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The molecular basis of amphibian limb regeneration: integrating the old with the new

David M. Gardiner*, Tetsuya Endo and Susan V. Bryant

Is regeneration close to revealing its secrets? Rapid advances in technology and genomic information, coupled with several useful models to dissect regeneration, suggest that we soon may be in a position to encourage regeneration and enhanced repair processes in humans.

Key words: limb / regeneration / pattern formation / urodele / fibroblast / dedifferentiation / stem cells

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Introduction

The study of amphibian limb regeneration has a rich experimental history. After many decades of research at the tissue and cellular levels, much is known about the phenomenology and the basic organizing principles of regeneration. In recent years, molecular analyses have begun to provide insights into the mechanisms controlling regeneration. It is already clear that regeneration involves complex molecular interactions between multiple tissues, and thus we can expect that there will be many important genes involved, rather than just a small number of 'regeneration genes'. With the recent input from the axolotl EST project to the databases, nearly 500 non-redundant 'regeneration' genes have been cloned and identified,¹ a number that likely will increase dramatically over the next few years. Consequently, the availability of cloned regeneration genes will not be a limiting factor to progress in understanding limb regeneration. The challenge will entail drawing on the wealth of information from

classical studies to guide the identification of the functions of this large set of genes.

The challenge of understanding the mechanisms controlling the biology of complex systems is hardly unique to regeneration biology. In recent years, techniques have become available to identify all the molecular components of a system, and to study the interactions between those components. Key to the success of such an approach is the ability to identify the molecules, while at the same time having an understanding of the cell and tissue level properties of the system. The goal of this review is to discuss key insights from the classical literature as well as more recent molecular findings. We focus on three critically important cell types: fibroblasts, epidermis and nerves. Each of these is necessary, and together they are sufficient for the regeneration of a limb. Although the final limb is composed of a variety of other cells and tissues, such as muscle, blood vessels and pigment cells, these other cell types do not appear to be necessary for the control of growth and pattern formation during regeneration, but rather they respond to signals from nerves, fibroblasts and the epidermis. Thus at this time, these three cell types pose both great challenges and promising opportunities for research directed toward developing an integrated view of the molecular interactions controlling limb regeneration.

Key insights from pre-molecular studies

Fibroblasts

During regeneration, growth and pattern formation are coordinately regulated by interactions between cells that are derived from fibroblasts of the connective tissues of the amputated stump (see References 2, 3). The function of connective tissue fibroblasts is demonstrated qualitatively by grafting studies to induce new or altered pattern and quantitatively by cell contribution studies. As a generalized conclusion,

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grafts of tissues that contain fibroblasts affect growth and pattern formation; whereas, grafts that do not contain fibroblasts do not.⁴ Among these various tissues, the dermis plays a particularly dominant role during regeneration.

A direct demonstration of the importance of the dermis comes from studies of regeneration in X-irradiated limbs. These limbs are inhibited from regenerating, but can be rescued by grafts of unirradiated skin.^{5–7} The regenerated limbs are formed from graft-derived dermal fibroblasts, and have a normal pattern of skeletal and connective tissues, blood vessels and nerves, though they lack muscles. Since the stump muscles are irradiated, precluding muscle precursor cells from migrating distally into the regenerate, it follows that myogenic cells are not required to build a normal limb pattern during regeneration, in common with similar findings in developing limbs.⁸ We conclude that the fibroblast-derived mesenchymal blastema that eventually reforms the cartilaginous skeleton and associated connective tissues, forms a blueprint that guides the migration and growth of nerves, blood vessels and myogenic cells.

Descriptive histological studies suggested that each tissue of the mature limb contributed cells in proportion to its availability in the stump,^{9,10} but subsequent lineage analysis does not support this interpretation.¹¹ The progeny of dermal fibroblasts account for between 19 and 78% of the cells of the early blastema (42% on average), even though dermal fibroblasts represent less than 20% of the cells of the stump, suggesting that the fibroblast population is subject to selective expansion in the blastema. Since dermal fibroblasts account for about half of all fibroblasts in the limb,¹² it is possible that essentially all of the early blastemal cells are derived from fibroblasts, even though these cells account for less than half of the cells of the mature limb. At present it is unclear whether there is a population of quiescent stem cells in the dermis that are activated during regeneration, or whether dermal fibroblasts become dedifferentiated by losing their differentiated phenotype and reversing their cell fate to become stem cells. Nevertheless, an important consideration for future experimental work on the biology of stem cells for limb regeneration is that such a population of cells likely will be isolated from the dermis.

Epidermis

Although epidermal cells do not contribute directly to the blastema (see References 11, 13), a covering

of specialized epidermis is critical for the success of regeneration. Epidermal cells function to enable outgrowth, and may also function in the control of pattern formation, as is the case during limb development. A specialized wound epidermis (WE) forms during the initial healing of the wound surface by the migration of an epidermal sheet of cells derived from the basal cells of the mature skin epidermis (see Reference 3). This epidermal layer subsequently thickens, as a result of continued cell migration, to form the apical epithelial cap (AEC), and acquires unique functions associated with regeneration. A new basal lamina is not reformed until relatively late during regeneration, which presumably is critical in allowing for epithelial–mesenchymal interactions (see Reference 3).

Treatments that affect the formation of the WE or the AEC alter the course of regeneration (see Reference 13). Formation of the WE is inhibited by a graft of mature skin over the amputation surface, in which case regeneration is inhibited. Removal of the WE or AEC after it has formed also inhibits regeneration. Conversely, experiments that relocate the position of the AEC induce limb outgrowth at the new position.

Nerves

The importance of nerves in regeneration has long been recognized, but the molecular mechanisms mediating neuronal influences are still largely unknown. Nerves are severed during amputation, begin to regenerate rapidly into the stump tissues at the amputation plane, and subsequently innervate the blastema and overlying epidermis (see Reference 3). If the limb is denervated during the early stages of regeneration, the limb fails to regenerate. In addition to axons, nerves contain connective tissue cells and Schwann cells. The function of nerves during limb regeneration is not considered to be associated with these non-neuronal cells since they are present whether or not the limb has been denervated. Consequently, the critical cell type is considered to be the neuron, which is hypothesized to produce a ‘neurotrophic factor’ required for the initiation and progression of the early stages of regeneration. Denervation of regenerating limbs at later stages inhibits further growth of the regenerate, but not redifferentiation of normally patterned limb tissues (see Reference 3).

The production of factors required for regeneration may be a normal function of nerves. Alternatively, this function of nerves may be acquired in response to amputation, as is the case for fibroblasts and the

epidermis. Presumably this function would be induced and maintained by interactions with blastema cells and epidermal cells, and would be coupled with the interactions that stimulate growth and pattern formation. Since the behavior of nerves during regeneration has not been well characterized at the molecular level, the nature of such interactions cannot be evaluated at the present time.

Understanding the function of nerves is critical to devising strategies to induce regeneration in humans, since nerves are the source of factors that are required for the recruitment and proliferation of limb stem cells. Progress in this area of research has been limited by the lack of efficient and appropriate functional assays. Many experiments involve denervation of the limb, which inhibits regeneration, followed by application of test treatments for their ability to rescue regeneration. Rescue is generally limited in nature (see References 3, 14) and rarely results in normal regeneration (however, see Reference 15). Such experiments provide a negative assay for a positive acting factor, and have yet to identify the elusive neurotrophic factor.

Building on the past: model systems for understanding limb regeneration

Adult urodele amphibians are unique among vertebrates in their ability to regenerate their limbs perfectly. These organisms thus offer the unique opportunity to discover the events and processes that occur, and the genes that are expressed during successful regeneration. Ironically, it is challenging to test the ability of a gene to induce a regenerative response in urodeles since regeneration is the default response. Experiments designed to inhibit regeneration yield negative results, which are difficult, if not impossible to interpret. The alternative approach of first inhibiting regeneration (e.g. by denervating the limb) and then testing the ability of a gene or factor

to rescue regeneration is again basically negative in design, and subject to experimental artifacts. Fortunately, as discussed next, there are a variety of experimental models that hold great potential to identify key signals controlling growth and pattern formation during regeneration. Some are based on relatively recent discoveries; whereas, others are based on classical studies that can be reinvestigated using modern techniques to analyze molecular function.

Molecular studies of normal regeneration in urodeles

Studies of gene expression in regenerating urodele limbs have led to the conclusion that there are at least three distinct phases of limb regeneration (Figure 1, Table 1). During phase I, the wound is healed, and patterns of gene expression are comparable for both amputation wounds and lateral skin wounds. However, when formation of the WE is prevented, expression of genes associated with normal limb regeneration are inhibited, indicating that the WE is necessary for expression of those genes.^{16–18} Phase II is unique to limb regeneration and involves the process that generates the population of undifferentiated, proliferating blastema cells, either by ‘dedifferentiation’, and/or by activation of quiescent stem cells. The divergence of phase II limb regeneration from the wound-healing pathway is first evidenced by the re-expression of *Hoxa-9/Hoxa-13*, preceding by many days the morphologically observable changes in stump tissues that will eventually lead to the accumulation and proliferation of blastema cells.¹⁹ These genes function in patterning the proximal–distal limb axis and their early co-expression suggests that the earliest event in regeneration is the specification of the distal tip of the pattern (see Reference 2). Thus the conditions are established at the outset for intercalation of the missing parts of the proximal–distal limb axis, an ability of regenerating limbs that has been appreciated for some time.^{20–24} Phase III of regeneration is characterized

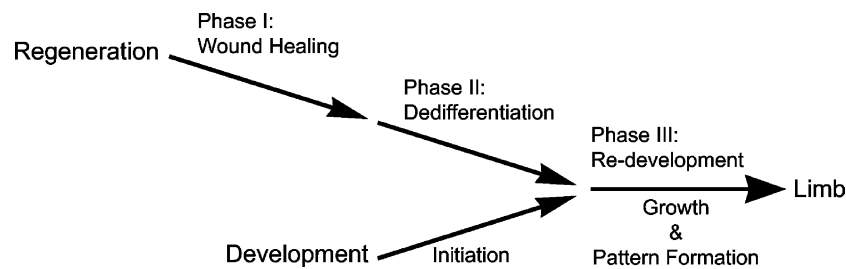


Figure 1. The converging pathways of limb regeneration and limb development.

Table 1. The three phases of limb regeneration

Phase I: wound healing	
Epidermal healing	Epidermal sheet migrates to cover the wound area within 1–2 h
Induction of gene expression	Genes common to wound healing and limb regeneration are expressed (e.g. <i>Msx-2</i> and <i>Mmp-9</i>)
Nerve dependency	Not dependent on nerves
Phase II: dedifferentiation	
Dedifferentiation	Cells in the stump tissues lose their specialized characteristics and become migratory
Blastema formation	Cells derived from fibroblasts migrate to form the blastema and begin to proliferate
Induction of gene expression	Spatial and/or temporal patterns of re-expressed genes differ from development; many are not expressed during phase I
Nerve dependency	Dependent on nerves
Phase III: redevelopment	
Growth and pattern formation	Responses to grafting are the same as in developing limbs; developing and regenerating limbs can cooperate to form a chimeric limb
Induction of gene expression	Expression and function of genes same as in developing limbs
Nerve dependency	Continued growth depends on nerves, but differentiation is nerve-independent
Positional dependency	Requires cells that are positionally diverse in origin

by growth and differentiation of the blastema. In this phase, studies of both gene expression and cell–cell interactions indicate that the blastema is functionally equivalent to the developing limb bud (see Reference 2), and thus phase III corresponds to the redevelopment of the limb. Each of the three phases has distinct characteristics that can be investigated individually in the accessory limb model discussed later.

Most of the genes expressed during phases II and III of regeneration are also expressed during limb development. Although the spatial and temporal patterns of expression may vary, their functions are conserved during regeneration and development (see References 2, 25). One exception concerns the expression of *Hoxc-10* in regenerating forelimbs of the axolotl. *Hoxc-10* is expressed in developing hindlimbs and tails, but not in developing forelimbs. During limb regeneration, *Hoxc-10* is reexpressed in hindlimbs and tails, and is also expressed in forelimbs.²⁶ Genes within the HoxC complex are involved in the specification of positional identity along the rostral–caudal axis of vertebrate embryos, and expression during axolotl development is similar to that observed in other embryos.^{26,27} Although the function of *Hoxc-10* in regenerating forelimbs is unclear at this time, its expression suggests the presence of signals in regeneration that are not present in development. Since *Hoxc-10* gene expression is responsive to these signals, which are likely to influence expression of a suite of genes, analysis of the regulation of this gene provides an opportunity to isolate and identify regeneration-specific signals.

Regenerative decline in Xenopus as a model for inducing limb regeneration

Although the limbs of most adult vertebrates do not regenerate, in all examples studied, developing limb buds can regenerate. Regeneration of amputated limb buds has been most extensively investigated in anuran amphibians, in particular, *Xenopus*. Early stage limb buds can regenerate perfectly; whereas, by the end of limb development, all regenerative abilities are lost. Loss of regenerative ability occurs progressively,^{28,29} and is associated with the progressive loss of the ability to reexpress genes involved in growth and pattern formation (e.g. *shh*³⁰). During this period, regenerative ability can be rescued to a limited extent by the application of exogenous FGF (FGF2 and FGF4 in chick and FGF10 in *Xenopus* (see References 31, 32). Exogenous FGFs presumably substitute for the normal function of the apical epidermis in the production of FGFs, an ability that is lost coincident with the loss of regenerative ability.^{33,34} Experimental treatment of amputated *Xenopus* limb buds with FGF10, which is normally expressed in the mesenchyme, induces a number of genes, including *fgf8*, in the epidermis, and partially rescues regeneration of late stage limbs that normally would not regenerate.³² A comparable result is observed in amputated chick limb buds that are supplied with either FGF or a grafted apical epidermis (see Reference 31). Relatively little is known about the expression and function of FGFs in either developing or regenerating limbs of urodeles. *fgf8* and *fgf10* are expressed in both the apical epidermis and

the mesenchyme of regenerating urodele limbs^{35,36} and likely have equivalent functions to those in developing limbs.

The epidermis is also involved in dorsal–ventral pattern formation during limb development, in which expression of *En-1* in the ventral epidermis restricts expression of *Wnt-7a* and *Rfng* to the dorsal epidermis. *Wnt-7a* in turn induces expression of *Lmx-1* in the dorsal mesenchyme (see Reference 37). During regeneration of early stage limb buds in *Xenopus*, the epidermis similarly controls the dorsal–ventral expression pattern of *Lmx-1* and the resultant DV limb pattern. Coincident with the ontogenetic loss of regenerative ability, the epidermis of later stage limb buds loses the ability to control *Lmx-1* expression and dorsal–ventral pattern.³⁸ It is unclear which genes expressed in the epidermis account for this function, since in *Xenopus* *Wnt-7a* and *Rfng* are uniformly expressed in both limb buds and regeneration blastemas.³⁹ The role of the epidermis in pattern formation during urodele limb regeneration has yet to be investigated, though expression of *Rfng* in developing and regenerating newt limbs is reported to be uniform as in *Xenopus*.⁴⁰

Dedifferentiation of c2c12 myotubes *in vitro*

c2c12 cells behave as pluripotent mesenchymal precursor cells that can be induced to undergo myogenesis, adipogenesis, chondrogenesis or osteogenesis in response to different growth and differentiation factors. Of particular significance to regeneration is the observation that when *Msx-1* is expressed in c2c12 myotubes, these multinucleate cells fragment to give rise to mononucleate cells.⁴¹ A similar response is observed when myotubes derived from an urodele (newt) cell line is exposed to serum,⁴² and both are being investigated as model systems for muscle ‘dedifferentiation’ and regeneration. Although the newt cells arrest in the cell cycle, the c2c12-derived mononucleate cells are proliferative and recapitulate the developmental potential of the parent cell line.⁴¹ The ability of *Msx-1* to induce this response *in vitro* is consistent with other lines of evidence indicating that *Msx* transcription factors are important for regeneration *in vivo* in amphibians as well as in mammals.^{16,43–47}

An extract of regenerating limb blastemas also induces formation of proliferative mononucleate cells from c2c12 myotubes *in vitro*,⁴⁸ indicating the presence of blastema factor(s) involved in the control of the differentiated state in a multipotent mesenchymal

stem cell population. Given that *Msx-1* is known to be causally linked to fragmentation,⁴¹ this may indicate that there are factors in blastemas that regulate *Msx* expression. Several signaling molecules are known to have this ability, including members of the FGF, Wnt, and BMP signaling pathways,^{32,49–54} which are obvious candidates for further studies.

Induction of accessory limbs as a model to test for regeneration signals

Classic experiments on the induction of accessory limbs in urodeles clearly demonstrate that fibroblasts, nerves and a WE are necessary and sufficient for limb regeneration.^{55–58} In addition, each of the three phases of regeneration discussed above can be distinguished and studied independently (Figure 2). If a piece of skin (epidermis and dermis) is removed from the lateral surface of a limb, the wound heals and the skin is regenerated (phase I). If a nerve is deviated to the site of a lateral wound, a symmetrical outgrowth composed of undifferentiated cells is induced, but it does not continue to develop, and eventually regresses (phase II). Finally, if a piece of skin from the opposite side of the limb is grafted to a lateral wound along with a deviated nerve, a well-patterned accessory limb develops at the site of the wound (phase III).

Although the phenomenology of accessory limb induction has been well characterized, little is known about the cellular and molecular mechanisms controlling growth and pattern formation. Early genes (*Msx-2* and *Mmp-9*) expressed in response to amputation (phase I) are also expressed in lateral wounds,^{16,18} and thus are not dependent on a deviated nerve supply. Studies are in progress to study gene expression and the origin of the cells that form symmetrical ‘bumps’ in response to a deviated nerve (phase II). Fibroblasts from local connective tissues presumably are the source of cells, and likely are induced to proliferate by factors supplied by the deviated nerve. The final component required to form a limb is a source of fibroblasts with positional characteristics that are distinct from those of the fibroblasts at the host site (skin graft from the opposite side of the limb). Interactions between fibroblast-derived cells with disparate positional values stimulate continued growth and pattern formation,^{20,21} leading to the development of the accessory limb. Phase III outgrowths express genes that are characteristic of late stage regenerating limbs (e.g. *Dlx-3* and *Hoxd-11*). The accessory limb model offers the advantage of being able to test candidate signals in a positive regeneration response assay, and allows

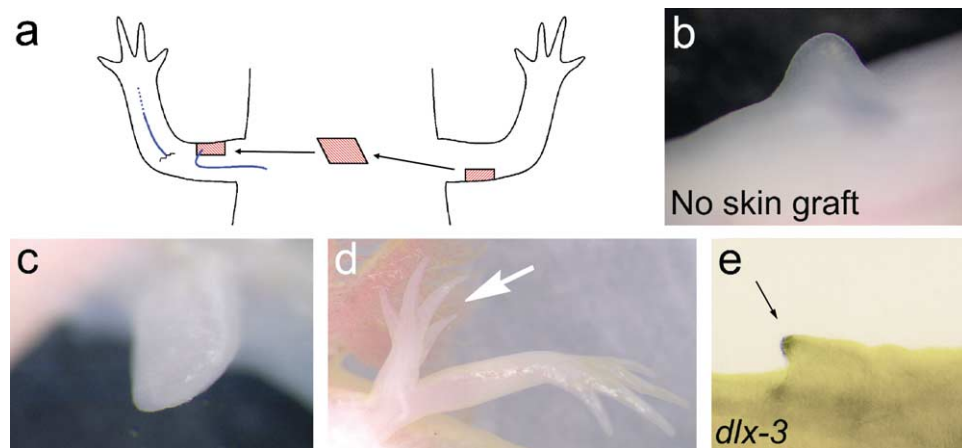


Figure 2. The induction of lateral limbs as a model system to study the three phases of limb regeneration. (a) Three types of lateral wounds can be generated. A piece of skin can be removed (type I), a nerve can be deviated to the site of the wound (type II), and a piece of skin from the opposite side of the limb can be grafted to the site of the wound/deviated nerve (type III). (b) Type II wounds form a symmetrical outgrowth that eventually regresses. (c–e) Type III wounds form an outgrowth that becomes asymmetrical (c) and continues to grow to form a normally patterned accessory limb (arrow in (d)). Type III outgrowths express genes that are characteristic of regenerating limbs (e).

for the identification of the signals that are unique to each of the three phases of regeneration.

Future prospects for understanding regeneration

In nearly every review of amphibian limb regeneration, the authors seem compelled to speculate about the potential for inducing regeneration in humans. For many generations, regeneration biologists have expressed optimism for what has proven to be an illusive goal. It is possible that the opportunities for this generation of biologists are unique in that we are no longer limited to describing the phenomenology of regeneration, but can now alter the biological processes directly. In addition there are techniques to discover all the genes involved in the processes of regeneration (e.g. Reference 1), and to test the function of these genes.^{17,59–61} Given these technological advances, combined with the rich experimental history and the availability of good experimental models, there is reason to be encouraged that we may yet realize the long dreamed of goal of stimulating regeneration in humans.

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