown by these three cell types could occur for different mesenchmal cell types recruited into the limb blastema. For example, a critical contribution to tissue patterning comes from the connective tissue fibroblasts of the dermis, and the degree of change in their differentiated status is still unclear (*61*, *62*).

The plasticity of differentiated cells presents an interesting alternative to the familiar perspective for mammalian regeneration based on embryonic and adult stem cells. Salamanders can sustain an indefinite number of successive cycles of limb regeneration, and the renewable unit is the combination of a differentiated cell type and its derivative blastemal cell (or several cells for multinucleate muscle) (*49*). One example of mammalian regeneration that depends on the plasticity of differentiated cells is in the liver (*63*), where the retention of function by cycling hepatocytes resembles that of cardiomyocytes in the salamander. Another example is the regeneration of peripheral nerve, which depends on the ability of Schwann cells to reenter the cell cycle, lose their differentiated properties such as myelin expression, and acquire a phenotype that facilitates axonal regeneration (*64*). The mammalian Schwann cell is a regenerative cell in the sense familiar in salamander regeneration, and the pathways leading to its reversal of differentiation are currently under investigation (*65*). Current interest in differentiated cells as a target for mammalian renewal and regeneration is exemplified by evidence for renewal of rennin synthesizing cells (*66*) or pancreatic β cells (*67*) and by evidence for the division of adult postmitotic auditory hair cells after the removal of the retinoblastoma gene (*68*). The retinoblastoma protein in salamanders is a critical target for inactivation and S phase reentry in myotubes (*59*, *60*), cardiomyocytes (*51*), and iris epithelial cells (*69*). Another approach has been to screen combinatorial libraries for small molecules that are able to reverse the differentiated state in cultured mouse cells (*70*). For example, this has led to the identification of myoseverin, a substituted purine able to fragment myotubes into viable mononucleate cells (*71*, *72*).

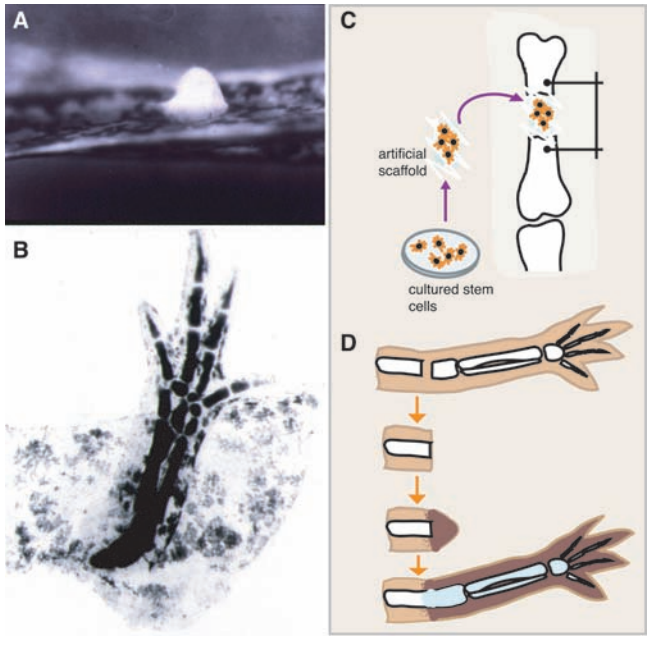

Fig. 3. Morphogenetic autonomy and its implications for regeneration. (**A**) A limb blastema from a salamander transplanted to the fin tunnel. (**B**) The limb structures formed from the blastema of (A). (**C**) Repair of a bone gap by grafting an artificial scaffold seeded with stem cells; an example of the approach of Fig. 1B. (**D**) Repair of a bone gap in a salamander by formation of a blastema and subsequent autonomous reconstruction of the distal skeletal elements.

Implementation in Mammals

These examples indicate that certain aspects of the regenerative mechanism in salamanders may become accessible in mammalian contexts, but what are the prospects for a more radical change in our potentiality, such as the regeneration of a limb? It is not understood why some animals are able to regenerate and others apparently are not (*73*, *74*); but even from our present limited perspective, there appear to be a number of differences between mammals and urodeles that prevent or limit regeneration, rather than any single defect or aberrant pathway. For example, mouse myotubes are refractory to the action of the thrombin pathway that leads to S phase reentry in newt myotubes, although mouse nuclei do respond in a mouse/newt heterokaryon (*75*). In another case, the Hox gene *C6* is turned off after limb development in the mouse, but its expression persists into the adult newt forelimb and limb blastemal cells (*76*–*78*). One aspect of adult wound healing in mammals that has been discussed in relation to the curtailment of regeneration is the occurrence of fibrosis, and also of immune and inflammatory responses (*79*, *80*). These are all potential targets for genetic and other manipulations. Existing variation in mouse strains and transgenics encompasses marked ability in tissue regeneration. For example, the MRL strain has the ability to heal punch wounds in the pinna of the ear (*81*), whereas transgenic mice expressing elevated levels of the muscle insulin-like growth factor–1 isoform

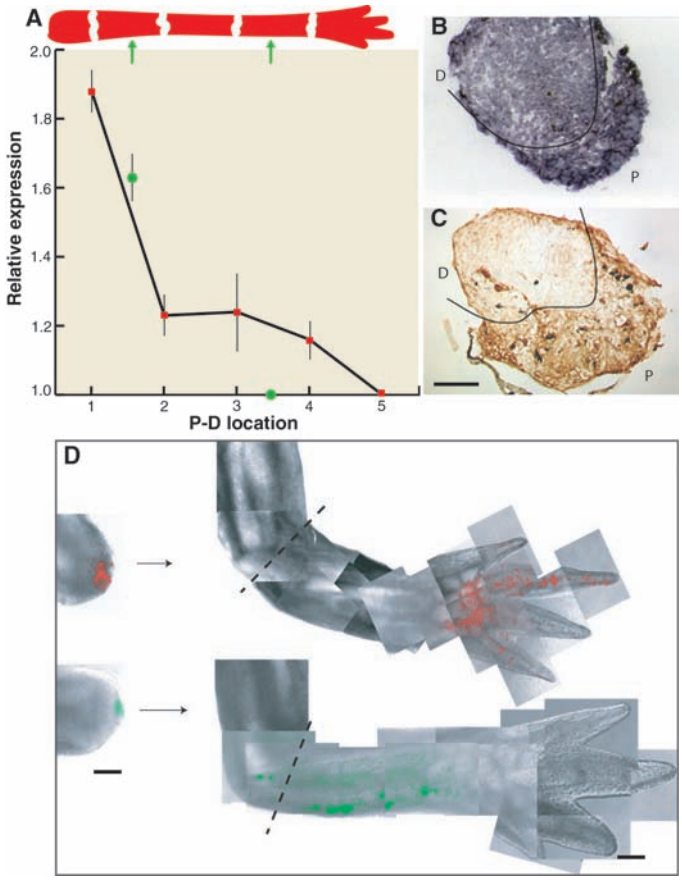

Fig. 4. Prod 1/CD59 as a local cue for PD identity in limb regeneration. (**A**) The graded expression of *Prod 1* mRNA along the PD axis in adult newt limb (outlined in red) is shown relative to the level in the hand (red points), whereas the expression in P and D blastemas is shown after amputation (green points) at the levels arrowed. (**B**) Expression of Prod 1 mRNA in P and D blastemas confronted in culture. (**C**) Expression of Prod 1 protein in confronted P and D blastemas. Scale bars in (B) and (C), 200 μm. (**D**) Elevated expression of Prod 1 converts distal blastemal cells to proximal. The left limb blastema of a larval axolotl (upper) was electroporated so as to express red fluorescent protein, and after regeneration, the labeled cells contribute to the hand. The right blastema (lower) was electroporated to express green fluorescent protein and Prod 1, and cells contribute to proximal tissue after regeneration, even to tissue proximal to the amputation plane (dashed line). Scale bars in the left panel, 200 μm; in the right panel, 1 mm. For experimental details, see (*89*). (B) and (C) are from (*33*) and (D) is from (*28*), with permission.

show enhanced recruitment of bone marrow cells and augmented repair mechanisms after injury (*82*). Nevertheless, it would be surprising if such approaches, even in combination, were to confer regeneration on a structure such as the limb.

Here we have outlined some of the distinctive properties of the limb blastema in salamanders, and a critical step forward for mammalian regeneration would be to engineer the equivalent of a founder blastemal cell. This goal should be facilitated first by increasing our understanding of stem cells in other contexts, including planarian regeneration (*83*) as well as limb development (*84*), which should help to define critical aspects of cellular regulation (*85*). Second, we need a better appreciation of how dedifferentiation operates to generate progenitor cells retaining local cues and specification. For example, the effects of cell cycle reentry in this process can be explored both in amphibian cells and in a mammalian context, such as the Schwann cell. In principle, it is possible that the blastemal phenotype could be approached either by modification of a generalized mesenchymal precursor, or by reversal from more differentiated cells. Third, we need a more extensive inventory of the properties of limb blastemal cells that takes advantage of the recently completed salamander expressed sequence tag (EST) projects (*86*, *87*). Finally, the approaches of systems biology should allow an integrated theoretical and experimental program to model the properties of blastmal cells. The value of such models as design tools has been noted previously (*88*), and this may allow for the derivation of a mammalian counterpart. This approach, although obviously challenging, seems more realistic than attempts to regulate externally the myriad processes of limb morphogenesis after beginning with relatively unspecified cells.

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