Int. J. Dev. Biol. 40: 785-795 (1996)



# The cellular basis of limb regeneration in urodeles

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**ABSTRACT** **Formation of a regeneration blastema on the amputated urodele limb involves changes in the gene activity of differentiated cells resulting in their histological dedifferentiation and their return to a proliferative state. This review summarizes studies in limb regeneration and in the related fields of tissue repair and limb development that provide new insights into regulatory mechanisms of likely importance in establishing the blastema. Factors required for epithelialization of the wound are briefly described, followed by what is known regarding the biochemistry of extracellular matrix remodeling in the regenerating limb. Cellular "dedifferentiation" is discussed, emphasizing variations in the process among major cell types that give rise to the blastema: fibroblasts, cells of skeletal tissue, muscle cells, Schwann cells, and vascular endothelial cells. Attention is drawn to evidence that cells of connective tissue have a special role in establishing the pre-pattern of the new limb in the early phase of blastema formation and that angiogenesis may be controlled differently during epimorphic regeneration than in the process of wound repair. Several possible sources of the mitogens which stimulate cell cycle re-entry during dedifferentiation are described, as well as evidence suggesting the importance in limb regeneration of one such class of mitogens, the fibroblast growth factors. The trophic effect of nerves required for cells of dedifferentiating tissues to progress through the cell cycle is summarized briefly, along with recent work suggesting how this neural influence is exerted. Finally, the critical role of the wound epithelium in the cellular events forming the blastema and factors that may mediate the epithelial effect are discussed.**

KEY WORDS: *regeneration, angiogenesis, fibroblasts, nerves, transferrin*

## Introduction

Urodeles have the greatest capacity of all vertebrates for regeneration of new body parts. This ability to regenerate has been described for many organs, including lens, retina, intestine, cardiac ventricle, upper and lower jaws, and tail, but the cellular interactions involved have been most thoroughly studied in amputated limbs (see Stocum in this issue). All larval amphibians probably have some ability to regenerate appendages, but the regenerative capacity in adults is restricted to the order Urodela, particularly the aquatic newts and salamanders (Wallace, 1981). The events that lead to perfect replacement for an amputated body part have fascinated developmental biologists for centuries (see Dinsmore in this issue), but current interest in limb regeneration can be attributed to the fact that it includes basic aspects of two very important and normally distinct developmental processes: post-traumatic repair of complex tissues and limb formation during embryonic development. Research on amphibian limb regeneration has provided insights into both of these processes.

This article will review the most important cellular changes and interactions during the early phases of limb regeneration and briefly summarize the current understanding of how the reparative events occurring initially in the amputated limb are extended and converted into the "epimorphic" process that culminates in a new appendage. Recent studies on mammalian tissue repair and embryonic limb formation are obviously relevant to an understanding of limb regeneration, and many inferences from such work are included here. The evidence that patterning mechanisms during amphibian limb regeneration are similar to those in limb development is reviewed by Stocum and by Bryant in this issue.

## What features characterize epithelialization of the wound?

Within an hour of limb amputation basal epidermal cells at the wound edge mobilize by removing desmosomes and extending pseudopodia (Repesh and Oberpriller, 1978, 1980). Migration of the epithelial sheet from the epidermis across the amputation surface depends on interactions between integrins of the

*Abbreviations used in this paper:* WE, wound epithelium; ECM, extracellular matrix; MMP, matrix metalloproteinase; GAG, glycosaminoglycan; PA, plasminogen activator; TIMP, tissue inhibitor of metalloproteinase; TGF, transforming growth factor; FGF, fibroblast growth factor; MRF, myogenic regulatory factor; Id, inhibitor of differentiation; GGF, glial growth factor; IGF, insulin-like growth factor; AER, apical ectodermal ridge.

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0214-6282/96/$03.00

keratinocytes with fibrin and fibronectin in the substrate (Donaldson and Mahan, 1983; Donaldson *et al.*, 1989). Such epithelialization occurs much more rapidly in newts than in humans, possibly because the relevant integrins are expressed constitutively in keratinocytes of urodeles (Donaldson *et al.*, 1995). Wound closure is generally complete within 24 hours of amputation. The newly formed epithelium thickens considerably in the next few days due to continued keratinocyte migration and proliferation, forming the wound epithelium (WE) or apical epithelial cap which is critically important for limb regeneration.

Cells of the WE do not immediately produce a new basal lamina, remaining in direct contact with the fibrin meshwork and with the extracellular matrix (ECM) and cells of the cut tissues (Repesh and Oberpriller, 1980). Ultrastructural evidence indicates roles for this epithelium in extruding inert material from the limb stump and in phagocytosis of fibrin and debris. This function, along with removal of bacteria, is augmented by the action of macrophages and neutrophils in the injured tissues (Singer and Salpeter, 1961; Repesh and Oberpriller, 1980).

## How is the extracellular matrix remodeled?

Amputation trauma elicits a localized process similar in some respects to inflammation in the wound healing response of other vertebrates. Blood loss is usually minimal, due apparently to very rapid vasoconstriction and blood coagulation in urodeles (Schmidt, 1968), and edema normally occurs following epithelial closure of the amputation surface. Leukocytes quickly begin to accumulate and prominent among these are macrophages associated with the injured tissues, the WE, and the subepithelial space (Schmidt, 1968).

Concomitant with the arrival of macrophages is the onset of histolysis and dissociation of injured muscle, connective tissue, nerve sheaths, skeletal elements, and other tissues. As described below, constituent cells revert to a mesenchymal, embryonic appearance as collagen, glycosaminoglycans (GAGs) and other extracellular matrix material of these tissues are degraded. Collagenolytic activity in the amputated amphibian limb was assayed in the pioneering work of Gross (reviewed by Stocum, 1995), but characterization of specific extracellular enzymes has only recently been accomplished. Yang and Bryant (1994) demonstrated that activities of five separate gelatinases or collagenases are elevated in axolotl limb tissues during regeneration. The largest and most rapid increase occurs with a 90-kDa gelatinase similar to mammalian matrix metalloproteinase 9 (MMP-9), an enzyme important for remodeling ECM in many developmental and neoplastic events. Formerly considered "the macrophage gelatinase," MMP-9 is now known to be secreted by several fibroblastic and epithelial cells (reviewed by Matrisian, 1992), suggesting multiple sources for the enzyme in the limb stump. The increase in urodele MMP-9 activity occurs independently of the WE and was also seen during repair of nonlimb tissues (Yang and Bryant, 1994).

As in tissues of other vertebrates, the inflammatory response in urodele limb stumps is likely to involve expression of plasminogen activators (PAs) by macrophages and other cells of the affected tissue (reviewed by Danø *et al.*, 1985; Matrisian and Hogan, 1990). PA secretion leads to the rapid generation of plasmin, a nonspecific serine protease, in the interstitial fluid. Plasmin not only readily degrades most extracellular proteins by itself, but also activates (by removal of "pro" domains) latent MMPs, including collagenases and stromelysins with broad substrate specificities. Thus, the appearance of plasmin produces a cascade of extracellular proteolytic activities which, together with lysosomal enzymes such as elastases and cathepsins, are capable of degrading both fibrillar and nonfibrillar proteins of the ECM (Danø *et al.*, 1985; Matrisian, 1992). Table 1 lists other degradative enzymes secreted by macrophages.

TABLE 1

**SECRETORY PRODUCTS OF MACROPHAGES**

| |
|---|
| Representative degradative enzymes |
| plasminogen activators (tissue- and urokinase-types) |
| matrix metalloproteinases for collagens I, II, III, IV, and gelatin |
| elastase |
| lysozyme |
| hyaluronidase |
| cathepsins B, L, H, N |
| amyloid proteinase |
| lipoprotein lipase |
| acid hydrolases |
| ß-galactosidase |
| ß-glucuronidase |
| acid phosphatases |
| sulfatases |
| amylase |
| Representative growth factors and cytokines |
| fibroblast growth factors |
| platelet-derived growth factor |
| transforming growth factor-ß |
| erythropoietin |
| granulocyte/monocyte colony stimulating factor |
| insulin-like growth factors |
| interleukins-1, -6, -8 |
| tumor necrosis factor -α |
| interferons α, ß, and γ |

From Adams and Hamilton, 1992.

Histolysis normally occurs only in distal areas of the limb stump, a restriction that may involve localization of the PAs to the surfaces of macrophages and other proteolytically active cells (reviewed by Saksela and Rifkin, 1988). As discussed by Woessner (1991), proteolytic activity in the ECM is also regulated by local production of protease inhibitors, including tissue inhibitors of metalloproteinases (TIMPs), PA inhibitors, and other well-characterized factors involved in mammalian tissue remodeling. Moreover, localized activation or secretion of regulatory factors such as transforming growth factor-ß (TGF-ß), which repress MMP expression and elevate levels of TIMP and a PA inhibitor, may also help restrict histolysis to distal tissues.

It is clear however that the accumulation of proliferating mesenchymal cells beneath the WE ultimately reverses the process of tissue dissociation and intensifies production of new ECM during tissue redifferentiation. This shift from degradation to synthesis of ECM components is probably due to the activity of the various growth factors that have been shown to regulate expression of PAs, PA inhibitors, many metalloproteases, and TIMPs (reviewed by Matrisian and Hogan, 1990). The wealth of data on the role of such regulatory factors in the control of ECM remodeling during

mammalian development strongly suggests that similar roles for these factors will be found during the transition from histolysis to growth in urodele limb stumps.

As collagen and other fibrillar proteins are degraded in distal stump tissues, multimeric glycoproteins of the ECM are removed from their characteristic locations and resynthesized more diffusely. This change in the concentration and location of factors important for cell attachment can be expected to have profound effects on the adhesivity, migration, and proliferation of the dedifferentiating cells (reviewed by Raghow, 1994). Fibronectin is removed from its normal sites during histolysis (Repesh *et al.*, 1982), but is expressed by the WE and most mesenchymal cells, becoming very abundant throughout the ECM during growth (Nace and Tassava, 1995). Tenascin, which is restricted primarily to the tendons, periosteum or perichondrium, and basal epidermal granules in urodeles, accumulates like fibronectin throughout the ECM during histolysis and growth (Onda *et al.*, 1990), being synthesized by essentially all the mesenchymal cells (Onda *et al.*, 1991). Laminin disappears as basal laminae of muscle fibers, blood vessels, and Schwann cells are broken down, and is not resynthesized until redifferentiation (Gulati *et al.*, 1983).

Hyaluronan is the principal GAG produced during the first phase of regeneration. Hyaluronan synthesis begins with the onset of histolysis, increases rapidly, and continues throughout the period of growth (Mescher and Munaim, 1986; Toole and Gross, 1971). Unlike the smaller sulfated GAGs, which are synthesized and attached to core proteins in the Golgi, hyaluronan is elaborated by cell membrane enzyme complexes and extruded as extremely large polymers directly into the extracellular space (Prehm, 1989). Moreover, production of hyaluronan is directly linked to the cells' proliferative activity (reviewed by Toole, 1991). In regenerating limbs hyaluronan synthesis is stimulated by certain mitogens (Mescher and Munaim, 1986) and by growing axons (Mescher and Cox, 1989). Histochemical studies indicate the presence of hyaluronan among the mesenchymal cells (Mescher and Munaim, 1986), in areas also rich in fibronectin and tenascin (Gulati *et al.*, 1983; Onda *et al.*, 1990), where its accumulation may be correlated with increased intercellular space (Mescher and Cox, 1988). Based on work in other systems, production of hydrophilic hyaluronan in the limb stump is expected to facilitate cell migration and increase the volume of the growing tissue (Toole, 1991). Synthesis of chondroitin sulfate and other uncharacterized sulfated GAGs also begins during histolysis, but does not become prominent until the onset of redifferentiation (Mescher and Munaim, 1986).

## How does the "dedifferentiation" process vary among cell types in the limb?

Cells of limb tissues undergoing histolysis and ECM remodeling revert to a mesenchymal appearance in a process referred to as "dedifferentiation." Such cells proliferate and accumulate beneath the WE to form a growing mass of cells, the blastema. Although they are relatively homogeneous cytologically, blastema cells are derived from different tissues, and heterogeneity can be detected immunohistochemically using monoclonal antibodies as cell markers (reviewed by Stocum, 1995).

The molecular basis of dedifferentiation is poorly understood, but this situation may change rapidly with new understanding of the interplay between proliferation and differentiation in mesenchymal cell lineages, particularly myoblasts, at the molecular level. Certain mitogens, such as the fibroblast growth factors (FGFs), have been shown to repress myogenesis by various functionally redundant mechanisms: by stimulating phosphorylation of myogenic regulatory factors (MRFs); by inducing expression of the helix-loop-helix protein Id *(inhibitor of differentiation)* which inactivates MRFs; and by inducing expression of certain immediate early gene products that also repress the effects of MRFs (reviewed by Olson, 1992). Mitogens released during histolysis in amputated urodele limbs (see below) are likely therefore to repress differentiation as well as elicit proliferation in cells derived from muscle and other tissues.

During dedifferentiation in newt limb stumps, expression of the homeobox gene *msx-1* is strongly upregulated, and MRF-4 expression in muscle is turned off until the onset of myogenesis in the regenerate (Simon *et al.*, 1995). As discussed below, *msx-1* is also expressed in the mesenchyme of embryonic limb buds under the influence of FGFs (Fallon *et al.*, 1994). Moreover, expression of *Id* colocalizes with that of *msx-1* in the developing limb (Muneoka and Sassoon, 1992). Current work strongly suggests therefore that both the signaling factors and the transcription factors involved in dedifferentiation and the renewal of cell cycling in limb regeneration are similar to those controlling the balance between growth and differentiation in limb buds.

Classic work in limb regeneration using grafts of marked tissue suggests that each tissue of the stump probably contributes most of the cells for the corresponding tissue in the regenerate, and, despite the embryonic appearance of blastema cells, there is no evidence that extensive metaplasia or transdifferentiation is involved in normal blastema development (reviewed by Wallace, 1981). Because crossover among the major cell lineages does not appear to be widespread during normal limb regeneration, it is instructive to review the information available on the origin of blastema cells from the individual limb tissues.

### *Fibroblasts*

Cells referred to collectively as fibroblasts populate connective tissue in the dermis, the sheaths of muscles and nerves, and the outer layers of large blood vessels. There is considerable evidence that certain fibroblast populations make the most important contribution to the initial regeneration blastema, both quantitatively and qualitatively. Using a triploid axolotl cell marker, Muneoka *et al.* (1986) found that cells from the dermis comprise nearly half of the cell population in upper limb blastemas. This cellular contribution to the blastema is more than twice that expected based on the percentage of dermal fibroblasts in the total number of mesodermal cells in the upper limb. The origin of the other blastema cells counted by Muneoka *et al.* (1986) could not be determined, but many of them are also likely to have arisen from stump connective tissues since the dermis contains only about half of all fibroblasts present in axolotl limbs at this level (Tank and Holder, 1979).

Schmidt (1968) compared the proliferation and migration of fibroblasts in forming the early blastema to the immigration of these cells in forming granulation tissue during mammalian tissue repair. The significance of the major fibroblast contribution to the blastema is not completely understood, but it is clear from grafting experiments that cells of connective tissues are major determinants of positional information during regeneration and are capable of position-dependent growth. Bryant *et al.* (1987) have reviewed

numerous studies on transplanted dermis and other limb tissues which indicate that fibroblasts alone have the capacity to provoke position-dependent formation of supernumerary limbs. These authors have proposed that fibroblasts migrating from stump connective tissues to form the early blastema interact with one another according to their positions, giving rise to an "outline" of the limb pattern (Gardiner *et al.*, 1986; Bryant and Gardiner, 1989). While establishing the pattern for the new growth, these cells would also interact with cells of the other lineages in the limb stump to direct their locations and differentiative pathways during histogenesis. According to this view, the framework of positional information produced by fibroblasts from the stump acts as a "scaffold" for the interaction of other cell types as the distribution of new tissues in the regenerate is established (Muneoka and Sassoon, 1992).

Clear evidence for the self-organizational capacity of dermal fibroblasts comes from studies using axolotl limbs with X-irradiated internal tissues and grafted normal skin (Holder, 1989). Most regenerates from such limbs lacked musculature either completely or partially, but nevertheless showed a remarkably normal pattern of connective tissue components, including skeleton, retinaculae, digital tendons and aponeuroses. Such work indicates that dermal fibroblasts can autonomously give rise to all connective tissue components in their proper arrangement and suggests strongly that such cells may play a role in patterning other tissues during regeneration.

***Vascular endothelial cells***

Chalkley (1954) observed that blood vessels of the newt limb stump contribute very few cells to the early regeneration blastema. Arteries, veins, and capillaries in the distal area of the stump dissociate during the histolytic phase of regeneration, with blood flow restricted to more proximal regions. Histological analyses and vascular injection studies with adult and larval urodele limbs agree that the distal preblastemic area of dedifferentiated cells and the early blastema are essentially free of functional blood vessels (Peadon and Singer, 1966; Iten and Bryant, 1973; Revardel and Chapron, 1975; Smith and Wolpert, 1975). The work of Peadon and Singer (1966) indicates that capillaries do not begin to penetrate proximal regions of the dedifferentiated zone until a growing blastema is well-formed (Fig. 1). Vascular ingrowth begins peripherally near the skin of the stump, then gradually continues toward the center of the blastema. The distal region near the WE is not vascularized until the cone stage of blastemal growth.

The relationship between the vascular supply and cellular survival and growth has been studied extensively in tumors, where it is well-established that growth beyond a specific size does not continue without invasion of capillaries from surrounding tissue. It is estimated that no cell in a rapidly growing tumor is more than about 100 μm from the nearest capillary (reviewed by Blood and Zetter, 1990; Sutherland, 1988). Extensive angiogenesis also characterizes normal tissue repair in mammals, transiently forming more capillaries than were present in the uninjured tissue to support the demands of the healing process for nutrients and oxygen (revs. Niinikoski, 1980; Arnold and West, 1991). Embryonic limb buds have well-developed capillary networks surrounded by a subectodermal zone of avascular mesenchyme (reviewed by Caplan, 1985).

Given the importance of angiogenesis for tissue growth, the early period of limb regeneration, in which distal areas most active in cell proliferation remain poorly vascularized and sprouting

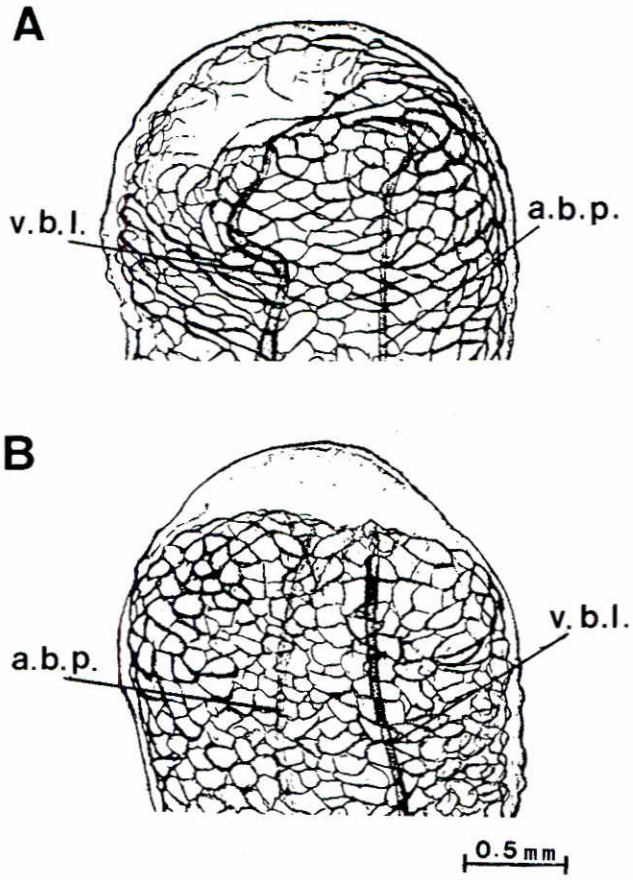


**Fig. 1. Drawings of whole-mount preparations of dye-injected and cleared regenerates of adult newt forelimbs, reproduced with slight modification from Peadon and Singer (1966).** *The scale is approximate and abbreviations are: a.b.p., artery brachialis profunda; v.b.l., vein brachialis lateralis.* **(A)** *Regenerate of right limb at late dedifferentiation stage (Iten and Bryant, 1973), tilted anterior view, showing blood vessels in the wound area arranged as the rim around a central "crater" of avascular tissue. The apparent crater is filled with dedifferentiated mesenchymal tissue and is capped by a thickened wound epithelium.* **(B)** *Regenerate of left limb in early bud stage (Iten and Bryant, 1973), flat anterior view, showing the avascular blastema and the edge of the rim of vessels at the approximate level of amputation. Vascular sprouting into proximal levels of the blastema has begun at this stage but is not visible from this angle.*

capillaries appear to "avoid" the rapidly growing blastema (Peadon and Singer, 1966), appears to be somewhat unusual. The blastema is however well-supplied with regenerating axons which emerge from nerves in the stump, course throughout the mesenchymal tissue and penetrate the overlying epithelium (reviewed by Singer, 1952). As described below, these axons are critical for blastemal cell proliferation, and recent studies indicate that they can supply growth requirements that are otherwise provided via capillaries (reviewed by Mescher, 1992). Release of trophic factors from axons, together with the respiratory exchange across the skin of aquatic urodeles, may explain the ability of the early blastema to grow rapidly in the absence of a blood supply.

Why the early blastema does not become vascularized like tissues undergoing simple repair is not known. Given the apparent similarities between the initial period after amputation and the inflammatory phase of mammalian wound healing, such as immi-

gration of leukocytes and remodeling of the ECM, it is likely that various factors are released in injured stump tissues that would normally stimulate capillary ingrowth (reviewed by Sunderkotter *et al.*, 1994). Yet angiogenesis is delayed until the blastema is well formed. The failure of capillaries to grow immediately into dedifferentiating tissues could involve localized accumulation of hyaluronan during this period. It is known that hyaluronan inhibits proliferation of vascular endothelial cells even in the presence of angiogenic factors (Arnold and West, 1991) and that local concentrations of hyaluronan are important in determining where blood vessels form during limb ontogeny (Brand-Saberi *et al.*, 1995). However, control of capillary growth during tissue repair involves a complex interplay of signaling factors and several ECM components, with a fine line apparently separating angioinhibition and angiogenesis (reviewed by Polverini, 1994). It is not surprising given the elementary state of knowledge of such processes in mammalian tissues that nothing is known of these activities in urodele repair or regeneration.

Delayed angiogenesis of distal areas during the early phase of limb regeneration could be important for subsequent epimorphic growth. The processes of dedifferentiation, reformation of the ECM to facilitate cellular migration, and "patterning" interactions among the cells of the early blastema may involve changes incompatible with endothelial cell differentiation. The result would be inhibition of capillary ingrowth until the cellular migration and interactions required to produce the regeneration blastema are accomplished.

### *Cells of skeletal tissue*

Following amputation the bones in adult urodeles and the cartilaginous elements of larval limbs are slowly eroded at the cut site by monocyte-derived osteoclasts or macrophages. Osteocytes and chondrocytes thus freed from their lacunae may contribute to the blastemal population, but probably only to a minimal extent (Muneoka *et al.*, 1986). Instead, the majority of cells that give rise to skeletal tissue in the new limb appear to arise from precursor cells in the periosteum or perichondrium (Chalkley, 1954; Hay and Fischman, 1961). These tissues undergo less complete destruction than the skeletal matrix and release cells that proliferate and migrate into the blastema. As blastema growth proceeds distally, these cells aggregate and differentiate as chondroblasts to form proximal cartilaginous elements continuous with the cartilage or bone in the stump. In adults cartilage elements of the new limb are gradually replaced by ossification to produce typical bones.

Under experimental conditions other cells of the limb can produce cartilage in the regenerate. If the humerus is removed prior to amputation through the upper arm, skeletal tissue is present in the regenerated limb but is not reformed in regions proximal to the level of amputation. In this situation chondroblasts producing the new skeleton apparently arise from "fibroblasts" in various connective tissues, including the dermis (Dunis and Namenwirth, 1977). Similarly, Holder (1989) has shown that cells of unirradiated dermis grafted onto X-irradiated limb stumps can give rise to the complete skeleton of the regenerate. It appears therefore that in the absence of viable skeletal elements in the stump, other connective tissue cells acquire the ability during "dedifferentiation" to become chondroblasts in the blastema.

### *Muscle cells*

Myogenic cells of the blastema are clearly derived from the stump musculature, possibly from the multinucleated fibers as well as from a population of muscle progenitor cells (Stocum, 1995). Histological analyses suggested that the proliferating myogenic cells arise from "mononucleated fragments" of dissociating muscle fibers (Thornton, 1968; Hay and Fischman, 1961), but Schmidt (1968) seriously questioned this interpretation of the data on technical grounds. Recent evidence in favor of this hypothesis has been reported by Lo *et al.* (1993) who doubly labeled multinucleate myotubes derived from newt limbs *in vitro* with $^{3}$H-thymidine and fluorescent dextran. After pelleting and implanting the myotubes into limb stumps, individual doubly-labeled cells could be detected histologically days later in the blastemas, primarily in the mesenchyme around the central chondrogenic mass. Label did not persist through the period of muscle fiber redifferentiation. The results suggest that partially differentiated myotubes from newt cells can revert to myoblasts, which is consistent with the concept that multinucleated muscle fibers dedifferentiate and give rise to myogenic cells during limb regeneration.

Hinterberger and Cameron (1990) have reviewed evidence indicating that muscle of the regenerated limb also develops from myogenic reserve cells resembling satellite cells, which are the primary source of new myotubes during muscle repair in other vertebrates. These putative reserve cells could be selectively labeled with $^{3}$H-thymidine in explanted newt limb muscle and their progeny traced during their fusion into myotubes (Cameron *et al.*, 1986). The observation that such myotubes are also labeled with a monoclonal antibody specific for both blastema cells and myoblasts further supports the hypothesis that cells similar to satellite cells can contribute to the myogenic population of the blastema.

### *Schwann cells*

Axons from nerves in the limb stump grow profusely throughout the developing blastema. The general dissociation of the injured tissues includes the nerves, with breakdown of the connective tissue layers and loss of the myelin sheaths covering axons. Macrophages that invade the dissociating nerves become engorged with lipid as they engulf myelin during dedifferentiation of the Schwann cells. Dedifferentiated Schwann cells and fibroblasts of nerves proliferate (Hay and Fischman, 1961) and contribute cells to the blastema (Chalkley, 1954), but probably remain associated with the regenerating axons and begin to form myelin again in nerves of the stump once the blastema is formed (Schmidt, 1968). Cells identified as Schwann cells through use of a monoclonal antibody marker are first seen only in proximal regions of the blastema in adult newts (Ferretti and Brockes, 1991).

## How is cell proliferation re-initiated?

During histolysis and dissociation of stump tissues, cells that will migrate distally and contribute to the blastema either re-enter the cell cycle or, in the case of larval limbs, increase their rate of cell cycling. Classic work demonstrated that proliferation is an integral part of cellular dedifferentiation in the urodele limb stump, and recent research has provided important insights into how cellular activities leading to either differentiation or proliferation can be regulated. As discussed earlier, investigations of lineage-specific transcription factors such as those of the MyoD family have indicated several mechanisms by which activation or maintenance of a differentiation program is opposed by signaling pathways triggered by mitogenic polypeptides (reviewed by Olson, 1992). The production of additional probes for urodele homologues of such regulatory factors can be expected to lead to rapid progress in

understanding the dedifferentiation process in limb regeneration.

The renewal of cell proliferation during dedifferentiation is triggered by the injury and does not depend on the tissue interactions required to produce the regeneration blastema. The percentage of cells replicating DNA during the first few days after amputation is not affected by removal of either the nerve supply (Tassava *et al.*, 1974; Mescher and Tassava, 1975; Maden, 1978) or the WE (Mescher, 1976; Loyd and Tassava, 1980). Specific factors stimulating the onset of proliferation in the limb stump have not been identified, but work on mammalian tissue repair suggests that several sources for such mitogens will be found.

First, various mitogenic factors are released during blood coagulation, including members of at least three growth factor families released during platelet degranulation, and kinins, which are derived directly from plasma kininogen within seconds of extravasation (Dvorak *et al.*, 1988). Kinins have been proposed as a stimulus for the transient rise in inositol phosphate levels that occurs in cells at the site of injury during the first five minutes postamputation in larval axolotl limbs (Tsonis *et al.*, 1991). The injury-triggered increase in inositol triphosphate, an important component for intracellular signaling, may be critical for subsequent mitogenesis or other initial activities in regeneration, since inhibiting this increase with beryllium ion administered for five minutes or less at amputation also blocks regeneration (Thornton, 1968; Tsonis *et al.*, 1991).

Growth factors are also stored within the ECM or in cells and are released during matrix breakdown and cell injury, becoming available to promote the proliferative aspects of tissue repair (reviewed by Raghow, 1994). FGFs and other heparin-binding growth factors are concentrated at pericellular matrix sites containing heparan sulfate proteoglycans throughout connective tissues and basal laminae. Degradation of matrix proteins during inflammation releases FGF bound to heparan sulfate to trigger growth in neighboring cells. That exogenous FGF can stimulate growth of blastema cells has been known for many years (reviewed by Gospodarowicz and Mescher, 1980). Boilly *et al.* (1991) have reported the presence of FGF-1 in extracts of both mesenchyme and WE from young axolotl blastemas. Immunohistochemistry using either antisera against bovine FGF-1 (Boilly, 1989) or against a synthetic decapeptide representing the amino terminal of FGF-2 (Mescher, unpublished) indicates the diffuse presence of this factor on cells and in the ECM throughout the blastema, the WE, and in proximal tissues of the limb. Using *in situ* hybridization on sections of newt blastemas, Poulin *et al.* (1993) demonstrated that the receptor specific for FGF-1, -2, and -4 is expressed in all mesenchymal cells, while cells of the WE and perichondrium express a receptor specific for FGF-1 and FGF-7 (keratinocyte growth factor). Collectively these data strongly suggest that FGFs have important roles as mitogens or regulators of other activities during regeneration.

A third source of growth factors during the initiation of limb regeneration is likely to be the macrophages infiltrating distal areas of the limb stump. In addition to secreting enzymes, many of which degrade ECM components and release matrix-bound mitogens, mammalian macrophages are one of the most important sources of growth factors in wound repair (reviewed by Rappolee and Werb, 1992). Some macrophage-derived growth factors are listed in Table 1. Although this has not been examined in urodeles, similar roles for macrophages can be expected in the amputated limb.

Many of the polypeptide growth factors released from platelets, the ECM, injured cells, and macrophages have been shown to stimulate proliferation in the same target cells. The fact that growth factors with apparently overlapping mitogenic effects are released from many sources during inflammation may reflect the adaptive value of such redundancy in promoting reliable and efficient wound repair during the evolution of multicellular animals.

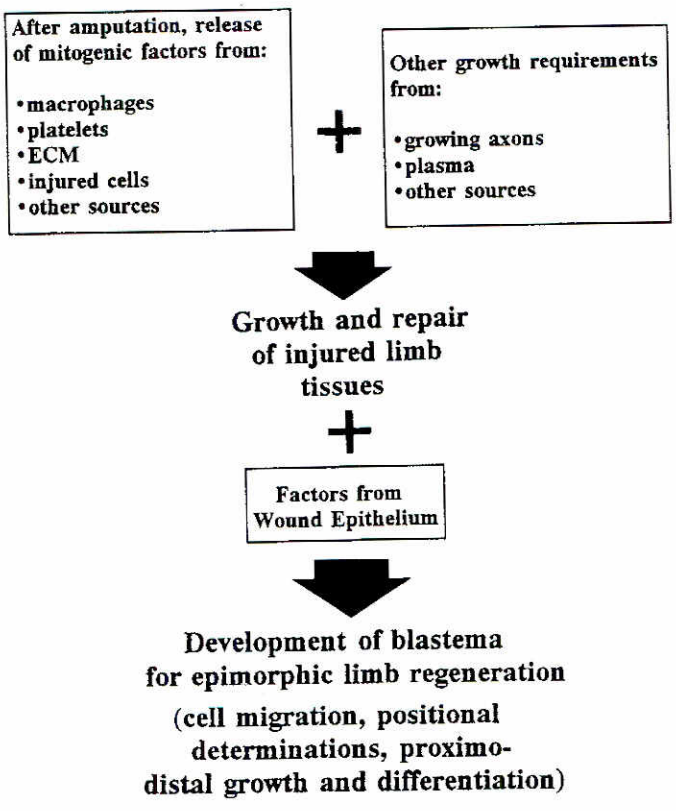


**Fig. 2. Sources of growth-promoting factors for tissue repair and blastema growth after amputation of urodele limbs.**

## How do nerves provide their trophic effect?

That urodele limb regeneration requires an adequate supply of nerves in the appendage has long been known. Work reviewed by Singer (1952, 1978) shows that any type of nerve can support limb regeneration and that nerves affect only mitotic activity in the blastema, not pattern formation or histogenesis. As indicated above, nerves are not required for dedifferentiation of cells in the stump. Moreover, once a sufficiently large blastema has been formed and redifferentiation has begun proximally, nerves can be removed and formation of a new limb will nevertheless proceed (reviewed by Singer, 1952). Results from a wide variety of studies indicate the growth-promoting activity is mediated by protein(s) transported and released from axons (reviewed by Carlone and Mescher, 1985; Wallace, 1981). Regeneration of limb buds and "aneurogenic" limbs (discussed below) suggest that synthesis of the protein is widespread in the embryo and is not unique to nervous tissue (Singer, 1978). The trophic factor(s) has not been purified nor its gene cloned, primarily due to the lack of a simple and reproducible bioassay for the effect on blastemal growth.

Important information about the nature of the neural agent emerged from work designed to elucidate more clearly the nerve-dependent activities in the cell cycle (reviewed by Tassava *et al.,* 1987). If adult or larval forelimbs are completely denervated by nerve transection near the brachial plexus, either at the time of amputation or a few days earlier to allow complete elimination of axonal factors, tissue dedifferentiation still occurs and a normal percentage of cells re-enter the cell cycle and incorporate $^3$H-thymidine (Mescher and Tassava, 1975; Maden, 1978). However the onset of mitotic activity is severely inhibited in such limb stumps and no blastema forms. DNA microspectrophotometry indicates that the $^3$H-thymidine incorporation does represent replication rather than repair and that cells do not accumulate in either $G_1$ or $G_2$ (i.e., with either 2C or 4C DNA content) (Mescher and Tassava, 1975; Loyd and Connelly, 1981). The data suggest that the dedifferentiated cells fail to progress through the S phase of the cell cycle. The lack of mitotic activity followed by the apparent removal of dedifferentiated cells in amputated larval limbs results in morphological resorption of the limb if the denervated state is maintained (reviewed by Singer, 1952), although the fate of cycling cells in limbs denervated at amputation remains unknown. When denervation is delayed until the late bud or cone stage of regeneration, blastema cells complete mitosis and accumulate in the $G_1$ phase of the cell cycle (Maden, 1979; Loyd and Connelly, 1981).

Taken together the blastema cell cycle studies suggest that the neural effect on cell proliferation in the early, nerve-dependent phase of regeneration involves substances needed to sustain cell cycling, rather than mitogens triggering cells to enter the cycle. This is consistent with Singer's (1978) conclusion, after reviewing the effects of denervation on metabolic and synthetic activity in blastema cells, that nerves affect the *rate* of cellular activity without directly influencing gene expression or the *nature* of what the cells are doing. Muneoka *et al.* (1989) reviewed other aspects of the neural effect on blastema growth and concluded that axons do not release a factor that *regulates* cell proliferation, but provide one or more growth requirements necessary for proliferation-competent cells to progress through the cell cycle.

The trophic effect of nerves has been proposed to involve various mitogenic factors associated with this tissue, such as FGFs (Gospodarowicz and Mescher, 1980) and glial growth factor (GGF; Brockes and Kintner, 1986). FGF-1 is abundantly expressed in both sensory and motor neurons (Elde *et al.,* 1991), but whether it is transported and secreted are not known (reviewed by Mason, 1994). Members of the neuregulin family, including GGF, have been shown to have various activities in developing nerves (Jo *et al.,* 1995). Present understanding of the roles of neural FGFs and neuregulins is very primitive, and it is unclear whether the functional repertoire of these signaling proteins includes the permissive or nutritive trophic effect suggested by analyses of nerve-dependent blastema activities (Muneoka *et al.,* 1989). Moreover, since several mitogenic factors including FGFs are thought to be released in the early regenerate from multiple sources, it is not clear how axonal release of additional, redundant mitogens could lead to the dependence on nerves for blastema growth.

Peripheral nerves, particularly regenerating nerves, also contain high concentrations of nonregulatory trophic factors required to sustain cell proliferation, such as insulin-like growth factors I and II (IGF-I, -II) (Hansson, 1993) and transferrin (Oh and Markelonis, 1984). Such proteins are also abundant in plasma and are delivered to cells by capillaries, although the IGFs are also paracrine factors synthesized by many cells in injured tissue (reviewed by Sara and Hall, 1990). We have suggested that release of such proteins from regenerating axons is important for local cell proliferation in avascular tissues with limited access to plasma-derived factors (Mescher and Munaim, 1988; Mescher, 1992). Dedifferentiated cells in the distal amphibian limb stump and the early blastema would be expected to have reduced access to plasma proteins because of the avascularity and proteolytic extracellular environment in these tissues. Axonal transport and release of plasma factors required for growth would explain both the nerve-dependence of proliferation in the early regenerate and the reduced neural dependence for growth during the late bud or cone stage, when proteolysis is inhibited and vascularization is underway throughout the blastema.

An absolute requirement for growth of all animal cells, transferrin is an 80 kDa plasma protein that transports two atoms of ferric iron. Iron is obtained by cells via receptor-mediated endocytosis of transferrin, which is followed by recycling and release of apotransferrin capable of further rounds of iron binding and delivery. Uptake of ferric iron is absolutely necessary for cell proliferation, primarily because this metal is required as a cofactor for activity of ribonucleotide reductase, the rate-limiting enzyme for DNA replication, and for synthesis of mitochondrial cytochromes (reviewed by Kühn *et al.,* 1990). In early embryos transferrin is synthesized locally and supplied in an autocrine or paracrine manner to cells of many developing organs, but as development proceeds production becomes increasingly restricted to the yolk sac and liver and to specific cells with "trophic" functions in organs having blood-tissue barriers, e.g., Sertoli cells, glial cells and ependyma (Welch, 1992). Several laboratories have shown that transferrin is abundant in peripheral nerves (reviewed by Mescher and Munaim, 1988).

Inhibiting uptake of iron-transferrin by growing cells *in vitro* causes the cells to arrest rapidly in a manner similar to that observed with cells of denervated early blastemas: in the S or $G_2$ phases of the cell cycle (Kühn *et al.,* 1990). The presence of transferrin in peripheral nerves and the similarity between this factor's role in cell proliferation and the neural influence on blastema cell growth have prompted several tests of the hypothesis that transferrin mediates the nerve's effect in regeneration.

Transferrin is present in neurons and Schwann cells of axolotl sciatic nerves and becomes concentrated over 20-fold in the nerve during limb regeneration (Kiffmeyer *et al.,* 1991). As predicted for the trophic factor, transferrin undergoes anterograde fast transport in axons and is released distally at growth cones (Mescher and Kiffmeyer, 1992). Denervation results in a 50% reduction of the transferrin content in the distal half of the blastema, concomitant with the decrease in proliferative activity (Kiffmeyer *et al.,* 1991). The blastema growth-promoting activity of brain tissue extracts *in vitro*, investigated by several laboratories (Carlone and Mescher, 1985), is completely lost by removing either iron (Munaim and Mescher, 1986) or transferrin (Mescher and Connell, unpublished) and is completely restored by their readdition.

Urodele limbs that develop in the absence of the nerve supply nonetheless regenerate, paradoxically, after amputation (reviewed by Wallace, 1981). Limb buds also regenerate with a nerve supply much smaller than that normally needed to promote blastema growth, suggesting that the factor(s) which nerves provide during limb regeneration is already present locally in the embryonic limb (Singer, 1978). One explanation for regeneration of "aneurogenic"

limbs therefore is that cells of these limbs, unlike those of normal limbs, continue to produce the required factor(s). We have found that transferrin is synthesized in developing limbs and that this synthesis is important for the regenerative ability of such limbs (Mescher, Overton and Clarkson, in preparation). Experiments are currently in progress to test the hypothesis that endogenous transferrin production is involved in the regenerative capacity of aneurogenic limbs.

The evidence reviewed briefly here makes transferrin an excellent candidate for a factor mediating the neural effect on blastema growth. IGF-I, another trophic factor stimulating cellular metabolism and cell cycle progression, is also transported in peripheral nerves (Hansson, 1993) and may be important in limb regeneration if released from growing axons and not otherwise available in the blastema. Moreover, since information about intercellular signaling during dedifferentiation and blastema growth is currently almost non-existent, the possibility remains that other mitogenic or regulatory factors released by axons may also play growth-promoting roles.

## What role does the wound epithelium play in blastema formation?

The tissue interaction that is perhaps most critical for epimorphic regeneration and which is unique to urodeles among adult amphibians is the effect exerted by the WE on the underlying mesenchymal cells. This epithelial influence changes the course of the proliferative activity in the injured tissues of the amputated limb from a process of repair to one of epimorphic regeneration. The WE causes cells to proliferate more extensively than required for simple repair (Mescher, 1976) and to accumulate distally beneath the epithelium (Thornton, 1968). In many respects this effect is similar to that of the apical ectodermal ridge (AER) in producing a progress zone for mesenchymal outgrowth during ontogenic limb development.

Secretion of FGFs has been strongly implicated in the AER effect. Both *fgf-2* (Savage *et al.*, 1993) and *fgf-4* (Niswander *et al.*, 1993) are expressed in chick AERs, and exogenous applications of FGF-2 (Fallon *et al.*, 1994) or FGF-4 (Niswander *et al.*, 1993) support continued growth and development of limb buds after removal of the AER. Niswander and Martin (1993) found with mouse limb buds that other FGFs also supported growth after ridge removal. FGF-2 application has also been shown to allow regeneration of complete limbs in buds amputated at the prospective zeugopodium (Taylor *et al.*, 1994).

Fallon *et al.* (1994) have shown further that FGF-2, like the AER, also promotes expression of the homeobox gene *msx-1* in neighboring mesenchymal cells of the limb bud. *Msx-1* is expressed in various embryonic regions where ectoderm and mesoderm interact to produce progress zones of proliferating cells. FGF-4 in combination with retinoic acid also activates expression of polarizing signals such as sonic hedgehog, which induces expression of other signaling factors such as bone morphogenetic protein 4 in mesoderm and FGF-4 in ectoderm. The data suggest the existence of a positive feedback loop operating between cells of the mesenchyme and the overlying AER to coordinate patterning events with growth in the limb bud (Laufer *et al.*, 1994; Niswander *et al.*, 1994). The chick AER also induces synthesis of IGF-I mRNA in the subridge mesenchyme, an effect that may also be produced by epithelial FGFs (Dealy and Kosher, 1995).

Similar mechanisms involving FGFs are likely to operate between the WE and blastema mesenchyme in urodele limb regeneration. Immunoreactive FGF is clearly present in the WE (Boilly, 1989; Mescher, unpublished) and implants of FGF-2 have been shown to maintain full mitotic activity of dedifferentiated cells in the absence of the WE (Chew and Cameron, 1983). Exogenous FGF-2 also stimulates hyaluronan secretion by dedifferentiated newt limb cells (Mescher and Munaim, 1986) and by mesenchymal cells of chick limb buds (Munaim *et al.*, 1991). Stimulation of a hyaluronan-rich matrix in adjacent mesenchyme thus represents a mechanism by which epithelial FGF could positively influence cellular migratory activity in addition to the effect on proliferation. Moreover, both mesenchymal cells of limb buds (Muneoka and Sassoon, 1992) and blastemas (Crews *et al.*, 1995; Simon *et al.*, 1995) express the *msx-1* homeobox gene and downregulate expression of myogenic factors during the period of limb outgrowth. In the chick distribution of *msx-1* transcripts is initially widespread throughout the limb bud, but soon becomes restricted to the region underlying the ridge. By analogy with the AER (Fallon *et al.*, 1994), *msx-1* expression in dedifferentiating cells and their progeny in the blastema is also likely to be controlled by FGFs derived from the WE.

As discussed by Simon *et al.* (1995), *msx-1* expression in embryonic progress zones including that of limb buds overlaps expression of *Id*, which represses activity of myoD-like transcription factors and inhibits myogenesis. Signaling factors of the FGF family secreted by the WE may therefore not only positively regulate cell proliferation, but also induce expression of transcription factors which prevent redifferentiation and maintain the "dedifferentiated" state. The ability of urodeles to heal amputated limbs with epithelia functionally similar to the AER of vertebrate limb buds is clearly of key importance in their capacity for epimorphic regeneration.

## Conclusions

Twenty years ago Tassava and Mescher (1975) summarized existing information on the cellular interactions leading to blastema formation in terms of the cell cycle. Dedifferentiation and the onset of proliferation in injured tissues of the limb stump were shown to be effects of the injury alone. The requirement for nerves was proposed to involve axonally released factor(s) that allowed growth-competent cells to progress through the cell cycle and complete mitosis. The role of the WE in this model was to promote continued proliferation leading to a blastema and to prevent cells from redifferentiating in a process of tissue repair. New knowledge acquired on several fronts, particularly on the signaling roles of polypeptide growth factors during tissue repair and limb development, has not only validated this model but has also elucidated mechanisms which could constitute the molecular basis of blastema formation. The major sources of factors important for regeneration are summarized in Figure 2.

Studies of tissue repair indicate that macrophages arriving at the site of injury are critically important for subsequent repair, and one can predict that similar roles will be found for these cells in the initial events of limb regeneration. Macrophages are a major source of enzymes and regulatory factors through which the ECM of injured tissue is remodeled in a controlled manner, as well as an important source of growth factors. Mitogens with activity apparently redundant to those secreted by macrophages are also released from platelets, injured cells, and the ECM. Remodelling of

the ECM and the onset of cell proliferation and migration lead to the mesenchymal appearance of distal limb stump tissues. New work on the molecular basis of myogenesis indicates that mitogens also induce expression of transcription factors which inhibit differentiative activity at the level of the gene.

The WE, which is functionally similar to the embryonic AER (Stocum, 1995), is likely to promote continued cycling and inhibit redifferentiation in neighboring cells by production of FGF signaling factors which maintain localized expression of transcription factors such as *msx-1*. The distal progress zone established under the influence of the WE remains avascular throughout the period of blastema growth. This avascularity, possibly abetted by extracellular protease activity, may lead to the dependence on local nerves for factors needed by blastema cells to complete the cell cycle. Axons regenerating in the blastema are an abundant source of transferrin, a requirement for cell cycle progression normally provided by capillaries.

The first cells in the blastemal progress zone appear to be fibroblasts migrating from dermis and other connective tissues. These cells are important repositories of positional information and interact to establish the prepattern or "scaffold" for proper patterning during subsequent blastema development (Muneoka and Sassoon, 1992). Though transdifferentiation of regenerating limb tissues can be observed in certain experimental situations, such as limbs from which bones have been removed, there is little evidence for metaplasia in normal regeneration. Cells of the myogenic, chondrogenic, and Schwann lineages, along with vascular endothelial sprouts, redifferentiate proximally in continuity with the proper tissues of the stump as growth extends the progress zone distally. The effect of the WE to induce and maintain the blastemal progress zone, in which patterning events occur that direct subsequent histogenesis, is critical for the unique regenerative capacity of urodeles.

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