Urodele Limb Regeneration: Mechanisms of Blastema Formation and Growth

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Introduction

The ability of larval and adult urodeles to regenerate the complex spatial organization of amputated limb segments has been known since the experiments of Spallanzani [1] in the sixteenth century. Limb regeneration is accomplished by the histolysis of tissues at the amputation site to release resident stem cells, as well as differentiated cells that undergo dedifferentiation to progenitor cells. These cells accumulate under the wound epidermis to form a regeneration blastema that grows and self-organizes into the tissue patterns and morphological shapes of the amputated structures. Blastema formation and growth requires early signals mediated by amputation and by the wound epidermis that lead to histolysis, as well as subsequent interactions between regenerating nerve axons and wound epidermis that drive blastema cell accumulation and proliferation. These signals and interactions are the subject of this chapter.

Stages of Limb Regeneration

Figure 1 illustrates the stages of a regenerating urodele (larval Ambystoma) limb [2]. Within 24 h after amputation (depending on limb size), epidermal cells migrate over the wound surface to provide a thin epithelial sheet that thickens within 3–4 days to form an apical epidermal cap (AEC) several layers thick in the center of the amputation surface. Undifferentiated mesenchymal cells derived by the histolysis of dermal, nerve sheath, and muscle and skeletal tissues accumulate under the AEC to form the accumulation blastema or early bud. The outer layers of the AEC are protective, whereas its basal layers appear to be equivalent to the outgrowth-promoting apical ectodermal ridge (AER) of amniote embryonic limb buds [3].

Regenerating motor, sensory and sympathetic axons, as well as capillaries, penetrate into the forming blastema, with sensory axons reaching the AEC by the early bud stage. Eventually regenerating motor axons will innervate developing muscle, and sensory and sympathetic axons will innervate the skin, skeletal structures, and blood vessels. Following reinnervation of the AEC, the early bud grows rapidly to a conical medium bud stage. As the blastema continues to grow through late bud and redifferentiation stages, its cells self-organize the patterns of differentiation that replicate the amputated limb parts. While the growth and differentiation of the blastema appears similar to embryonic limb bud development, the requirements for mesenchymal proliferation in the two are not the same. Blastema cell proliferation is dependent on signals generated by interaction between the AEC and the regenerating nerves, whereas proliferation of limb bud mesenchymal cells relies solely on signals from the AER, the counterpart of the AEC in the regenerating limb.

The tissues of the new limb parts derived from the blastema redifferentiate in continuity with their parent tissues. Differentiation and morphogenesis of the blastema take place in a proximal to distal and anterior to posterior sequence, except that in the proximodistal (PD) axis, the digits begin differentiation prior to the carpals or tarsals. Differentiation in the dorsoventral (DV) axis appears to take place simultaneously across the axis. The remainder of the regenerative process consists of growth to match the size of the unamputated limb.

Light and electron microscopic studies have suggested that myofibers of the limb cellularize to produce mononucleate cells that dedifferentiate to form blastema cells [4]. Recently, using the satellite cell-specific transcription factor Pax-7 as a marker, satellite cells were shown to contribute to the limb regeneration blastema and develop into new muscle

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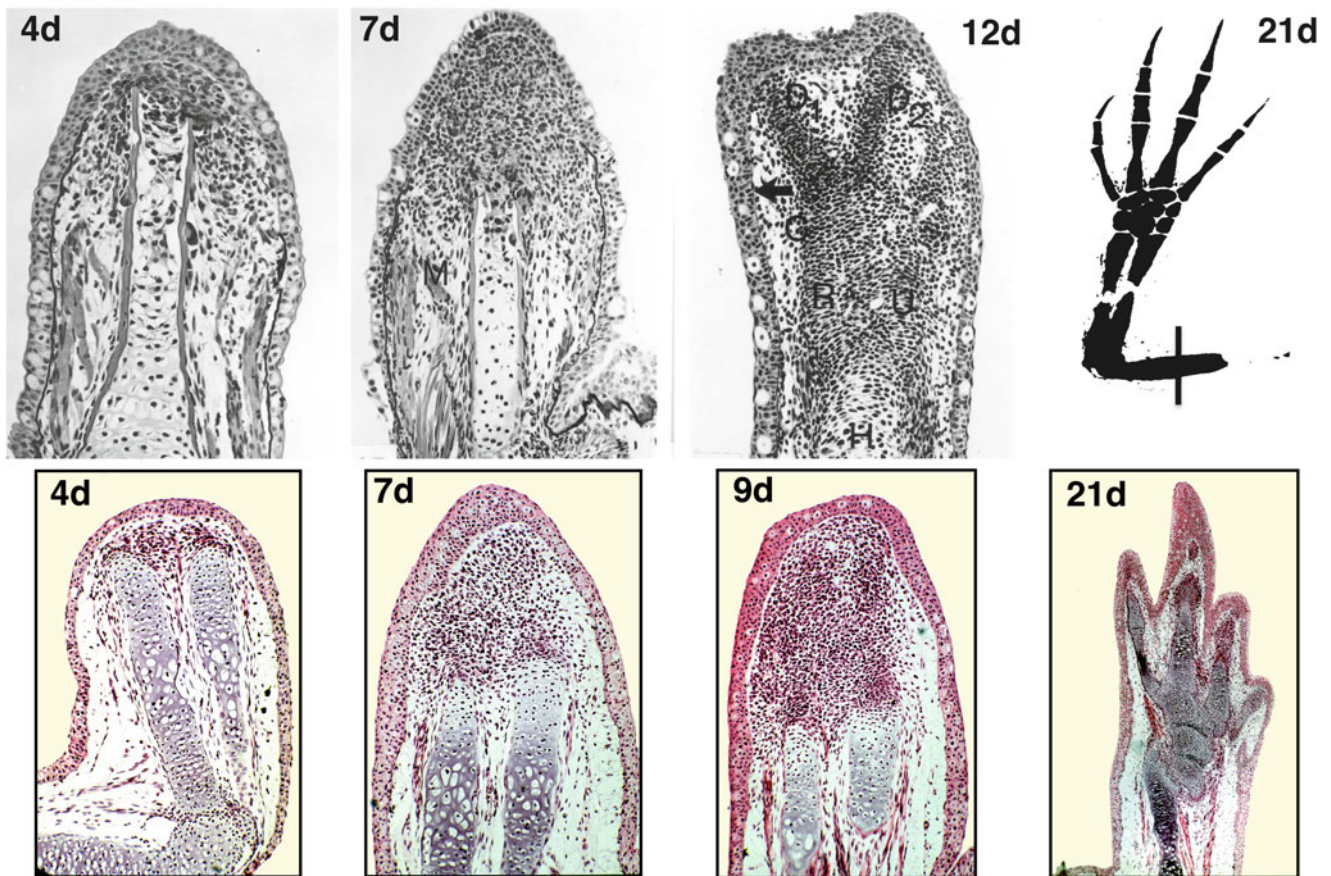
Fig. 1 H & E-stained longitudinal sections of larval Ambystoma forelimbs regenerating from (**a**) the mid-humerus and (**b**) the distal tips of the radius/ulna. Sections of upper arm regenerates are shown for 4, 7, and 14 days postamputation, along with a methylene-blue-stained whole mount of a 21-day regenerate (*line* indicates amputation level). Sections of distal R/U regenerates are shown for 4, 7, 9, and 21 days postamputation. The length of time required to regenerate from these two levels is approximately the same. The blastemas pass through an initial accumulation stage (4 days, early bud), then a conical (7 days, medium bud) stage, and followed by stages of progressive differentiation and morphogenesis (late bud and fingerbud)

[5]. By grafting individual limb tissues from transgenic GFP-expressing axolotls in place of their unmarked host counterparts, Kragl et al. [6] showed that blastema cells derived from muscle, fibroblasts, cartilage, and Schwann cells retained an epigenetic memory of their origin and redifferentiated into their preexisting parent cell types but that dermal fibroblasts also undergo transdifferentiation into chondrocytes and tenocytes, confirming earlier results [7–9]. Dermal fibroblasts contribute nearly half the blastema cells of the amputated axolotl limb and contribute the majority of the regenerated chondrocytes [10]. In the axolotl, dermis represents ~19 % of the limb tissue, while cartilage represents 6 %. However, grafts of triploid dermis to diploid limbs contributed an average of 43 % of the blastema cells and grafts of triploid cartilage only 2 %. Thus, dermal fibroblasts are overrepresented in the blastema by more than a factor of 2, and cells from cartilage are underrepresented by a factor of 3.

These results show that cartilage and muscle of the regenerate are each derived from two sources, cartilage from dedifferentiated chondrocytes and transdifferentiated fibroblasts and muscle from dedifferentiated myofibers and satellite stem cells. However, it is unclear what the proportional contributions of dedifferentiated mononucleate cells vs. satellite cells to regenerated muscle might be. Satellite cells could be the sole source of muscle in regenerating limbs, or the proportions of satellite cells and dedifferentiated myofibers might change with age, metamorphosis, or species of animal. Inducible genetic marking of myofibers and/or genetic ablation of satellite cells in transgenic animals might provide answers to these questions. Assuming that myofiber dedifferentiation is real, regeneration of the urodele limb involves the simultaneous use of four different mechanisms: dedifferentiation and redifferentiation, differentiation of adult stem cells, transdifferentiation, and regrowth of single cells, in this case axons of neurons.

Mechanisms of Blastema Formation

Early Signals: IP3, DAG, and Ionic Flux

Two early signals for blastema formation are inositol triphosphate (IP3) and nitric oxide (NO). IP3 and diacylglycerol (DAG) are the products of PIP2, which in turn is derived from inositol. IP3 synthase, a key enzyme for the synthesis of inositol from glucose-6-phosphate, is highly upregulated during blastema formation in regenerating axolotl limbs [11]. IP3 stimulates a rise in cytosolic Ca^{2+} that results in the localization of protein kinase C (PKC) to the plasma membrane, where it is activated by DAG and regulates transcription. During blastema formation, there is a general downregulation of proteins involved in Ca^{2+} homeostasis, which suggests that IP3 might signal a rise in cytosolic Ca^{2+} in regenerating limbs by this mechanism [11]. Other studies have shown that IP3 is generated from PIP2 within 30 s after amputation in newt limbs and that beryllium inhibition of IP3 formation prevents blastema formation [12]. PKC rises to a peak by the accumulation blastema stage [13]. The enzyme that catalyzes NO synthesis, nitric oxide synthase 1 (NOS1), is strongly upregulated in the wound epidermis of amputated axolotl limbs by 1 day postamputation [11]. NO has a wide variety of signaling functions [14]. It is produced by macrophages and neutrophils as a bactericidal agent and has a role in activating proteases known to be important effectors of histolysis in regenerating limbs.

Na^+ influx in the amputated newt limb and H^+ efflux in the amputated tail of *Xenopus* tadpoles generate ionic flow across the skin and wound epidermis. Na^+ influx is via sodium channels [15], while H^+ efflux is driven by a plasma membrane ATPase in the epidermal cells [16]. H^+ efflux is likely to be important in limb regeneration as well, since a gene encoding a v-ATPase was the most abundant clone in a suppressive subtraction cDNA library made from dedifferentiating axolotl limb tissue [17]. These ion movements are obligatory for regeneration, since drug-induced inhibition of either Na^+ in limbs or H^+ movements in tails during the first 24 h or so after amputation results in failure of blastema formation [16, 18].

The timing of IP3 and DAG synthesis, the probable rise in cytosolic Ca^{2+}, the upregulation of NOS1, and the subsequent movements of Na^+ and H^+ across the wound epidermis suggest that these molecules and ions may be the earliest signals that initiate blastema formation. The details of how their activity is translated into histolysis and dedifferentiation, however, are unknown. Campbell et al. [19] have carried out a comparative microarray analysis of gene activity between the epidermis that re-covers limb radial skin wounds and the epidermis that re-covers amputation wounds. They identified 125 genes with higher expression in the wound epidermis of amputated limbs, indicating that these genes are specific to a limb regeneration response as opposed to general wound healing. Quantitative PCR data showed significantly higher expression and changes in expression overtime for several genes, including a gene encoding an mRNA similar to a methyltransferase. Study of the function of genes revealed in this way will help further understand how the wound epidermis promotes the early events of regeneration.

Apoptosis May Be Obligatory to Initiate Limb Regeneration

Apoptosis is minimal in the axolotl and newt limb 24 h after amputation and beyond [20, 21], but observations have not been made earlier than this. A transient wave of apoptosis has been shown to occur in the first 24 h after amputation of Xenopus tadpole tails [22] and tails of the knifefish *Apteronotus leptorhynchus* [23, 24]. This apoptosis is obligatory for Xenopus tail regeneration because when prevented by caspase inhibitors, regeneration fails. Whether apoptosis is obligatory for knifefish tail regeneration is unknown, but apoptosis of neurons remains elevated at the regenerate/stump interface, suggesting that integration of new neurons into circuits at that level requires substantial cell pruning [23]. Whether there is a relationship between apoptosis and ionic currents is unknown. Apoptosis and its potential role in regeneration should be examined in regenerating urodele limbs.

Histolysis

The cells that form the blastema, whether stem cells or progenitors derived by dedifferentiation, are released from their tissue organization by degradation of the extracellular matrix (ECM) and cellularization of myofibers, a process called histolysis. The liberated cells undergo dedifferentiation to mesenchyme-like blastema cells with large nuclei and sparse cytoplasm that exhibit intense DNA, RNA, and protein synthesis. Histolysis and dedifferentiation are visible histologically within 2–3 days postamputation in larval urodeles and within 4–5 days in adults [4].

Degradation of tissue ECM is achieved by acid hydrolases and matrix metalloproteinases (MMPs) [25, 26]. Acid hydrolases identified in regenerating urodele limbs include cathepsin D, acid phosphatase, β-glucuronidase, carboxyl ester hydrolases, and *N*-acetyl-glucosaminidase. Osteoclasts degrade bone matrix via hydrochloric acid, acid hydrolases, and MMPs. Upregulated MMP transcripts include *MMP-2* and *MMP-9* (gelatinases) and *MMP-3/10a* and *b* (stromelysins) [27–29]. In the newt limb, the basal layer of the wound epidermis transcribes *MMP3/10a* and *b*, as well as a novel

MMP with low homology to the others [30]. Chondrocytes express *MMP-2* and *MMP-9* transcripts in the newt limb, and these enzymes are proposed to diffuse outward from the degrading skeletal elements [30]. The importance of MMPs to regeneration is underscored by the failure of blastema formation in amputated newt limbs treated with the MMP inhibitor GM6001 [31].

An important function of the MMPs encoded by the basal layer of the wound epidermis is thought to be the prevention of basement membrane reassembly beneath it, thus maintaining communication between the wound epidermis and subjacent mesenchymal cells. Loss of such communication, either by removing the wound epidermis [32] or conditions under which a pad of connective tissue becomes prematurely interposed between wound epidermis and blastema cells [33], inhibits regeneration. MMPs from the basal wound epidermis might also diffuse into the underlying tissues to participate in the degradation of other ECM components. Histolysis continues through the medium bud stage of blastema growth and then ceases due to the activity of tissue inhibitors of metalloproteinases (TIMPS) [34]. TIMP1 is upregulated during histolysis when MMPs are at maximum levels and exhibits spatial patterns of expression congruent with those of MMPs in the wound epidermis, proximal epidermis, and internal tissues undergoing disorganization.

The levels and temporal expression patterns of the MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, and MMP-13 proteins during blastema formation are different in regeneration-competent wild-type axolotls vs. regeneration-deficient *short-toe* axolotls and Xenopus froglets [35], suggesting that these differences play a role in the abnormal histolysis and thus availability of cells for dedifferentiation noted in regeneration-deficient Xenopus limbs [36].

Dedifferentiation

Dedifferentiation involves the epigenetic reprogramming of limb cells that alters their global pattern of transcriptional activity to produce a less differentiated state. The activity of differentiation genes is suppressed and genes associated with stemness (*msx-1*, *nrad*, *rfrng*, *Notch*) are activated [37]. Carlson [38] showed that inhibition of this transcriptional shift by actinomycin D does not affect histolysis, but does prevent or retard dedifferentiation, leading to regenerative failure or delay. This suggests that at least some of the protease expression involved in histolysis is not regulated at the transcriptional level, but that dedifferentiation is regulated primarily at the transcriptional level. Dedifferentiated cells express a more limb bud-like ECM in which type II collagen synthesis and accumulation are reduced, collagen I synthesis is maintained at a steady level, and fibronectin, tenascin, and hyaluronate accumulate [39].

Mammalian adult fibroblasts have been reprogrammed to pluripotency (induced pluripotent stem cells, iPSCs) equivalent to that of embryonic stem cells (ESCs) by transfecting them with four of six transcription factor genes (*Oct 4*, *Sox 2*, *c-myc*, *Klf-4*) [40] and *Oct 4*, *Sox 2*, *Nanog,* and *Lin 28* [41]. Three of these six genes (*klf4*, *Sox2*, *c-myc*), but not the others, are upregulated during blastema formation in regenerating newt limbs and also during newt lens regeneration [42]. Upregulation of the microRNA-processing protein Lin 28 was detected during blastema formation in regenerating axolotl limbs [11]. Further studies are needed to comprehend the role of these and other transcription factors, as well as understanding the changes in promoter and histone methylation, histone acetylation, and microRNAs that determine the extent and course of epigenetic erasure and rewriting involved in dedifferentiation, redifferentiation, and transdifferentiation. Studies on such changes have begun for the transdifferentiation of newt lens regeneration [43, 44] but have not yet been reported for limb regeneration beyond the observation that the long-range limb specific *shh* enhancer, a conserved sequence called mammals-fishes-conserved-sequence 1 (MFCS1), which is located in a noncoding region of the LMBR1 gene [45], is hypermethylated in Xenopus vs. moderately methylated in the axolotl and newt [46]. This hypermethylation is associated with the lack of *shh* expression on the posterior side of the blastema and regeneration deficiency of the amputated Xenopus limb.

Blastema Cell Accumulation

The AEC directs the migration of mesenchymal cells to form the accumulation blastema beneath it [4]. This was shown by experiments in which shifting the position of the AEC laterally caused a corresponding shift in blastema cell accumulation, and transplantation of an additional AEC to the base of the blastema resulted in supernumerary blastema formation. Nerves that innervate the AEC do not appear to physically guide blastema cells, since similar experiments on aneurogenic limbs also resulted in eccentric blastema formation. The redirected accumulation of blastema cells under an eccentric AEC may be due to the migration of the cells on repositioned adhesive substrates produced by the AEC. TGF-β1 is strongly upregulated during blastema formation in amputated axolotl limbs [47]. A target gene of TGF-β1 is fibronectin, a substrate molecule for cell migration that is highly expressed by basal cells of the wound epidermis during blastema formation [3, 11]. Inhibition of TGF-β1 expression by the inhibitor of SMAD phosphorylation SB-431542 reduces fibronectin expression and results in failure of blastema formation [47], suggesting that fibronectin produced by the AEC may provide directional guidance for blastema cells.

The Structure and Function of the Wound Epidermis Is Nerve-Dependent

Neither denervation nor deprivation of wound epidermis prevents histolysis, dedifferentiation, and entry of blastema cells into the cell cycle, but blastema cells do not accumulate under the wound epidermis and disappear (Fig. 2). Thus, transection of the brachial nerves at the level of the shoulder or preventing the formation of a wound epidermis by inserting the amputated limb tip into the coelom or grafting full thickness skin over the amputation surface prevents formation of the accumulation blastema [32, 48–51], showing that both nerve and AEC are required for its formation.

Maintenance of AEC structure and function is dependent on innervation by regenerating axons [52], but the nature of this dependency has not been clear. In experiments making a wound in the skin of axolotl limbs, the regenerated epidermis developed a thickening comparable to the AEC that subsequently regressed. However, if a nerve was deviated into the wound, the epidermal thickening was maintained, and a blastema-like growth was formed from the underlying tissues [53]. This growth is equivalent to a blastema formed by amputation in terms of morphology and expression of MMP-9, Msx-2, Hox A-13, Prx-1, and Tbx-5 [54]. In other experiments on amputated axolotl limbs, the nerves were shown to induce expression of the zinc finger transcription factor *Sp9*

in the wound epidermis, which is associated with epidermal dedifferentiation [55]. Collectively, the results imply that in a normally innervated limb, the AEC forms independently of the nerve, but its structure and function are not maintained unless the AEC becomes innervated by regenerating axons, an implication that fits the timing of AEC formation and initiation of axon regeneration into the wound epidermis during the formation of the accumulation blastema.

A Neural-Epidermal Circuit Is Required for Blastema Cell Proliferation

A great deal of evidence indicates that blastema growth requires the action of a signaling circuit between limb nerve axons and the wound epidermis/AEC.

Effects of Denervation and Deprivation of Wound Epidermis/AEC

During formation of the accumulation blastema, the DNA-labeling index of blastema cells is high, indicating that a substantial percentage of dedifferentiating cells enter the cell cycle. However, the frequency of cells undergoing mitosis is very low (~0.4 %), suggesting that most blastema cells temporarily arrest in G_2 after completing DNA replication [50,
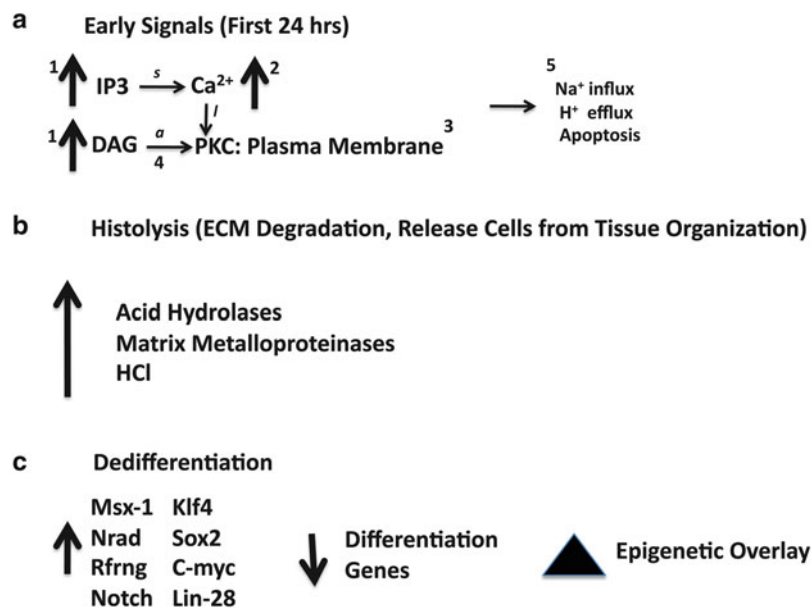
Fig. 2 Elements of the mechanism of blastema formation. (**a**) Early signals within the first 24 h postamputation. 1–5 are steps in signaling (see text for details). *s* stimulates, *a* activates, *l* localizes. (**b**) Molecules active in regulating the degradation of extracellular matrix (ECM) during histolysis of tissue organization. (**c**) Dedifferentiation of liberated cells. A group of genes associated with stemness (*left*) is upregulated, while differentiation genes are downregulated (*middle*). The *triangle* (*right*) represents change in the pattern of histone acetylation and methylation, DNA methylation, microRNAs, and Polycomb and Trithorax proteins constituting the epigenetic overlay that stabilizes transcription

56–60]. Further indirect evidence for G_2 arrest is the strong upregulation of the ecotropic viral integration factor 5 (Evi5) throughout formation of the accumulation blastema in regenerating axolotl limbs [11]. Evi5 is a centrosomal protein that accumulates in the nucleus during early G_1 in mammalian cells and, in concert with Pin1, prevents them from prematurely entering mitosis by stabilizing Emi1, a protein that inhibits cyclin A degradation by the anaphase-promoting complex/cyclosome [61, 62]. At G_2, Emi1 and Evi5 are phosphorylated by Polo-like kinase 1 and targeted for ubiquitin-driven degradation, allowing the cell to enter mitosis. The high levels of Evi5 during blastema formation, which takes significantly longer than the ~50 h cell cycle [63], may restrain dedifferentiated cells from entering mitosis until they have accumulated sufficiently to constitute a blastema [11]. While this hypothesis remains to be tested, it is significant that a high proportion of the fibroblasts of the ear tissue of the MRL/lpj mouse, which regenerates after punch injury, are arrested in G_2, suggesting that fibroblasts of this tissue are poised for mitosis upon injury [64].

Once the accumulation blastema has formed, it enters a growth phase where the mitotic index increases tenfold or more [51, 58]. In both larval and adult limbs, denervated growing blastemas are nerve-independent for morphogenesis and patterned differentiation. The regenerates formed by these blastemas, however, are much smaller than control regenerates [65–67]. This is because denervation at any stage of blastema growth leads to the reduction of blastema DNA synthesis [57, 68] and decreases the mitotic index to zero [69] due to disruption of the AEC mitogenic function. Direct evidence that proliferation during blastema growth stages requires the AEC is that DNA synthesis and mitosis of epidermis-free newt limb blastemas cultured in the presence of dorsal root ganglia are reduced three- to fourfold [70, 71]. These observations are compatible with the hypothesis that nerve axons induce and maintain a cell cycling function of the AEC that operates throughout regeneration.

The growing blastema may also be dependent on the AEC for proximodistal patterning and morphogenesis. Medium bud and later stage blastemas of larval *A. maculatum* denuded of wound epidermis and grafted into dorsal fin tunnels form smaller than normal skeletal elements with a distally truncated pattern, the degree of truncation being proportional to the developmental stage of the implant [72]. A complete proximodistal sequence of smaller than normal elements is formed by blastema implants positioned so that their distal tip becomes covered with fin wound epidermis. The small size of the skeletal elements in both types of implant is consistent with the lack of a nerve-dependent mitogenic function of the AEC. The PD truncation of implants denied regeneration of epidermis at their distal tip, however, suggests that the AEC may have a PD patterning function distinct from mitogenesis, although it remains to be established that the trunca-

tion is not due simply to death of apical cells destined to form distal structures.

Blastema Cell Proliferation Becomes Nerve-Dependent During Digit Stages of Limb Development

Amputated urodele limb buds are able to regenerate in the absence of innervation until they reach digital stages of development. At these stages, the limb bud becomes heavily innervated, whereupon regeneration becomes nerve-dependent and will not take place if the limb is denervated [73]. Nerve dependence is not acquired, however, if the limb never becomes innervated [74, 75]. This was shown by para-biosing two early embryos and excising the neural tube from one of them so that the fully differentiated limbs were aneurogenic. These limbs require only the wound epidermis/AEC to regenerate normally. Aneurogenic limbs can be oscillated between nerve-independent and dependent states. When grafted in place of innervated host limbs, they become innervated and nerve-dependent for regeneration by 10–13 days posttransplantation, but nearly half of the cases become nerve-independent again if re-denervated and maintained in a denervated state for 30 days [76].

These results can be explained by assuming that the outgrowth-promoting function of the limb bud apical epidermis [77] during limb development is either autonomous or depends on signals from the subjacent mesoderm as observed for chick limb buds [78]. As nerves grow into the limb, however, the epidermis becomes dependent on ("addicted to") neural factors to maintain its outgrowth-promoting function during regeneration [79]. This dependency never develops in aneurogenic limbs, and the AEC maintains its original functional capacity after limb amputation.

What Are the Mitogenic Factors for Blastema Cell Proliferation?

A protein has been identified that can substitute for the nerves in denervated and amputated adult newt limbs [80]. The protein is the anterior gradient protein (AGP), a ligand for the blastema cell surface receptor Prod1. Prod1 is a member of the Ly6 family of three-finger proteins anchored to the cell surface by a glycosylphosphatidyl inositol linkage [81–83]. As assessed by co-expression of the Schwann cell marker HNK1, AGP is strongly expressed in the distal-most Schwann cells of regenerating newt limbs at 5 and 8 days postamputation, when histolysis and dedifferentiation are underway [80]. AGP expression is abolished by proximal nerve transection, indicating that it is induced in the Schwann cells by axons. The function of the Schwann cell AGP is not clear, however.

By 10 days postamputation, when the newt accumulation blastema is forming, AGP expression shifts from Schwann cells to subepidermal secretory gland cells of the AEC [80]. The wound epithelium of the axolotl does not have subepidermal gland cells, and here AGP expression is observed in the Leydig cells of the AEC. Both sets of gland cells appear to discharge secretions by a holocrine mechanism [84]. The expression of AGP by gland cells is also axon-dependent, as shown by the fact that it is abolished in denervated limbs. AGP has been shown to be a complete mitogen for blastema cells in vivo. When electroporated into denervated newt limbs at 5 days postamputation, the AGP gene supported regeneration to digit stages. Conditioned medium of Cos7 cells transfected with the AGP gene stimulated BrdU incorporation into cultured blastema cells, and antibodies to Prod1 blocked this incorporation [80]. Collectively, these results suggest that nerve axons induce the AEC to express AGP, which is then secreted and acts through Prod1 on subjacent blastema cells to stimulate their proliferation, thus giving the nerve dependence of AEC function a molecular basis. Further persuasive evidence for this idea is that AGP is downregulated in the apical epidermis of the limb bud during its acquisition of nerve dependence, but remains high in the apical epidermis of aneurogenic limbs throughout development and during regeneration [85].

Factors other than AGP that promote blastema cell proliferation in vitro and in vivo have been detected in the wound epidermis of the regenerating axolotl limb, primarily members of the fibroblast growth factor family (FGFs 1, 2, and 8) [86, 87]. Blastema cells express fibroblast growth factor-10, which is essential for maintaining FGF-2 expression by the AEC in regenerating Xenopus limb buds [88, 89]. The role these epidermal factors play in regeneration in vivo is not clear, but one hypothesis would be that FGFs, while not essential for blastema cell proliferation, synergize with AGP to augment their mitosis, or even that FGFs are the essential mitogens for blastema cells, but require AGP for their synthesis. Examining the effect of denervation on synthesis of these factors by the AEC would help to reveal their function. If their expression is eliminated by denervation and they fail to rescue regeneration after exogenous delivery to denervated blastemas in vivo, the hypothesis that they are essential mitogens would be unlikely. FGFs made by the wound epidermis/AEC might also play an essential role in axon and capillary regeneration into the blastema.

What Are the Axon Factors that Stimulate AGP Expression?

A major question is the identification of the factors produced by axons that induce AGP expression by Schwann cells and the AEC. Glial growth factor 2 (GGF-2, neuregulin) fulfills the criteria to be a candidate for the axon stimulus. It is expressed by neurons, is present in the blastema, and is lost from the blastema upon denervation [90, 91]. GGF-2, along with other growth factors produced by platelets and macrophages (FGFs, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), interleukins (IL) 1, 2, 6), has been shown to be mitogenic for Schwann cells in transected mammalian peripheral nerves [92]. The GGF-2 gene is expressed in newt dorsal root ganglia, and recombinant human GGF-2 infused into denervated axolotl limb blastemas was reported to maintain the DNA-labeling index at control levels and to support regeneration to digit stages [93], similar to the rescue of denervated blastemas by implants of spinal ganglia [94]. However, little detail was supplied in support of the ability of GGF-2 to promote complete regeneration in these experiments. Furthermore, there is no experimental data to show that GGF-2 actually induces the expression of AGP in the AEC. KGF (Fgf-7) has been shown to be expressed in axolotl dorsal root ganglion cells and to induce expression of the *Sp9* gene when administered in beads under a wound epidermis in the absence of the nerve [55]. KGF stimulates the mitosis of keratinocytes and thus the thickening of the wound epidermis in mammalian skin wounds [95], suggesting that it might also play this role in AEC formation.

Neurons synthesize other factors that directly promote the proliferation of blastema cells in vitro and in vivo. Denervation reduces protein synthesis by regeneration blastemas, but addition of neural extracts to blastema explants partially restored protein synthesis [96, 97]. The activity of the extracts was abolished by trypsin treatment and heating, but not by RNase, suggesting that the active molecules are proteins [97]. Spinal cord extracts from axolotls undergoing limb regeneration stimulated the mitosis of cultured blastema cells at twice the level of extracts from unamputated animals, and blastemas explanted next to cultured dorsal root ganglia or spinal cord segments that had regenerated many neurites had a mitotic index substantially higher than control cultures [70, 98]. Specific neural factors that promote blastema cell proliferation include transferrin, FGF-2, and substance P [99–101]. FGF-2 is the only factor shared with the AEC. With the exception of transferrin levels, which are reduced by 50 % in vivo, the effect of denervation on loss of these factors from the blastema has not been tested, and none has been shown to support the full course of regeneration. The function of these mitogens is thus unclear. They might synergize with GGF-2 in an augmentative but nonessential role to enable the function of the AEC, or along with the FGFs made by the AEC, they might be synergistic with AGP but be nonessential for mitosis. Singer [102] showed that the axon requirement for regeneration is quantitative and independent of the motor or sensory quality of the axons. It would be interesting to examine AGP synthesis in the AEC
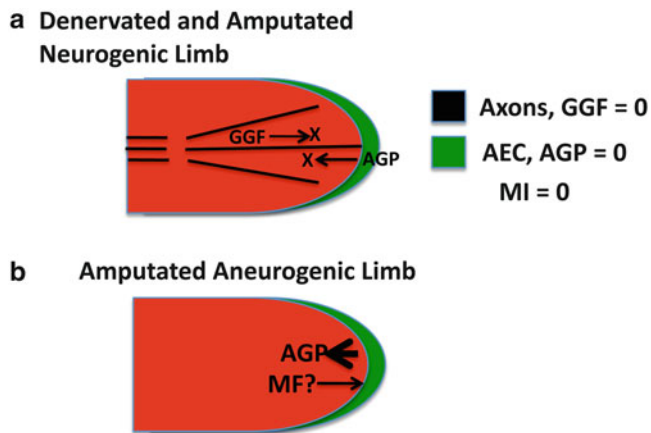
Fig. 3 The neural/epidermal circuit that drives proliferation of blastema cells. (**a**) Axons (*black lines*) secrete GGF-2 (hypothetical) that stimulates the AEC (*green*) to secrete the mitogen AGP, which binds to its ligand Prod1 on the surface of blastema cells to promote cell cycling. Upon denervation (*break* in lines), GGF-2 is no longer delivered to the AEC (X), and its secretion of AGP is terminated, causing a rapid fall in the mitotic index (MI) to zero. (**b**) The epidermis of a neurogenic limb acquires dependence on the nerve for AEC maintenance and AGP secretion during late stages of limb bud development when axons are ramifying throughout the limb tissues. The tissues of an aneurogenic limb never encounter axons, and the AEC formed after amputation maintains the capability of the limb bud apical epidermis to make AGP and blastema cells proliferate normally. An unresolved question is whether blastema cells undergo mitosis during their accumulation in an amputated aneurogenic limb or exhibit a very low level of mitosis like neurogenic limbs and whether blastema cells of a regenerating aneurogenic limb signal the AEC via a maintenance factor (MF) to secrete AGP

of regenerating limbs with selectively denervated motor or sensory components.

The relationships of the tissues and molecules that comprise the nerve/epidermal circuit are summarized in Fig. 3.

Blastema Cells Promote Axon Regeneration

As the blastema grows, axons must continually elongate to innervate differentiating tissues. Schwann cells provide most of the soluble factors (nerve growth factor, brain-derived growth factor, neurotrophic factors 3 and 4, ciliary neurotrophic factor, and glial-derived neurotrophic factor) and some adhesive factors required for neuron survival and axon elongation after transection of peripheral nerves [103]. Regeneration of axons from amphibian spinal cord neurons is promoted in vitro by co-culture with limb regeneration blastema mesenchyme [104]. Brain-derived neurotrophic factor, neurotrophic factors 3 and 4, glial-derived growth factor, and hepatocyte growth factor/scatter factor can substitute for blastema tissue in promoting this axon outgrowth [105]. Axon outgrowth was significantly more vigorous with blastema tissue, suggesting that blastema cells produce other

factors that support neuron survival and axon outgrowth. One of these factors may be retinoic acid [106]. In cultures of newt spinal cord, retinoic acid added to the culture medium not only evoked the extension of a greater number of axons than in control cultures, the length of the axons was 4 times greater. Axon outgrowth was enhanced even more by co-culture with blastemas in the absence of exogenous retinoic acid. However, treatment of the co-cultured blastemas with the retinoic acid inhibitor citral reduced axon outgrowth, suggesting that retinoic acid is an axon outgrowth-promoting molecule made by the blastema. It would be of interest to know whether the wound epidermis/AEC also produces axonotrophic and angiogenic factors.

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