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Nerves and Proliferation of Progenitor Cells in Limb Regeneration

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Abstract

Nerves, in conjunction with the apical epidermal cap (AEC), play an important role in the proliferation of the mesenchymal progenitor cells comprising the blastema of regenerating urodele amphibian limbs. Reinnervation after amputation requires factors supplied by the forming blastema, and neurotrophic factors must be present at or above a quantitative threshold for mitosis of the blastema cells. The AEC forms independently of nerves, but requires nerves to be maintained. Urodele limb buds are independent of nerves for regeneration, but innervation imposes a regenerative requirement for nerve factors on their cells as they differentiate. There are three main ideas on the functional relationship between nerves, AEC and blastema cells: (1) nerves and AEC produce factors with different roles in maintaining progenitor status and mitosis; (2) the AEC produces the factors that promote blastema cell mitosis, but requires nerves to express them; (3) blastema cells, nerves, and AEC all produce the same factor(s) that additively attain the required threshold for mitosis.

Introduction

Peripheral nerves, along with neurons of the central nervous system (CNS), blood vessels, immune cells, and endocrine organs, have historically been viewed as part of an integrated signaling system that regulates the biochemical and biomechanical functions of the body. More recently, nerves have been shown to play important roles in tissue and appendage regeneration in invertebrates and vertebrates, and in the progression of some cancers by stimulating the growth of stem and progenitor cells (reviewed by Kumar and Brockes, 2012; Boilly et al, 2017). A widely used experimental model to study the role of nerves in regeneration is the regenerating urodele limb (Dinsmore, 1991, 1998). The present review summarizes recent research results and ideas on how

nerves and the wound epidermis promote the proliferation of progenitor cells in regenerating urodele amphibian limbs, and on the identification of the molecular agents involved.

Formation of the Regeneration Blastema

Urodele limbs regenerate via a blastema of undifferentiated progenitor cells released by the proteolytic histolysis of mesodermal limb tissues. These cells aggregate under a wound epidermis that is thickened into a ridge called the apical epidermal cap (AEC). There are two mechanisms of tissue contribution, by the release of resident progenitor cells from muscle, periosteum and perhaps as yet undefined fibroblast populations, and the dedifferentiation of nucleated myofiber fragments, fibroblasts, and Schwann cells (reviewed by Stocum, 2017). The proportion of regenerate muscle contributed by release of resident progenitors (satellite cells) and by dedifferentiation (myofiber fragmentation) varies with species and developmental status (Sandoval-Guzman et al, 2014; Tanaka et al, 2016). The contribution of each tissue to the blastema also varies, with dermal fibroblasts contributing over half the blastema cells and cartilage contributing little or none (Muneoka et al, 1986; McCusker et al, 2016). Redifferentiation is lineage-specific except for blastema cells derived from dermal fibroblasts, which differentiate not only into new fibroblasts, but also transdifferentiate into the new chondrocytes of the skeletal elements, congruent with the minimal contribution of cartilage to the blastema (Kragl et al, 2009). The wound epidermis/AEC does not contribute to the progenitor cell population, but redifferentiates as epidermis. **Figure 1** illustrates the phases and stages of urodele limb regeneration.

Role of the nerve in blastema cell proliferation

In the 1940s, a series of denervation studies by Singer and colleagues on regenerating newt limbs revealed that a threshold quantity of axons per unit area of limb tissue is required for regeneration.

Singer synthesized his results into the neurotrophic hypothesis (Singer, 1952, 1964, 1965), which states that sensory, motor and autonomic nerves provide the early blastema with a quantitative threshold of neurotrophic factor(s) required for blastema growth. The normal sensory innervation, but not the motor or sympathetic innervation, is sufficient to meet the threshold requirement. There is no qualitative difference in the ability of nerve type to promote regeneration, for if the motor nerve supply is augmented by deviating motor nerves such that they could branch into denervated sensory endoneurial tubes, regeneration takes place (Sidman and Singer, 1960; Thornton, 1960).

The nerve is not required for histolysis and the initial accumulation of blastema cells, but is essential for blastema cell proliferation. Blastema cells enter the cell cycle and synthesize DNA after denervation of an amputated limb (Mescher and Tassava, 1976), but mitotic index is reduced to zero by denervation at any stage of blastema formation (Goldhamer and Tassava, 1987). Once the blastema has grown to a critical size, it becomes independent of the nerve for differentiation and morphogenesis, but remains nerve-dependent for blastema cell mitosis, as evidenced by the fact the blastema forms a miniaturized regenerate (reviewed by Thornton, 1968; Stocum and Cameron, 2011; Pirotte et al, 2015; Boilly et al, 2017, Stocum, 2017). Regeneration of axons following amputation is stimulated by factors expressed by blastema cells, as shown by *in vitro* co-culture experiments (Richmond and Pollack, 1983; Boilly and Bauduin, 1988; Bauduin et al, 2000). Several known neurotrophic factors such as brain-derived neurotrophic factor (BDNF), neurotrophins 3 and 4 (NT3, 4), glial derived neurotrophic factor (GDNF) and hepatocyte growth factor/scatter factor (HGF/SF) can substitute for blastema tissue in promoting axon outgrowth *in vitro* (Tonge and Leclere, 2000). These factors are produced by Schwann cells and promote neuron survival and axon outgrowth in the regenerating peripheral nerves of mammals (reviewed by Fu and Gordon, 1997), raising the question of whether they might be produced by the subpopulation of blastema cells derived from Schwann cells. Retinoic acid has been shown to be produced by the blastema of the

regenerating newt limb, and to induce outgrowth of spinal cord axons toward the blastema via the RAR β receptor (Dmetrichuk et al, 2005). Regardless, axon outgrowth is significantly more vigorous with blastema tissue, suggesting that blastema cells produce other, as yet unidentified, factors that encourage neuron survival and axon outgrowth. A comparison of genes expressed by axolotl dorsal root ganglia (DRG) cells in the presence and absence of blastema cells revealed 27 DRG genes that were differentially expressed in the presence of blastema cells (Athipozhy et al, 2014). **Figure 2** depicts the relationship between nerves and blastema growth, and also the influence of blastema cells on regenerating axons.

Recently, studies of zebrafish caudal fin regeneration have defined a role for H₂O₂, a reactive oxygen species (ROS), in regeneration (reviewed by Meda et al, 2017). Injured nerves produce high levels of H₂O₂ through activation of the Hedgehog (Shh) pathway. The H₂O₂ appears to provide an environment conducive to blastema formation and also stimulates axon regrowth. Denervation prevents this production and inhibits regeneration. ROS production has not as yet been investigated in the nerves of regenerating amphibian limbs. Macrophages are essential for blastema formation in regenerating amphibian limbs (reviewed by Mescher, 2017), and produce bactericidal H₂O₂ during mammalian skin wound repair that might also contribute to axon regeneration and other aspects of wound repair and blastema formation (reviewed by Clark, 1996, Stocum 2017).

Transcriptomic analysis has revealed that expression of genes specific to wound repair or muscle regeneration do not differ in amputated control and denervated limbs, but transcription of genes regulating proliferation is rapidly reduced in denervated limbs coincident with the start of blastema growth (Monaghan et al, 2009). This is reflected in a 50-70% reduction of protein synthesis after denervation without any effect on the amino acid precursor pool, rate of protein degradation, or

rate of translation (Dresden, 1969; Lebowitz and Singer, 1970; Manson et al, 1976; Choo et al, 1978). Changes in the protein profile are quantitative (Singer and Ilan, 1977; Singer, 1978) and are particularly notable in the synthesis of extracellular matrix (ECM) proteoglycans, collagen, and collagen-associated glycosaminoglycans (GAGs). Hyaluronate is the major GAG synthesized during blastema formation in adult newt limbs (Smith et al, 1975) and is reduced the most by denervation (Mescher and Munaim, 1986; Young et al, 1989). The effect of denervation on the regulation of a wide array of individual genes and proteins revealed by global transcriptomic and proteomic analysis of regenerating amphibian limbs (Monaghan et al, 2009; Looso et al, 2013; Rao et al, 2009, 2014; Voss et al, 2015) can now be investigated, facilitated by the sequencing, assembly, and analysis of the axolotl genome (Nowoshilow et al, 2018) coupled with CRISPR (clustered regularly interspaced short palindromic repeats) gene editing technology (Charpentier and Doudna, 2013; Fei et al, 2014, 2016; Flowers et al, 2014).

Role of the AEC in blastema cell proliferation

The apical ectoderm of anuran, avian, and reptilian limb buds is thickened into a cap called the apical ectodermal ridge (AER) that is indispensable for outgrowth of the limb bud mesoderm via a reciprocal mesenchymal (Fgf10): epithelial (Fgf8) induction (Gilbert and Barresi, 2016). The apical ectoderm of urodele limb buds does not form an AER, but it has the same crucial outgrowth-promoting function (reviewed by Stocum, 1975, 1995). However, the wound epidermis of the regenerating urodele limb is configured within a few days after amputation into the AEC that is rich with sensory innervation and is essential for blastema cell proliferation (reviewed by Thornton, 1968; Stocum, 2017).

The AEC synergizes with the nerve to promote the mitosis of mesenchymal progenitor cells. Endo et al (2004) showed that the initial formation of the AEC is independent of nerves, but that its

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maintenance is nerve-dependent. Other experiments have shown that preventing contact between the wound epidermis or AEC and underlying tissues after amputation leads to regenerative failure, even in the presence of innervation (Mescher, 1976; Chew and Cameron, 1983; reviewed by Stocum and Cameron, 2011; Stocum, 2017). Blastemas deprived of the AEC during their growth phase and implanted into a dorsal fin tunnel such that they cannot contact wound epidermis form miniature regenerates, but unlike denervated blastemas, these regenerates are also truncated distally (Stocum and Dearlove, 1972). Positioning the mesenchyme so that its distal tip protrudes from the tunnel and is re-covered by fin wound epidermis also produces a miniature regenerate, but one that is complete in the PD axis, as in denervated limbs (**Fig. 3**). In these experiments, the blastema mesenchyme is also denervated, but innervation by nerves of the dorsal fin is likely to be quantitatively insufficient. Cell proliferation was not directly assessed in these experiments, but miniaturization again suggests that the AEC plays a role in mitosis. More direct evidence for a mitogenic role of the AEC is that there is a 3-4 fold reduction in DNA synthesis and mitosis of AEC-free blastema cells cultured *in vitro* transfilter to dorsal root ganglia or with brain neurons (Globus et al, 1980; Smith and Globus, 1989) (**Fig. 3**). The molecular effects of AEC deprivation on subjacent blastema cells have not been assessed, but 125 genes that are highly up regulated in the AEC have been identified by transcriptome analysis (Campbell et al, 2011).

Hypotheses Regarding the Functional Relationship of Nerve and AEC in Blastema Cell Proliferation

Three major ideas have been put forward as to the nature of the nerve: AEC functional relationship in promoting blastema cell proliferation (Stocum, 2017) (**Fig. 4**). These are (1) the nerve and AEC provide separate factors with different roles in the cell cycle; (2) the AEC provides all factors necessary for the cell cycle, but is nerve-dependent to express them; (3) multiple tissues all express the same mitogen that drives blastema growth.

Nerve and AEC have different but complementary roles in the cell cycle

The results of experiments on DNA synthesis and mitosis by blastema cells in innervated vs. denervated axolotl and newt limbs have suggested different but synergistic roles for nerves and AEC in the blastema cell cycle (reviewed by Tassava and McCullough, 1978). Labeling of amputated limbs deprived of nerves or wound epidermis with [³H]-thymidine showed that DNA synthesis in nascent blastema cells is independent of both these tissues, but that in the absence of either one the labeled cells were arrested in G₂, undergo apoptosis and are removed by macrophages (Mescher and Tassava, 1976; Loyd and Tassava, 1980; Mescher et al, 2000; Yun et al, 2015). These results led Tassava and Mescher (1975) to propose that injury stimulates blastema cells to enter the cell cycle and that the AEC maintains the cells in an undifferentiated state that renders them responsive to mitogenic signals supplied by the nerve. This idea is consistent with the results of the *in vitro* trans-filter experiments by Globus et al (1980) and Smith and Globus (1989) in which adult newt blastema cells grown opposite dorsal root ganglia or brain cells fail to undergo mitosis in the absence of the wound epidermis and withdraw from the cell cycle, and differentiate as cartilage, whereas in the presence of both epidermal cells and neural tissue, they are maintained in an undifferentiated state and proliferate.

The AEC is dependent on the nerve to express blastema cell mitogens

The neural requirement for regeneration is imposed on the urodele limb bud only as it becomes innervated at late stages of its development (Fekete and Brockes, 1988). Urodele limbs rendered aneurogenic by extirpating the neural tube during early embryogenesis do not acquire nerve-dependence for regeneration (Yntema, 1959a, b), but presumably remain dependent on the wound AEC for blastema cell mitosis, suggesting that innervation does not alter the requirement of blastema cells for mitogenic factors, but rather decreases the capacity of the AEC to provide them.

Thus, a second hypothesis to explain blastema cell proliferation in both aneurogenic and neurogenic limbs is that the AEC provides diffusible mitogens, but in neurogenic limbs nerve axons impose a dependency for their trophic factors on the AEC to express them (Stocum, 2011). This hypothesis is also compatible with the *in vitro* experiments of Globus et al (1980).

Multiple tissues produce the same mitogenic factor

Nerve dependence/independence for regeneration can be oscillated back and forth. Nerve dependence of aneurogenic larval limbs can be instituted by transplanting them to neurogenic larvae. If the limbs are then denervated for a period of time, they can regain nerve independence (Thornton and Thornton, 1970). Even adult newt limbs showed some capacity for nerve-independent regeneration when maintained in a denervated condition after grafting them to the back (Singer and Mutterperl, 1963). Singer (1965) explained the ability of limb buds and differentiated aneurogenic limbs to regenerate by postulating that all their tissues express the neurotrophic factor. Production of the factor is suppressed and is taken over by axons as they innervate the developing limb bud or aneurogenic limb. Thus, the nerve itself forces changes to the differentiating tissues of the urodele limb that render the progenitor cells of the blastema nerve-dependent.

Candidate AEC and neural factors

Considering all three models, candidates to be AEC and neural factors should meet several criteria (Brookes, 1984). First, they should be expressed by the AEC or dorsal root ganglion (DRG) cell bodies. Further criteria to be an AEC mitogen are expression of the mitogen's receptor in the blastema mesenchyme, loss of mitogen expression by denervation, ability of the mitogen to support regeneration of denervated or AEC-deprived limbs from early blastema formation to digit stages,

and expression of the mitogen by the AEC of regenerating aneurogenic limbs. Neural factors should be transported from DRG cell bodies along limb sensory nerve axons to the AEC where they bind to their receptor, denervation should prevent blastema cell mitosis by abolishing expression of AEC factors, and the nerve candidates should support regeneration to digit stages in denervated limbs.

Candidate AEC factors: Fibroblast growth factors and the anterior gradient protein (AGP) are candidates expressed by the AEC *in vivo* (Christensen et al, 2001; 2002; Han et al, 2001; Kumar and Brockes, 2007). Blastema cells express the *bek* (FGFR2) receptor for Fgfs (Poulin et al, 1993; Poulin and Chiu, 1995) and the AGP receptor Prod1 (Kumar and Brockes, 2007). Fgf1 elevates the mitotic index of cultured blastema cells (Albert et al, 1987; Albert and Boilly, 1988; Boilly et al, 1991), and Fgf2 elevates the mitotic index of blastema cells in amputated limbs covered by full-thickness skin (Chew and Cameron, 1983). The only AEC candidate factors so far reported to be down regulated *in vivo* by denervation and to substitute for the nerve in supporting the regeneration of denervated limbs to digit stages are Fgf2 (Mullen et al, 1996) and AGP (Kumar and Brockes, 2007). Fgf2 was administered in beads only to late stage blastemas.

AGP is strongly expressed in the Schwann cells surrounding the axons of regenerating newt limbs at 5 and 8 days post-amputation, when histolysis and initial dedifferentiation is under way. By 10 days post-amputation, AGP expression shifts to the gland cells of the AEC, coincident with formation of the accumulation blastema. Denervation abolishes AGP expression by Schwann cells and in the AEC, indicating its dependence on axons. The AGP gene supports regeneration to digit stages when electroporated into denervated newt limbs five days post-amputation. Conditioned medium of Cos7 cells transfected with the AGP gene stimulates BrdU (bromodeoxyuridine) incorporation into cultured blastema cells. Incorporation is blocked by antibodies to Prod1, suggesting that AGP acts

directly on blastema cells through Prod1 to stimulate their proliferation (Kumar and Brockes, 2007).

Finally, AGP is expressed by the AEC of regenerating aneurogenic limbs (Kumar et al, 2011),

supporting the idea that AGP is a blastema cell mitogen that requires neurotrophins to be expressed in regenerating neurogenic limbs. Whether Fgf2 is expressed in the aneurogenic AEC has not been investigated.

Nerve candidate factors: Factors expressed by DRG neurons that promote blastema cell proliferation *in vitro* include transferrin (Mescher and Kiffmeyer, 1992; Mescher et al, 1997), substance P (Globus and Alles, 1990; Globus et al, 1991), and Fgf2 (Mullen et al, 1996). Combinations of Fgf8 and BMP (bone morphogenetic protein) have also been tested as neurotrophic factors. Both are expressed in DRG neurons and are detectable in peripheral limb nerve axons *in vivo* (Sato et al, 2016). Furthermore, they can substitute for the nerve in the outgrowth of a supernumerary axolotl limb (Makanae et al, 2014). There is now evidence that glial growth factor 2 (Ggf2, neuregulin) (Law et al, 2004), is produced by nerves and blastema cells during blastema growth. Neuregulin is mitogenic for Schwann cells (Davies, 2000) and was suggested over 30 years ago as a nerve factor for limb regeneration (Brockes, 1984; Brockes and Kintner, 1986). It is expressed by DRG neurons, is present in the blastema, and is decreased by denervation. A new clone of the Ggf2 gene was briefly mentioned to rescue regeneration to digit stages in denervated axolotl limbs when injected intraperitoneally during blastema formation (Wang et al, 2000).

A more detailed study of neuregulin 1 (NRG1) in regenerating axolotl limbs (Farkas et al, 2016) showed that transcripts of *nrg1* and its receptors *erb2* and *erb3* are expressed by the basal cells of the AEC and by 56% of the blastema mesenchyme cells. Antibody staining revealed expression of NRG1 and ErbB2 in dorsal root ganglia and peripheral limb nerves. Denervation of 16-day blastemas

decreased the number of *nrg1*-expressing mesenchymal blastema cells by 26%. Western blotting for NRG1 shows only a slight drop in intensity in denervated blastemas, but the percentage of BrdU+ cells co-localizing with NRG1 was diminished by 20%, a statistically significant reduction. Inhibition of NRG1/ErbB2 signaling by immersion of animals in mubritinib abolished blastema formation in amputated innervated limbs. Treatment of 16-day innervated blastemas resulted in miniature regenerates, equivalent to the regenerates obtained by delaying denervation until a well-established blastema has formed. NRG1-soaked beads implanted under the wound epithelium of denervated limbs at seven days post-amputation induced blastema formation. Bead implants every four days from 19-36 days post-amputation supported regeneration to digit stages, though not to the same degree as in innervated controls.

These results support Singer's neurotrophic hypothesis in which multiple limb tissues have the capacity to produce a mitogenic factor essential for blastema cell mitosis. They suggest a synergistic relationship between nerve and blastema cells in which blastema cells autonomously express NRG1 in the absence of nerve, but at a level that is insufficient for mitosis. NRG1 from motor neurons would stimulate blastema cells destined to form Schwann, skeletal and muscle cells to increase their own NRG1 expression. The nerve and blastema cells would thus work synergistically to express a single molecule, NRG1, to attain a threshold level sufficient for mitosis. Although the effect of denervation on NRG1 expression by the AEC was not reported by Farkas et al (2016), expression of NRG1 by the AEC could be incorporated into the model by postulating that NRG1 expression by sensory innervation would stimulate NRG1 production by the AEC for diffusion into the underlying mesenchymal cells. This kind of synergism would explain why increasing motor innervation in the absence of sensory innervation enables complete regeneration, because the required threshold level of NRG1 could be reached in the absence of sensory nerves. The addition to nerve for regeneration

that arises during limb development is thus interpreted as a nerve-induced quantitative increase in the requirement by blastema cells for NRG1, which conforms to Singer's original ideas.

In any event, this model predicts that (1) sensory innervation alone can maintain the threshold expression of NRG1, whereas sensory denervation will reduce the expression of NRG1 below threshold; and (2) augmenting motor innervation in the absence of sensory innervation will maintain the threshold expression of NRG1. Experiments to test these predictions will hopefully be undertaken, and to investigate the AEC as another possible source of NRG1.

Several questions remain about the synergistic relationship between nerve, AEC, and blastema cells that require further research. (1) Can we label AEC and neural candidate factors and show that they move into the blastema and bind to receptors on blastema cells? (2) Are neural factors such as NRG1 and Fgf2 expressed in limb buds or the blastemas of amputated aneurogenic limbs? (3) If they are not, does exogenous administration of these factors render aneurogenic limbs nerve-dependent?

Another relevant question is whether the epithelial: mesenchymal interaction that characterizes urodele limb bud development and aneurogenic limb regeneration is maintained in the regeneration of neurogenic limbs or is completely replaced by a nerve: AEC synergy. Does the blastema mesenchyme produce a non-neural factor such as Fgf10 necessary to maintain the AEC in addition to neural factors? Growth factor-mediated epithelial: mesenchymal interaction in urodele limb bud development and regenerating aneurogenic limbs has not been sufficiently investigated. The expression of AGP in aneurogenic limbs suggests that AGP might be part of an epithelial: mesenchymal interaction in urodele limb bud development. Fgf8 and Fgf10 are both expressed in urodele limb buds and regeneration blastemas of neurogenic limbs (Han et al, 2001; Christensen et

al, 2001), but it has not been shown that expression of these growth factors is interdependent as it has for *Xenopus* limb bud development and limb regeneration (Yokoyama et a, 2000; 2001).

Conclusion

Progenitor cells of the regeneration blastema in urodele amphibians produce factors that support the regeneration of axons into the blastema. In turn, nerves, in conjunction with the AEC, produce neurotrophic factors essential for the mitosis of progenitor cells during growth of the blastema.

There are three main hypotheses on how these two tissues interact to drive blastema growth, but the available evidence fits all three. Further experimentation will be required before favoring one idea over another. Elements of all three hypotheses may be required to explain how the proliferation of blastema progenitor cells is promoted during limb regeneration, and there might be multiple redundant and synergistic circuits composed of different combinations of neural and AEC factors.

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Legends for Figures

Fig. 1: Summary of blastema formation, which results in the formation of an accumulation blastema, and development, which involves growth and patterning of the blastema. This phase can be divided into several morphological stages, a conical medium bud, a larger late bud initiating cartilage differentiation, the emergence of fingerbuds and growth to complete regeneration. **A-D**, longitudinal sections through (A) the accumulation blastema stage, (B) the medium bud stage, (C), the late bud stage, (D), the two-fingerbud stage. **E, F**, whole mounts stained for cartilage with methylene blue. E, two-fingerbud stage; F, the finished regenerate. Reproduced from Stocum, Mechanisms of urodele limb regeneration. *Regeneration* 4: 159-200, Open Access.

Fig. 2: Reciprocal relationship between blastema (b) and nerves (black lines and arrows). Limb amputated through the mid-stylopodium of forelimb (s). **(A)** As the blastema forms, it produces factors (yellow arrow) that promote axon regeneration. **(B)** Regenerating axons provide neurotrophic factors that promote blastema growth and result in the regeneration of a normal limb. z = zeugopodium; c = carpals. **(C)** Denervation (x) at medium bud stage does not inhibit differentiation and morphogenesis of the blastema, but mitosis is inhibited and the blastema forms a miniature regenerate.

Fig. 3: Experiments by Globus, et al, *Dev Biol* 75:358-372, 1980, demonstrating effect of the AEC on mitosis of blastema cells. **(A)** A medium bud stage blastema (red) implanted deep in a dorsal fin (DF) tunnel forms a miniature, distally truncated regenerate, whereas the same stage blastema implanted so that its distal tip protrudes from the tunnel and is re-covered by fin epidermis (yellow line) forms a PD-complete miniature regenerate. VC = vertebral column; VF = ventral fin. **(B)** Blastemas cultured on the opposite side of a nanopore filter (dashed red line) from spinal ganglia (gold). Left, blastema mesenchyme (blue) with AEC (green). The blastema cells undergo mitosis. Right, blastema mesenchyme without AEC. The blastema cells withdraw from the cell cycle and differentiate into chondrocytes.

Fig. 4: Models of functional synergy between nerve, AEC, and blastema cells. **(A)** The AEC (yellow) produces factors (black arrows) that maintain the blastema cells (blue) in an undifferentiated state. The nerve produces separate factors (red arrow) that stimulate mitosis of the blastema cells. **(B)** The AEC produces factors necessary to maintain the undifferentiated state of the blastema cells, but depends on nerve factors to carry out this function. **(C)** The nerve, AEC and the blastema cells themselves all produce the same factors (red arrows, red dots).







