Regeneration

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REVIEW

The axolotl limb blastema: cellular and molecular mechanisms driving blastema formation and limb regeneration in tetrapods

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Abstract

The axolotl is one of the few tetrapods that are capable of regenerating complicated biological structures, such as complete limbs, throughout adulthood. Upon injury the axolotl generates a population of regeneration-competent limb progenitor cells known as the blastema, which will grow, establish pattern, and differentiate into the missing limb structures. In this review we focus on the crucial early events that occur during wound healing, the neural–epithelial interactions that drive the formation of the early blastema, and how these mechanisms differ from those of other species that have restricted regenerative potential, such as humans. We also discuss how the presence of cells from the different axes of the limb is required for the continued growth and establishment of pattern in the blastema as described in the polar coordinate model, and how this positional information is reprogrammed in blastema cells during regeneration. Multiple cell types from the mature limb stump contribute to the blastema at different stages of regeneration, and we discuss the contribution of these types to the regenerate with reference to whether they are "pattern-forming" or "pattern-following" cells. Lastly, we explain how an engineering approach will help resolve unanswered questions in limb regeneration, with the goal of translating these concepts to developing better human regenerative therapies.

Keywords

Ambystoma mexicanum, blastema, limb, positional information, regeneration

Introduction

The axolotl is one of the few adult vertebrate model systems capable of complete and faithful regeneration of missing body parts throughout life (Carlson 2007). The axolotl is a member of the Urodele group of amphibians that includes salamanders and newts, which also are robust regenerators. Much research has focused on what makes these amphibian species capable of regenerating while other vertebrates such as the amniotes retain limited regenerative capacity as adults. One hypothesis to explain this divergence is based on the observation that some Urodeles such as the axolotl are paedomorphic (i.e., they become sexually mature while externally retaining juvenile characteristics [Tompkins 1978]), and thus they are capable of regenerating because they do not complete metamorphosis and their cells retain some embryonic-like characteristics (Galliot & Ghila 2010). This idea is consistent with data from anuran amphibian species, such as the African clawed frog, which can regenerate robustly at early larval stages (e.g., limb buds) but progressively lose this ability in association with differentiation and the initiation of metamorphosis (Suzuki et al. 2006). In contrast, the ability to regenerate is maintained in Urodeles such as newts that complete metamorphosis endogenously (Iten & Bryant 1973) and in axolotls that have been induced to complete metamorphosis in response to experimental activation of thyroid hormone signaling (Tompkins & Townsend 1977; Rosenkilde et al. 1982). Thus, while metamorphosis is the regeneration-restricting developmental step in frogs, this does not appear to be the case for Urodeles.

Another hypothesis to explain the difference in regenerative capacity is based on the observation that Urodeles have a simpler adaptive immune system than amniotes, and thus their ability to regenerate is suggested to be dependent on a

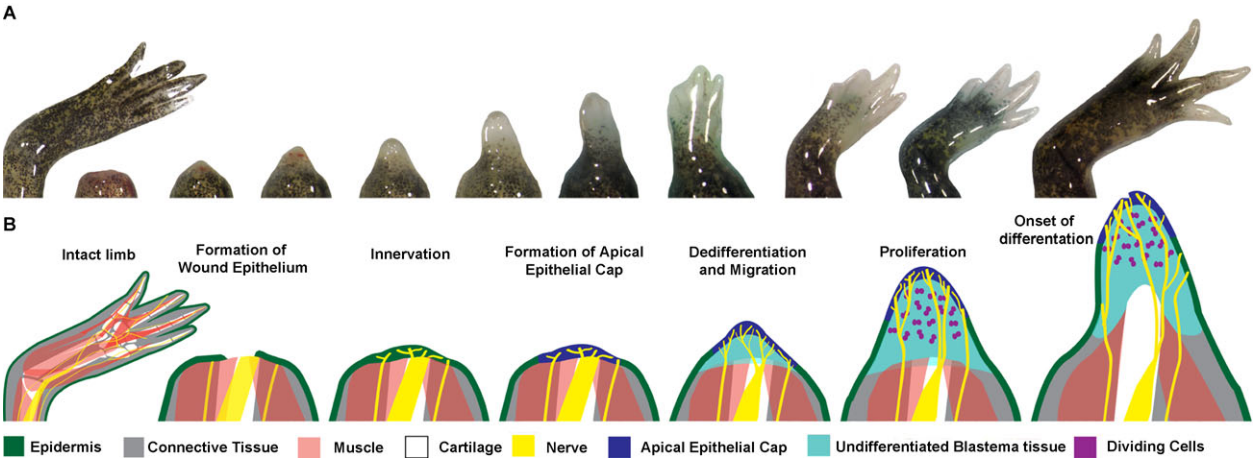

Figure 1. Axolotl limb blastema development. (A) Live images of the time course of limb blastema development showing an intact limb (left) and 1 day, 7, 9, 11, 13, 15, 17, 21, 25, and 31 days post amputation (consecutively to the right). (B) The key steps in the regenerative process are highlighted during blastema development. The tissue components are indicated by the color key. The intact limb is composed of multiple tissues including epidermis, connective tissue, cartilage, muscle, and nerves, which are organized in a specific way to generate functional structures (left). Within hours following an amputation, a wound epithelium covers the severed edge of the limb. Within days, this wound epithelium becomes innervated, and becomes a specialized signaling center known as the apical epithelial cap (AEC). The AEC induces dedifferentiation in the underlying stump tissue and attracts cells, which accumulate below the AEC. It is likely that positional interactions occur in the "dedifferentiation and migration" stage, because if insufficient positional disparity is present the blastema will continue to grow for a limited time but will not form limb structures. At later stages of development, the cells in the basal region of the blastema (closest to the stump) begin to differentiate, while the cells at the apical tip of the blastema remain in a proliferative and undifferentiated state (right). Over time, the blastema cells progressively differentiate into limb tissues from the basal to the apical regions of the blastema.

weak inflammatory response (Mescher & Neff 2005). Observations on tetrapod species that correlate a depleted immune response with increased regenerative capacity are consistent with this hypothesis (reviewed in Mescher & Neff 2005; Godwin & Brockes 2006). However, other seemingly conflicting observations on amphibians and reptiles show that the immune system plays a positive role in the regenerative response (reviewed in Godwin & Brockes 2006). While similar hypotheses based on comparative biology between animals that can or cannot regenerate have been proposed, it will probably be very difficult to assess the extent to which these differences are functionally at the root of the observed disparities in regenerative ability in these highly diverged species (Bely & Nyberg 2010). Our goal, in research and in the present article, is to understand the basic mechanisms underlying the regenerative response in the axolotl and to identify commonalities with regeneration-restricted species such that potential targets to enhance their regenerative capacity can be pinpointed.

Most studies of axolotl regeneration have focused on the limb, and to a lesser extent the tail, but many other parts of the body are capable of faithful regeneration, for example parts of the eye, brain, and internal organs. Although other vertebrates can replace missing parts, in many cases the new structures are not the same as the original. For example, when lizards regenerate their tails, the new structure serves the same function as the original but it develops by different mechanisms and its structure is a simplified version of the original (Bryant & Bellairs 1967; Gilbert et al. 2013). We are greatly interested in understanding the axolotl limb blastema because it represents vertebrate regeneration at its best.

Formation of the blastema is the critical event leading to successful regeneration of lost structures through the process of epimorphic regeneration (Goss 1969). Although blastema formation appears to be a unique process, once the blastema has formed it exhibits all the behaviors of the limb bud that formed the limb during embryogenesis (Bryant et al. 2002). Hence the mechanisms controlling the later events of regeneration (after the blastema has formed) appear to be conserved between regeneration and development, and one of the challenges for inducing an endogenous regeneration response is to discover how to induce blastema formation.

Blastema formation requires an adequate nerve supply (Singer 1974), a permissive wound epithelium (WE) just as in limb development (Wallace 1981), and cells of connective tissue origin that encode different positional identities (French et al. 1976; Bryant et al. 1981) (Fig. 1). Signaling between the nerve and the WE functions largely to recruit regeneration-competent cells that are equivalent to the undifferentiated cells of the limb bud. The blastema cells with

positional information interact with each other and with cells in the stump to control pattern formation to ensure that the regenerated tissues reform in the right place (positional information). When these early events are initiated and orchestrated correctly, a blastema forms (Fig. 1). By the late bud stage, all the necessary cells and information necessary to reform the limb are present such that when grafted to an ectopic site the blastema will continue to develop into an ectopic limb (Milojević 1924; Weiss 1925; Schwidefsky 1934; Iten & Bryant 1975; Stocum 1980). In this review we examine what is known about the requirements for the establishment of a functional blastema.

Early Response to Injury

Limb regeneration in a salamander is initiated by injury that leads to wound healing. In response to pro-regenerative signals, the cells in and around the wound are recruited to form a blastema (Gardiner et al. 1986; Endo et al. 2004), which grows and undergoes pattern formation to replace the missing limb structures. Although wound healing precedes blastema formation, not all wounds that heal will progress to form a blastema; for example, wound healing in a mammal leads to formation of scar tissue. Thus understanding the relationship between wound healing and blastema formation is important in order to be able to manipulate a non-regenerative wound such that it can form a blastema.

Wounds that do not lead to blastema formation either can heal and regenerate the skin or can form scars. In axolotls, the process of wound healing eventually leads to restoration of normal skin architecture rather than scar formation (Seifert et al. 2012). This process involves a transient phase of fibrosis that is not unlike that seen in skin wounds in mammals, but in contrast to mammals, fibrosis in axolotls is transient and is followed by remodeling of the fibrotic tissue leading to the restoration of normal skin structure (Neufeld & Day 1996; Endo et al. 2004). The process of skin regeneration itself has not been exploited experimentally, but presumably it could provide insights into how fibrotic tissue can be remodeled and ultimately into scar-free wound healing. Salamander skin regeneration is known to depend on nerve signaling because skin wounds on limbs that have been denervated heal with dense scar-like connective tissue and epidermal appendages are not reformed (Salley & Tassava 1981; Mescher et al. 2000).

The distinguishing feature of a scar is the persistence of excess fibrotic tissue. Although mammals typically heal skin wounds by formation of scar tissue, examples of scar-free wound healing do exist (Gurtner et al. 2008). These include wounds in human fetal skin prior to the third trimester as well as the skin of other mammalian embryos at comparable stages, and scar-free wound healing is associated with differential regulation of transforming growth factor β (TGFβ) signaling. In contrast to the transient fibrotic response of axolotl wounds (Endo et al. 2004), post-embryonic mammalian wounds continue to accumulate collagenous fibers, and the normal skin architecture is never restored. There is an obvious relationship between scar-free wound healing and the ability to regenerate complex tissues, even in mammals, where in the one well-studied case of digit tip regeneration in both mice and humans skin is regenerated when mammalian digit tips regenerate (Muneoka et al. 2008). Conversely, treatments that lead to the formation of dense connective tissues covering wounds that normally would lead to regeneration in a salamander (e.g., denervation or grafting of uninjured dermal tissue to cover the wound [Salley & Tassava 1981]) prevent regeneration. In summary, formation of excess fibrotic tissue is associated with regenerative failure, whereas modulation and remodeling of an initial fibrotic response is pro-regenerative.

Initiation of the Blastema

Although pro-regenerative wound healing is required for eventual regeneration, by itself it is not sufficient for blastema formation. This is demonstrated most directly in the accessory limb model (ALM), which is an in vivo gain-of-function assay for the signaling necessary for development of a blastema capable of generating a new limb (Endo et al. 2004). In this assay, an ectopic blastema that is equivalent to an amputation-induced blastema is induced on the side of the arm by making a small full-thickness skin wound and surgically deviating the brachial nerve to the wound site (Endo et al. 2004; Satoh et al. 2007). If a nerve is not deviated, the wound heals without scar formation and the normal skin architecture is restored, as discussed above, but no blastema forms (Endo et al. 2004). This indicates that blastema formation requires a threshold level of nerve signaling, that in the case of amputation is provided by the severed nerves of the limb but which is absent in the case of the ALM skin wound without a deviated nerve. It is therefore possible using the gain-of-function ALM assay to compare the differential regulation of signaling pathways and the response of cells to those signals in wounds that are induced to form a blastema and those that have the ability to form a blastema but have not been induced to do so. Hence the ALM has made clear that signaling from the nerve and the WE, as well as the presence of both dermal cells and the extracellular matrix (ECM), are needed for blastema formation and subsequent pattern formation leading to regeneration of a limb de novo (Endo et al. 2004; Satoh et al. 2007; McCusker & Gardiner 2013).

Regardless of whether or not a blastema forms, after injury there is a cascade of events leading to wound healing. The wound surface is covered rapidly by an epithelium derived from keratinocytes around the wound periphery (Ferris et al. 2010). Rather than the cells migrating across the wound

surface, this sheet of epithelial tissue is pushed from behind as cells at the periphery take up water and expand in volume (Tanner et al. 2009). Over the next few days there are a number of changes in both the stump tissues and the WE that precede the initial appearance of a blastema and the onset of blastema cell proliferation. The WE is induced to form the apical epithelial cap (AEC) in response to nerve signals (Singer & Inoue 1964), and this transition is associated with the basal keratinocytes becoming non-proliferative and starting to produce signals that lead to the recruitment of connective tissue fibroblasts to form the early blastema.

Cells in the stump also undergo changes prior to blastema formation, including changes in gene expression (Gardiner et al. 1995; Satoh et al. 2008b, 2011) and the ability to migrate into the wound bed to form the blastema (Gardiner et al. 1986; Endo et al. 2004). The response of cells in the stump depends on the extent of the injury that occurs when the wound is made. In the case of an amputation, much of the response of stump cells is associated with the extensive damage that occurs. Many of these responses such as inflammation and necrosis are also observed in comparable injuries in mammals, yet they are not sufficient to induce blastema formation. Nevertheless some of these responses appear to be necessary for blastema formation; for example, if the inflammatory signals from macrophages are inhibited limb regeneration is inhibited much like what is observed in denervated limbs (Godwin et al. 2013). Thus there are necessary early signals derived from the nerve and/or inflammatory cells that induce downstream changes in the WE/stump that are necessary for the regeneration cascade to progress to the point of blastema formation (Endo et al. 2004). As has been suggested for many decades, this cascade of events appears to be dependent on the interaction between nerves and the newly formed WE that induces formation of an AEC that is functionally equivalent to the AEC/AER (apical ectodermal ridge) of limb buds in developing vertebrate embryos (Singer & Inoue 1964).

Importance of Nerves

In contrast to amputations, the wounds made in the ALM cause minimal or no damage to the underlying stump tissues; nevertheless a blastema forms that is equivalent to a blastema that forms on an amputated limb (Endo et al. 2004; Satoh et al. 2007). Thus many of the events of wound healing associated with healing an amputated limb wound are not necessary for blastema formation. Of the signaling mechanisms that are sufficient for blastema formation, those associated with the nerve are the most obvious, which is consistent with classical studies demonstrating that nerve signaling is required for regeneration (Singer 1946, 1974; Singer & Inoue 1964). The role of signaling associated with inflammation in the ALM has not been investigated to date. In the ALM, a deviated nerve induces ectopic blastema formation but, as alluded to above, in the absence of a deviated nerve there are still sensory nerves present, and thus these wounds also receive nerve signals, although at a lower level than in wounds with a deviated nerve. Since denervated limbs form a dense connective tissue cap distally and fail to regenerate (Salley & Tassava 1981), it appears that at least a low level of nerve signaling is required for scar-free wound healing, but it is not quantitatively sufficient to induce blastema formation.

Regeneration is known to require signaling above a threshold level from the nerve (Singer 1974). This level of signaling is necessary for both initial blastema formation and for growth and development of the blastema during the early and mid stages of regeneration (Fig. 1). In addition, rather than the type of nerve (e.g., motor versus sensory), it is the quantity of nerves that is important in regeneration. The latter phenomenon is important given that it is the interaction between sensory nerves and the WE/AEC that is important for controlling scar-free wound healing and blastema formation. This early interaction involves changes in gene expression that initially are common to both processes but are persistent in the pathway leading to blastema formation. For example, the transcription factor Sp9 is involved in the regulation of fibroblast growth factor (FGF) signaling and is expressed in the AEC/AER of developing limb buds (Kawakami et al. 2004; Satoh et al. 2008a). It is re-expressed within 24 h at high levels in the WE keratinocytes during both scar-free healing and blastema formation. Expression is transient during scar-free healing, but persists and becomes localized to AEC basal keratinocytes during blastema formation and subsequent stages of regeneration (Satoh et al. 2008a). Thus it appears that nerve signaling induces keratinocytes of the WE to dedifferentiate as evidenced by the re-expression of the embryonic gene *Sp9*, and that high levels of nerve signaling maintain these cells in an undifferentiated state in the blastema.

Blastema formation requires that the keratinocytes of the WE undergo dedifferentiation to reacquire the functional properties of the limb bud AEC. Similarly, as discussed below, connective tissue fibroblasts dedifferentiate as they give rise to the mesenchymal cells of the early blastema (Muneoka et al. 1986; Kragl et al. 2009; McCusker & Gardiner 2013; Nacu et al. 2013). Mechanistically dedifferentiation must involve epigenetic modifications to reactivate earlier developmental signaling pathways (Stewart et al. 2009; McCusker & Gardiner 2013, 2014). The functional role of epigenetic modifications in dedifferentiation and transdifferentiation has begun to attract considerable research attention in recent years, and as with other clinical applications such as cancer, epigenetic modifications associated with the initiation of regeneration are potential targets for therapeutic intervention (Christen et al. 2010; Sancho-Martinez et al. 2012).

A number of factors have been proposed and investigated as the "neurotrophic factor" that is produced by nerves and is required for blastema formation and regeneration. Many potential candidates, including neuropeptides, organic molecules, cyclic nucleotides, growth factors, and even bioelectric signals have been investigated (Singer 1978; Wallace 1981; Mescher 1996; Bryant et al. 2002; Satoh et al. 2009). One approach has been to focus on the signaling pathways that function during limb development to control migration, proliferation, and differentiation of limb bud cells. For example, as in limb development FGF signaling is important during limb regeneration and is associated with nerve signaling. FGFs are expressed in the apical blastema where blastema mesenchymal cells interact with the AEC (Mullen et al. 1996; Han et al. 2001; Christensen et al. 2002). Nerve-dependent expression of the transcription factor *Dlx3* is rescued in denervated axolotl limbs by implanting FGF2 soaked beads (Mullen et al. 1996). Keratinocyte growth factor (FGF7) expression is induced by injury to nerves, and FGF7 soaked beads induce the expression of *Sp9* in basal keratinocytes of the axolotl WE when grafted into wounds (Satoh et al. 2008a). Most importantly, a cocktail of recombinant human growth factors that includes FGF (FGF2, FGF8 plus GDF5/BMP2) can substitute for a deviated nerve and induce blastema formation in the ALM (Makanae et al. 2013, 2014).

Another signaling molecule that has been implicated in nerve signaling during salamander regeneration is the newt anterior gradient (nAG) protein (Kumar et al. 2007). This molecule is expressed in association with Schwann cells of nerves and with skin glands, and can rescue regeneration in partially innervated newt limbs (Kumar et al. 2007). This factor appears to function at later time points in regeneration, after the initial wound has already been induced by nerve signals to progress along the blastema formation pathway (Endo et al. 2004). Although nAG protein appears to activate a newt-specific signaling pathway, the recent discovery that axolotl wounds can be induced to form a blastema in response to human growth factors (Makanae et al. 2014) is consistent with the hypothesis that the critical pathways involved are conserved between salamanders and humans.

Since the nerve itself continues to regenerate and innervate the WE/AEC as the blastema forms and grows, there is presumably a feedback loop in the signaling pathways between the nerve and WE/AEC (Stocum 2011). A recent study of the molecular response of the regenerating nerves (dorsal root ganglion) to signaling from blastema cells has identified a number of signaling pathways that are conserved between axolotls and mammals (Athippozhy et al. 2014). Among these is the bone morphogenetic protein (BMP) signaling pathway that has been shown to be necessary for successful mouse digit regeneration (Muneoka et al. 2008). To understand the quantitative regulation of this and other pathways associated with reciprocal nerve−blastema signaling, we have been working to optimize organotypic slice culture for axolotl blastemas (work in progress). Although the response of nerves to signaling from the blastema has not been exploited experimentally, it presumably would lead to insights into mechanisms for inducing and patterning peripheral nerve regeneration.

Role of Nerves in the Recruitment of Blastema Cells

The outcome of neuro-epithelial interactions during salamander wound healing is the recruitment of connective tissue cells from the stump and surrounding dermis to form the early blastema (Gardiner et al. 1986; Muneoka et al. 1986; Endo et al. 2004; Hirata et al. 2010; Nacu et al. 2013). The onset of cell migration is delayed for a couple of days after wounding, presumably as a consequence of the necessity to degrade the ECM surrounding these cells (Yang et al. 1999). The direction of migration is controlled by localized signaling from the interaction of the nerve and WE/AEC such that repositioning the WE/AEC or the nerve repositions where the blastema forms (Thornton 1960; Thornton & Thornton 1965). The directed migration of the early blastema cells towards the center of the AEC is consistent with the hypothesis that FGFs produced by the nerve/WE/AEC serve as early pro-regenerative signals. This idea is supported by the finding that the distal migration of limb bud cells can be redirected toward implanted beads soaked in FGF (Li & Muneoka 1999). Although muscle stem cells (satellite cells) are activated and begin to proliferate soon after limb amputation (Cameron et al. 1986), reentry into the cell cycle by the blastema progenitor cells (connective tissue fibroblasts) does not occur until the cells have migrated into the center of the wound, several days after injury (Gardiner et al. 1986). Thus blastema formation is initiated by nerve/WE/AEC dependent and directed cell migration, followed by proliferation of the undifferentiated blastema cells.

It appears that blastema cells arise by two different mechanisms. For some tissues (e.g., muscle) there are well characterized adult stem cells (satellite cells) that are activated and proliferate to repair and regenerate the damage and missing muscles (Carlson 1970; Cameron & Hinterberger 1984; Cameron et al. 1986). These muscle-lineage committed cells are recruited into the blastema after it has already formed, which is comparable to the temporal pattern of myoprogenitor cell migration into the limb bud during embryonic development (Kieny & Chevallier 1979). Although it appears that muscle regeneration in some salamanders (i.e., newts) occurs by fragmentation and dedifferentiation of preexisting myotubes (Brockes 1997; Sandoval-Guzmán et al. 2014), this does not occur during regeneration of muscle in axolotls (Sandoval-Guzmán et al. 2014). Thus the mechanism

of muscle regeneration is conserved between axolotls and other vertebrates, including humans.

It is noteworthy that in the ALM when a de novo limb is induced to form it contains normally patterned limb structures, including muscles. Since there is no injury to the underlying muscle tissues when the original skin wound is made, there presumably is signaling from the nerve/WE/AEC or the early blastema cells that activates and recruits myoprogenitor satellite cells in the absence of injury to the muscle. De novo limb formation can also be induced to form on newt limbs, again without injury to the underlying muscle tissues (Makanae et al. 2014), suggesting that satellite cells in newt muscle can also be activated in the newt. Further investigation of the mechanism of satellite cell activation in the ALM would probably provide insights into therapies for inducing muscle regeneration given the conserved mechanisms shared by axolotls and humans (Sandoval-Guzmán et al. 2014).

As discussed in more detail below, the second mechanism for blastema cell formation is referred to as dedifferentiation. This process is defined as cells being reprogrammed to a more embryonic-like state so as to acquire increased developmental potential (Han et al. 2005; Satoh et al. 2008a). This process is evident in the re-expression during regeneration of a large number of genes that had previously functioned during embryonic development (Bryant et al. 1987; Gardiner & Bryant 1996). Until recently, little was understood about how this occurs mechanistically, but with the advances in cellular reprogramming the feasibility of therapies based on this approach has been well established. As with the activation and recruitment of adult stem cells, signaling from the nerve/WE/AEC is required to induce dedifferentiation of connective tissue fibroblasts to form regeneration-competent blastema cells (Satoh et al. 2010). Mechanistically, nerve/WE/AEC induced dedifferentiation appears to involve FGF signaling (Makanae et al. 2013).

Stimulation of Blastema Growth Leading to Pattern Formation

When a salamander limb is amputated, a number of molecular and cellular events occur prior to the overt presence of the blastema. As discussed above, these include wound healing and formation of the WE/AEC in response to nerve signals. The AEC in turn begins to provide a number of important signals, including FGFs, BMPs, and Wnts as well as enzymes that degrade the ECM molecules in the adjacent mesoderm tissues, thereby freeing the cells to respond to attractive signals from the AEC. Compared to the relatively early onset of proliferation of myoprogenitor cells (Cameron & Hinterberger 1984), the reentry of blastema progenitor cells from the connective tissues into the cell cycle is a relatively late event in blastema formation. After a delay of a couple days, cells from the dermis (fibroblasts) around the limb circumference begin to migrate along with connective tissue fibroblasts surrounding the internal structures of the stump (muscles, bones, and nerves) and begin to accumulate in the center of the wound beneath the AEC. These cells and their progeny are known to have different positional identities in both the proximal distal axis and around the circumference, and as they gather in the center of the wound they encounter neighbors with disparate positional identities, which stimulates reentry into the cell cycle and regeneration (French et al. 1976; Bryant et al. 1981).

While the presence of nerve/WE/AEC signals is sufficient to induce formation of a blastema, blastema growth will not be sustained without the added diversity of positional information provided by cells arising from the opposite sides of the limb (French et al. 1976; Bryant et al. 1981; Endo et al. 2004). We know that in humans positional information is encoded in the fibroblasts throughout the adult body including the limbs via an epigenetic program of region-specific genes, such as the *Hox* genes (Chang et al. 2002). We predict that this same mechanism is used to program positional identity in fibroblasts in the axolotl, although the tools needed to test this idea (i.e., assembled genomic sequences of the *Hox* loci) are currently being developed. The presence and importance of positional information for growth and pattern formation in the regenerate were first illustrated clearly by grafting experiments in Urodeles that bring cells together from different parts of the limb circumference, or from different positions along the proximal−distal axis. From such studies it is known that regeneration of a limb requires a complete circumference of positional information, composed of cells from anterior, posterior, dorsal, and ventral positions, to be present at the base of the blastema. If a small part of the circumference is absent, the missing part can be filled in by interactions between the cells at the wound margin that stimulate proliferation and regeneration of the missing positional information by the process known as "intercalation" (Bryant et al. 1981).

These experiments, and analogous ones carried out in cockroach legs and Drosophila imaginal discs, led to the development of the polar coordinate model (PCM), which provides a formalized set of rules that predict the behavior of blastema cells and the pattern of the limb structures they will form (Fig. 2) (French et al. 1976). The premise of the PCM is that, in a vertebrate limb, fibroblasts in the connective tissue under the epidermis and surrounding the tissues of the stump (e.g., muscles, bones, blood vessels, and nerves) encode information about their positions in the proximal−distal axis and around the circumferential axis (anterior, dorsal, posterior, and ventral) (Fig. 2A). After amputation, these cells migrate towards the wound center and accumulate to form the blastema, as discussed above. In the blastema, cells from different parts of the limb circumference with different positional identities begin to interact with one

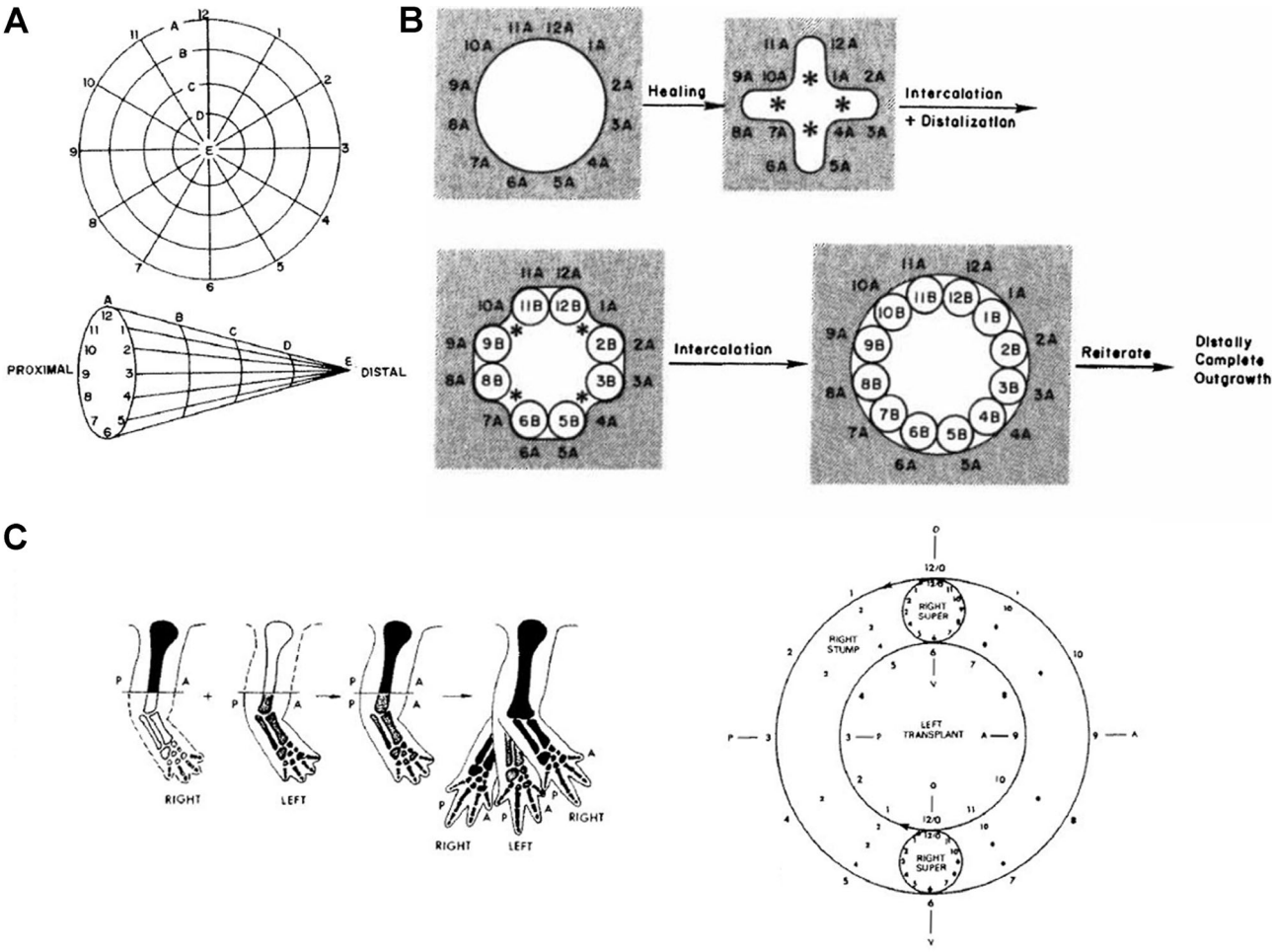

Figure 2. The polar coordinate model of regeneration. (A) The polar coordinate model for regeneration is based on the idea that cells in the limb field know their position relative to the circumferential axes of the limb (i.e., anterior/posterior and dorsal/ventral or coordinates 1–12) (top), and along the proximal–distal limb axis (A–E) (lower). (B) When the limb is amputated proximally (level "A"), a complete circle of positional values is present at the circumference of the wound. After the wound heals, cells from the different locations on the limb interact with cells that they do not normally interact with, stimulating a growth response that generates new cells with the intermediate (along the "shortest-route") circumferential positional values that correspond to the next proximal/distal level. The processes of intercalation and distalization are reiterated until all of the positional discontinuities are resolved and the pattern of the limb is completed. (C) If a "complete circle" of positional information is present in a regeneration-competent environment, then a limb structure will form. This is exemplified by the formation of supernumerary limbs when the stylopod from a left forelimb is rotated 180° and grafted to the stylopod of the right forelimb, which generates positional discontinuities that result in the intercalation of complete circles of positional values at the dorsal/ventral poles (French et al. 1976; Bryant et al. 1981).

another. This interaction between cells with disparate positional information stimulates growth and the genesis of new cells that adopt intermediate position information, a process referred to as "intercalation." Intercalation continues until all positional disparities have been eliminated, that is, the limb structure with normal pattern is completely regenerated (Fig. 2B). Growth of the blastema is also dependent on permissive factors provided by nerves and the WE/AEC (Singer 1946; Singer & Inoue 1964); however, without positional interactions between cells from different positions around the circumference of the limb, regeneration will not be successful.

Subsequent modifications of the PCM addressed the issue of what happens if there is not enough positional disparity to regenerate the entire limb pattern. When symmetrical limb stumps are created by removing one half of the upper arm (e.g., the anterior half) and grafting in its place the posterior half from a donor limb, thereby creating a symmetrical (double-posterior) limb upper arm (Bryant et al. 1981), amputation through this symmetrical limb leads to growth of a

symmetrical structure of variable complexity and length. This result again highlights the critical role of positional information in blastema formation and limb regeneration. When an amputation is made through a normal limb stump (containing all the circumferential positional values) cells from the limb circumference migrate onto the wound surface under the permissive influence of the wound epidermis and interact to regenerate the missing parts of the limb. In the case of symmetrical double half limbs, more than half of the information around the limb circumference is missing. In that situation, intercalation still occurs but the positional information is progressively lost along the symmetrical boundary, leading to a symmetrical tapering spike (Bryant et al. 1981).

Plasticity and Reprogramming of Positional Information in Blastema Cells

In order for a limb regenerate to form, the cells from a more proximal location in the limb generate blastema cells that will need to acquire the positional information of the missing distal structures (Rose 1962). For this to occur, positional information must be reprogrammed in these cells and their progeny during the regenerative response. The observation that the expression of genes from the *HoxA* locus is reactivated in the early blastema and late blastema is consistent with this hypothesis because it suggests that the establishment of new positional information is occurring in the regenerate (Gardiner et al. 1995; Roensch et al. 2013). Evidence also indicates that this new positional information is initially plastic, and gradually becomes stabilized in blastema cells as they differentiate (Singer 1952; Niazi et al. 1985; McCusker & Gardiner 2013). For example, the positional information in undifferentiated blastema cells of the early blastema and the apical tip of the late blastema can be reprogrammed to a more proximal location on the limb when exposed to exogenous retinoic acid (RA) (Niazi et al. 1985). However, differentiated cells in the stump tissue or the basal region of the late bud (LB) blastema are resistant to positional reprogramming by RA (Niazi et al. 1985; McCusker et al. 2014). The retinoic acid receptor that is responsible for positional reprogramming (*RAR-δ_2*) is present (Ragsdale et al. 1989, 1992, 1993; Pecorino et al. 1996) and is activated (Monaghan & Maden 2012) in both positionally plastic and stabilized cell populations in the limb. Thus, the presence of an activated receptor is not enough for positional reprogramming (Ragsdale et al. 1993). Rather, it appears that something about the plastic state of undifferentiated blastema cells, potentially because of their "opened" chromatin state (Hay 1959), renders them sensitive to positional reprogramming (McCusker & Gardiner 2014).

Recently published observations from our laboratory also support the idea that the positional information in undifferentiated blastema cells is plastic because grafts of these tissues (early bud [EB] and the apical region of LB blastemas) to new host locations do not induce the formation of supernumerary structures on either the proximal−distal or anterior−posterior axes of the limb (Fig. 3A) (McCusker & Gardiner 2013). In addition, grafted EB blastema cells lose the expression of a positional marker from their original location (*Tbx5*) and gain the expression of a marker that corresponds to the new host location (*Tbx4*) (McCusker & Gardiner 2013). In contrast to EB and apical LB blastemas, grafts of differentiated blastema cells (basal LB) and stump tissue do induce the formation of supernumerary structures. These results are consistent with the hypothesis that positional information is progressively stabilized in the blastema, starting first in the regions that are closest to the stump and furthest from the AEC (Fig. 3B) (McCusker & Gardiner 2013).

It appears that signaling from the nerves regulates the plasticity/reprogramming potential of undifferentiated cells in the blastema (McCusker & Gardiner 2013). In contrast to the innervated (un-manipulated) EB and apical LB grafts, when the regenerating limb is denervated these grafted regions of the blastema do induce the formation of ectopic limb structures (Fig. 3), which we interpret to indicate that they are no longer positionally plastic (McCusker & Gardiner 2013). The mechanism of this regulation currently is unknown, but it is probably an indirect result of the neuro-epithelial feedback loops that are required to maintain the function of the AEC (Singer & Inoue 1964; Mullen et al. 1996; Satoh et al. 2007; Monaghan et al. 2009; Athippozhy et al. 2014).

It is likely that signaling downstream of the nerve−AEC interactions is required to maintain plasticity in blastema cells until the positional information of the missing limb structures has been reestablished. Consistent with this hypothesis is the observation that denervation completely inhibits the ability of an EB blastema to form new limb structures (Singer 1952). This would not be expected if the pattern of the entire regenerate were established at an early stage of blastema development, and thus the missing pattern presumably is intercalated as the blastema grows (Gardiner et al. 1995). If the blastema is denervated at late stages of regeneration when the entire blastema is undergoing differentiation, and apparently has stabilized its positional information, a normally patterned (yet small in size) limb regenerate forms (Singer 1952). All together, these results suggest that the nerve is required to maintain positional plasticity in blastema cells that have not completely established the pattern of the missing structures (Fig. 3).

The Contribution of Cells With and Without Positional Memory to the Limb Regenerate

At the onset of limb regeneration, cells from multiple tissues in the stump lose the distinct morphological characteristics of their differentiated tissue type and acquire the fairly

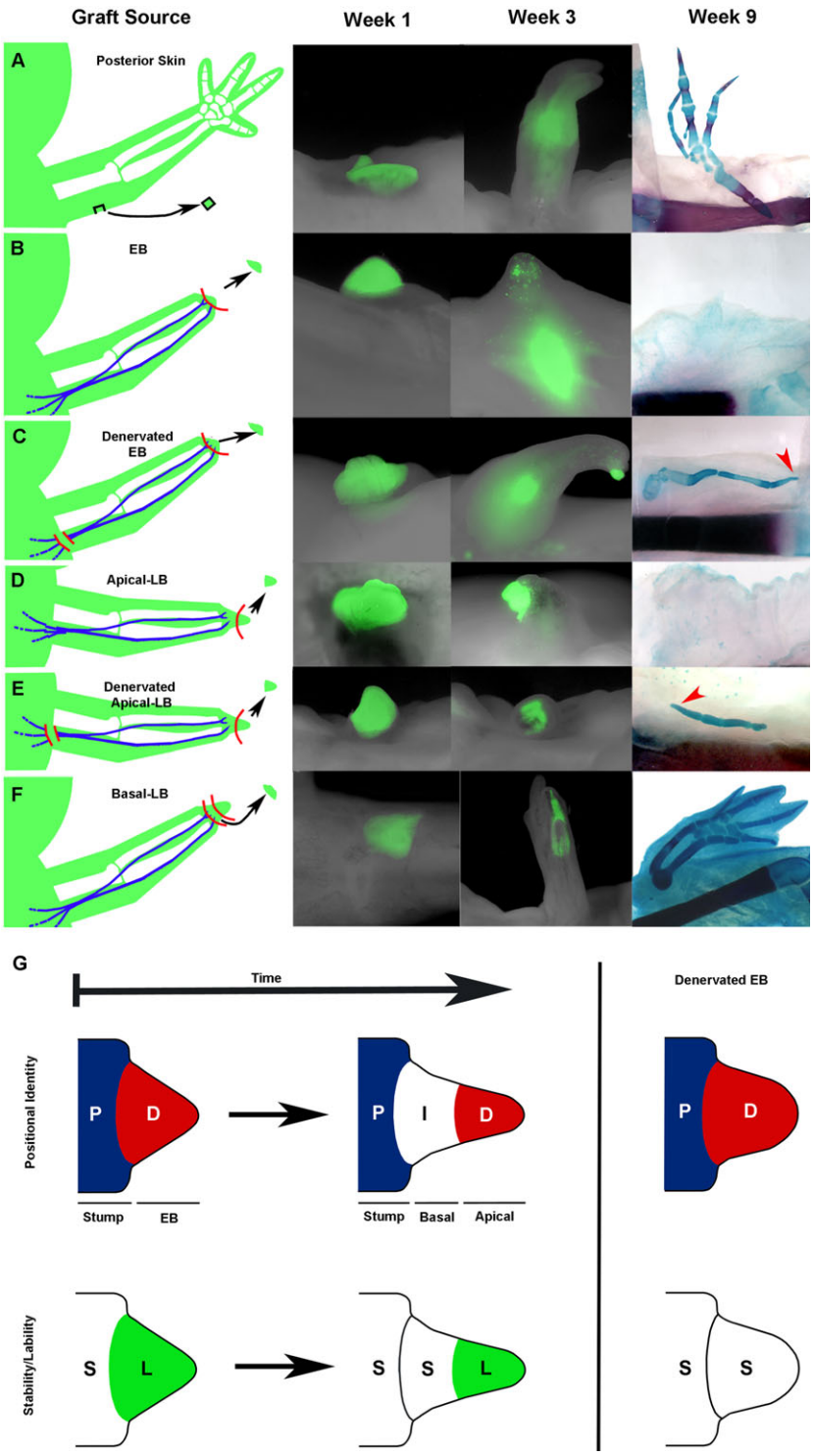

Figure 3. The stabilization of positional information in the blastema is controlled by nerve signaling. (A)–(F) Illustrations describing the origin of the grafted cells (left), live images of the grafted cells 1 and 3 weeks post-grafting (middle), and whole mount skeleton staining (right) are provided to summarize the ectopic growth response when different blastema tissues are grafted into an accessory limb model (ALM). (A) Ectopic limbs are generated in the ALM when posterior skin is grafted into an anteriorly located wound site with a deviated nerve. (B) Grafts of early blastemas (EBs) do not induce the formation of ectopic limbs when grafted into an ALM; however, grafting an EB from a limb 3 days after it was denervated results in the formation of a segmented but incomplete regenerate (C). (D) Grafts of the apical tip of the late blastema (LB) do not induce the formation of ectopic limbs. (E) Similar to denervated EBs, denervated apical-LBs induce the formation of segmented but incomplete regenerates. (F) Grafts of the basal region of the LB result in the formation of limb regenerates with completely patterned anterior/posterior and dorsal/ventral structures. (G) Our current model is that the positional information in the EB and the apical LB is labile or plastic, while the positional information has been stabilized in the stump and basal LB. If signaling from the nerves is removed, the positional information in the labile/plastic blastema cells prematurely stabilizes before the complete pattern of the missing structures has been intercalated, resulting in an incomplete regenerate (see C and E) (McCusker & Gardiner 2013). P, proximal; D, distal; I, intermediate; S, stable; L, labile.

uniform mesenchymal characteristics of "blastema cells." The stump cells that contribute to the blastema are local in origin, arising from tissues less than 1 mm from the amputation plane (Hertwig 1927). Although it was once thought that the different tissues contribute cells to the blastema in proportion to their relative abundance in the stump, we now know that the cells that retain positional memory (derived from connective tissue) contribute proportionately more cells to

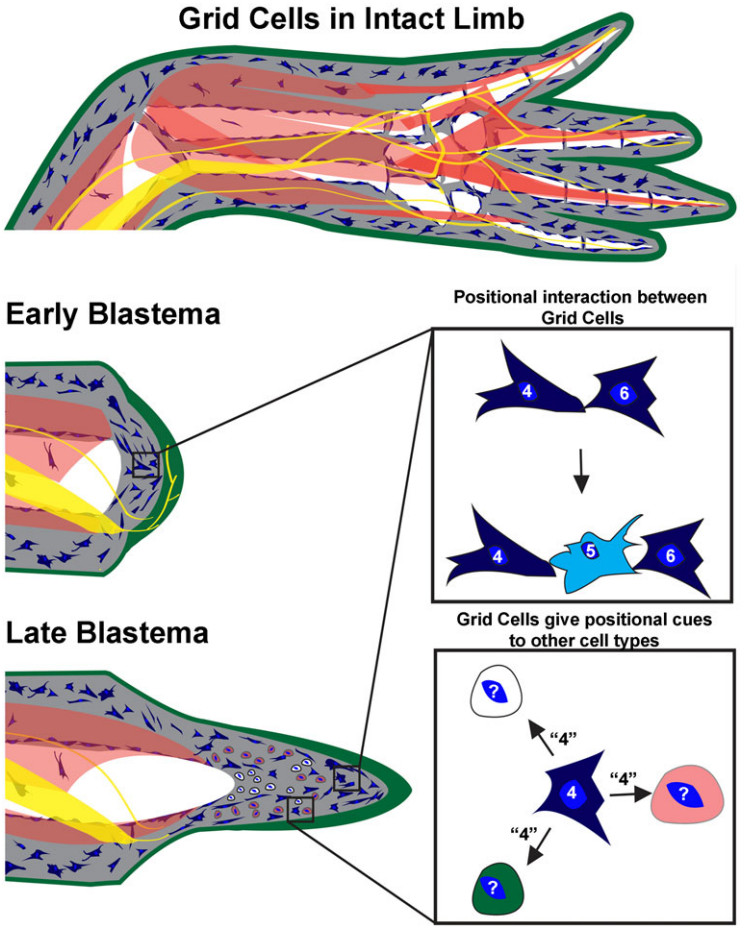

Figure 4. The pattern-forming grid cells guide the behavior of pattern-following cells. The cells that retain positional memory (dark blue) are located in the connective tissues that line all of the structures in the intact limb. When the limb is amputated, a regeneration-competent environment is generated through nerve–epithelial interactions, which generate the apical epithelial cap (AEC) that dedifferentiates and recruits the grid cells from the tissues in different locations on the limb to accumulate below the AEC and interact (early blastema). The grid cells with differencing positional information (e.g., 4 and 6) induce an intercalary response to generate cells with the missing positional information (i.e., 5). At later stages of development (late blastema), the basal region of the blastema begins differentiating, and the grid cells provide positional cues to guide the behavior of other pattern-following cell types (e.g., muscle, epithelial, and Schwann cells) that do not retain positional memory. At the same time, positional interactions continue to occur in the apical tip of the blastema to generate the pattern of the more distal structures in the regenerate.

the blastema (Muneoka et al. 1986). These cells that have and retain positional memory are the ones that establish the pattern of the missing limb structures (Fig. 4) (French et al. 1976; Bryant et al. 1981). Other cell types (e.g., muscle and Schwann cells) do not have positional memory, but respond to cells that do, and contribute proportionately fewer cells to the early bud (EB) and medium bud (MB) blastema (Muneoka et al. 1986). Obviously, at the end of regeneration, the relative proportion of the cells associated with the various tissues is restored. Below we shall discuss the contribution of the different tissue types in the stump to the blastema with reference to whether they are the "pattern-forming" cells with positional memory or "pattern-following" cells that lack positional memory.

The cells with positional memory establish the pattern of the missing limb structures during normal regeneration as well as establishing supernumerary structures when cells with different positional information are juxtaposed, as in the PCM and the ALM. Thus the ability to induce formation of de novo pattern is the experimental test of whether a specific cell or tissue type retains positional memory. By juxtaposing skin from different positions on the limb and observing the induction of new limb structures in a regeneration-competent environment, multiple studies have shown that this tissue contains cells that retain positional memory (Glade 1963; Carlson 1975; Tank 1981; Rollman-Dinsmore & Bryant 1982; Muneoka et al. 1986; Mescher 1996; Endo et al. 2004; Satoh et al. 2007). Removal of the epidermal layer does not affect this inductive capacity (Glade 1963; Tank 1981), and the reorientation of the epidermal layer does not affect the orientation of the regenerate suggesting that the pattern-forming cells in the skin are located in the dermis (Carlson 1975). The dermal layer of the skin consists primarily of fibroblasts and pigment cells (Holder & Glade 1984), and since pattern regulation occurs in limbs without pigment cells it is assumed that fibroblasts are the pattern-forming cells in skin tissue.

Cell types throughout the tissues from the central regions of the stump also have differing capacity to induce ectopic structures. For example, Schwann cells do not induce the formation of new structure and thus appear to be pattern-following cells (Kragl et al. 2009). On the other hand, cells in the limb muscle tissue do have this capacity (Carlson 1975). Skeletal muscle is composed of a variety of cell

types including myoblasts, satellite cells and connective tissue cells. Cells of the muscle lineage do not induce the formation of new pattern when grafted into a new limb environment and follow the positional cues in the host environment (Kragl et al. 2009; Nacu et al. 2013), suggesting that the connective tissue cells associated with the muscle are the pattern-forming cells in this tissue.

Seemingly conflicting results on the pattern-inducing ability of skeletal tissue are present throughout the literature (Goss 1956; Eggert 1966; Wallace et al. 1974; Carlson 1975). Given the variability in how these experiments have been performed (different proximal/distal regions of the limb, different sized/aged animals, and whether or not peri-skeletal connective tissues were included in the grafts), this ambiguity is not surprising. By grafting the humerus, radius, or ulna with the peri-skeletal tissue from large animals beneath the skin at different positions around the circumference of the contralateral limb, it was determined that positional information is asymmetrically distributed along the proximal−distal axis of the limb skeleton (Gardiner & Bryant 1989). In this experiment, humerus and radius grafts only formed supernumerary limb structures when grafted to posterior and dorsal locations, whereas the ulna induced the formation of supernumerary structures when grafted into anterior, posterior, dorsal, and ventral locations. Thus, the limited inductive capacity of the humerus elements to generate supernumerary structures when rotated in the stump is probably the result of insufficient positional diversity in this confrontation (Goss 1956; Carlson 1975; Wigmore & Holder 1985, 1986; Wigmore 1986), rather than the lack of positional memory in this tissue.

In addition to differences between skeletal elements, the ability to induce supernumerary pattern also depends on the age of the donor and the presence of the peri-skeletal tissues. Grafts of skeletal elements from small (young) animals (Wallace et al. 1974), as well as grafts that include peri-skeletal cells, have inductive properties (Gardiner & Bryant 1989), while grafts from older animals with the peri-skeletal tissue carefully removed do not (Wallace et al. 1974; Maden & Wallace 1975; Muneoka et al. 1986). In addition, cartilage cells have been reported to contribute to cartilage in the regenerate (Steen 1968; Kragl et al. 2009). This capacity may be related to the young age of the animals from which the donor tissue was obtained since in comparable experiments with older/larger donor animals these grafts had a limited contribution to the regenerate (Muneoka et al. 1986). These observations suggest that cartilage cells from young animals retain positional memory, and that they either lose their positional memory or their ability to communicate it as the animal ages. On the other hand, peri-skeletal connective tissue seems to always retain positional memory.

The extent to which cells contribute to the early blastemas appears to be related to whether or not they have positional memory. For example, almost half of the blastema population is derived from cells of dermal origin even though they account for less than 20% of the cells in the uninjured limb (Muneoka et al. 1986). Since the interaction of these cells in the early blastema stimulates position-dependent proliferation (intercalation), the progeny of these cells would be expected to be over-represented in the early blastema. Similarly, cells derived from grafted skin to an irradiated limb stump are capable of forming an entire regenerated limb without the contribution of other tissues from the irradiated stump (Dunis & Namenwirth 1977; Holder et al. 1979; Lheureux 1983). These regenerated limbs form a normally patterned skeleton with associated connective tissues, including the muscle sheaths and their connections to the skeleton (Dunis & Namenwirth 1977; Holder et al. 1979); however, the muscle sheaths do not contain muscles. This result shows that muscle tissue is not required for regeneration, and exists as a separate lineage within the limb. Dermal fibroblasts contribute to both connective tissue and skeletal elements in the regenerate. Subsequent studies with labeled dermal grafts into normal limbs have also shown that this tissue contributes to both connective tissue and cartilage in the regenerate (Muneoka et al. 1986; Kragl et al. 2009; Hirata et al. 2010).

In contrast to the pattern-forming cells, the contribution of pattern-following cells in the regenerate seems to be more straightforward in that they re-differentiate into the same tissue-type of their somatic origin. Grafted epidermal cells only contribute to epidermis in the regenerate (Dunis & Namenwirth 1977), and Schwann cells in the stump only contribute to Schwann cells in the regenerate (Kragl et al. 2009). Myoprogenitor cells arise from either adult stem cells (satellite cells), as in the axolotl and other vertebrates, or by fragmentation and dedifferentiation of myotubes as in the newt (Namenwirth 1974; Dunis & Namenwirth 1977; Lheureux 1983; Kragl et al. 2009; Nacu et al. 2013; Sandoval-Guzmán et al. 2014). As new tools are generated to label cells with specific somatic identities it will be possible to trace the contribution of specific cell types in the uninjured limb to the population of blastema cells with higher resolution.

Aside from whether or not the cells that contribute to the blastema have positional information/memory, they do retain memory of their somatic cell origin and are restricted to that lineage as they differentiate into the regenerated limb structures (Kragl et al. 2009; Nacu et al. 2013). It is worth noting that progenitor cell types that do not have positional information (epidermis, Schwann cells, and muscle) are restricted to re-differentiating into the same tissue from which they arose (Dunis & Namenwirth 1977; Kragl et al. 2009; Nacu et al. 2013; Sandoval-Guzmán et al. 2014). In contrast, blastema cells that arise from connective tissue cells in the dermis, which have positional memory, can differentiate into connective tissues throughout the limb and cartilage (Kragl et al. 2009; Hirata et al. 2010). Thus, cells that did have

positional memory at one time (fibroblasts) can differentiate into cartilage, which may or may not have positional memory. In the future it will be important to resolve the issue of whether cartilage has positional memory because if it does then it should contribute to the formation of pattern in the regenerate. Alternatively, if fibroblast-derived limb progenitor cells lose their positional memory (or their ability to communicate it) as they differentiate into cartilage, then the connective tissue cells are solely responsible for patterning the regenerate.

Induction of Ectopic Blastemas and the Supernumerary Response as Assays for Pro-regenerative Signaling

The challenge of understanding how regeneration works has been to understand the behavior of regeneration-competent cells in order to assay for the pro-regenerative signals that they respond to. Conceptually, there is a language that cells use to communicate with each other, and therapies to induce regeneration will necessitate learning how to use that language. We already know the alphabet of this language since the genomes of many animals have been sequenced, and it is not a very complex alphabet. The same signals (e.g., FGF, BMP, WNT, TGFβ, SHH) are used over and over again in development and in regeneration. We have begun to put some of the letters together to make words that make sense to the cells. Historically, the ability to test these words is based on the ability to induce ectopic (supernumerary) pattern formation, which still continues today. The idea is that you do an experiment (graft cells or mis-express a gene) and if nothing happens then you did not make up or supply a word that the cells understand. However, if you get new pattern (e.g., in response to grafting the Zone of Polarizing Activity (ZPA) or mis-expressing *Shh*) then the signal you provided did make sense to the cells. As we learn more words, the challenge is to put the words into sentences that tell the cells what, when, and where to do what we need them to do.

The important starting point for talking to regeneration-competent cells is the principle that the signals and pathways involved in development and regeneration are largely conserved. Therefore it is not necessary to rediscover what we already know from the extensive studies of developmental genetics. We can start with the known signals and use them to orchestrate the behavior of cells in time and space. Historically, as is still true today, the goal of this approach is to induce the cells to make new pattern, whether it is to replace missing pattern or to make supernumerary pattern (Fig. 5).

For regeneration, the mechanism by which supernumerary pattern is induced experimentally is the process of "intercalation" (French 1978; Bryant et al. 1981). As discussed above, cells with positional information are localized within the connective tissue and use this information to communicate with each other (French et al. 1976; Bryant et al. 1981). If a positional discontinuity between cells is created (e.g., by grafting posterior skin to an anterior wound as in the ALM), the cells begin to proliferate and their progeny adopt new, intermediate positional identities (French et al. 1976; Bryant et al. 1981). In some instances, intercalation results in the formation of the normal pattern, for example when the limb is amputated and intermediate pattern is intercalated between the stump and the distal tip of the limb (Fig. 5A) (Gardiner et al. 1995). Thus experimentally the stimulation (or lack of stimulation) of supernumerary structures by intercalation is an in vivo assay for the presence and distribution of positional information encoded by cells within the limb (Fig. 5B,C).

It has recently been suggested that intercalation is not the patterning mechanism underlying "normal" limb regeneration, although intercalation and other cell−cell recognition events probably play a role in certain grafting situations (Roensch et al. 2013). This conclusion is reminiscent of suggestions in the past that experimentally moving cells from one position to another to create positional disparities is an experimentally induced epiphenomenon, and is not indicative of what is "normal" (Stocum 1991). Such interpretations logically lead to the conclusion that regeneration cannot be studied because the act of experimentation itself may alter the response being studied. As noted previously (Muneoka & Sassoon 1992), while it is impossible to disprove this Draconian view, there are numerous examples in which experimentally induced perturbations lead to changes in the normal pattern of gene expression that is predictive of the final outcome. By our view, the experimental perturbation is affecting the normal mode of development or regeneration, rather than eliciting a distinct and unrelated injury response. For example, the interpretation that the proximal−distal limb pattern is regenerated by intercalation between the stump and distal tip (Maden 1977; Gardiner et al. 1995; McCusker & Gardiner 2013) is consistent with recent genetic analyses of FGF signaling in developing mouse limbs (Mariani et al. 2008).

Related to the distinction between "normal" and "experimentally induced" is the question of whether or not ALM blastemas form through a "normal" regenerative response since there is no amputation. While amputation is an obvious model for studying regeneration, and has been for over a century, its utility is limited largely to describing the process, and experiments are inherently loss-of-function since the control amputations always regenerate. For example, regeneration can be prevented by denervation of the limb (loss-of-function) followed by attempts to rescue inhibited regeneration. Such experiments have provided some insight into the requirement for nerves (discussed above), but do not address the mechanisms of the regenerative process. In addition, there is extensive damage that activates a complex

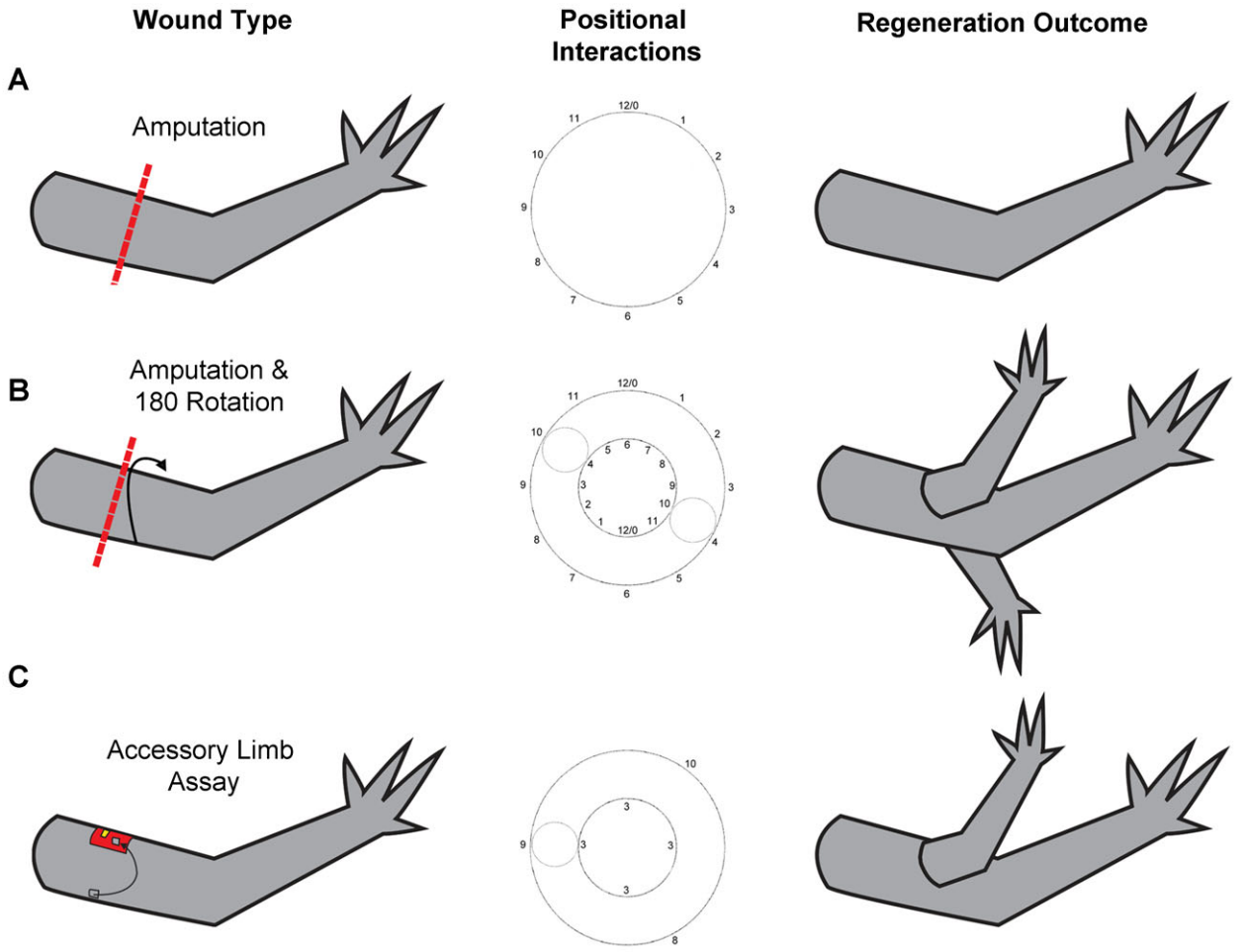

Figure 5. Amputation-induced and supernumerary blastemas. (A) Amputation-induced blastemas have a complete circle of positional information, which results in the formation of a complete limb. (B) 180° rotation of the limb results in the formation of blastema tissue with two complete circles of positional information, and thus results in the generation of two supernumerary limbs. (C) Grafting posterior skin into an anterior wound site results in the formation of a blastema with a complete circle of positional information, forming a single supernumerary limb.

injury response that is not associated with the necessary and sufficient signals and cellular responses leading to blastema formation and limb regeneration.

Although there may or may not be differences in the mechanisms by which a limb is formed in the ALM compared to regeneration of an amputated limb, none is evident at this point. Characterizations of ectopic blastemas (ALM) have demonstrated that the same molecular pathways are regulated as in an amputation-induced blastema (Endo et al. 2004; Satoh et al. 2007). Similarly, the cellular behaviors that have been analyzed are the same; for example, an early event is the migration of cells from the wound margin to the center of the wound (Gardiner et al. 1986; Endo et al. 2004) in response to signaling from the severed nerve and WE (Singer & Inoue 1964). Most importantly, experiments that result in changes in the positional information of cells in the blastema (e.g., as a result of grafting of tissues or treatment with RA) result in the same predicted patterns of supernumerary limbs (French et al. 1976; Bryant et al. 1981; Endo et al. 2004; McCusker et al. 2014). Thus induction of a limb de novo in the ALM most likely occurs as a consequence of providing the appropriate temporal and spatial cues associated with limb amputation, rather than eliciting a distinct and unrelated injury response.

In the end, the relevance of studying regeneration in an axolotl is the opportunity to discover the shared pathways and conserved cellular behaviors that can be targeted in order to orchestrate a regenerative response in humans. Therefore, amputation may be more natural, but that does not make it a more appropriate experimental model for understanding how regeneration works.

Future Study

With the advances in genomics, the future for studying axolotl regeneration (in all organs, not just limbs) is bright. Sequence of the first axolotl chromosome is soon to be available (Voss, pers. comm.), and brings forth the exciting possibility of resolving the genetic regulation of endogenous cell reprogramming and the events leading to regeneration. Newly generated transgenic axolotl lines have been used extensively as general cell lineage tracers (Sobkow et al. 2006; Kragl et al. 2009; McCusker & Gardiner 2013), as well as enabling the study of the role of the specific molecular pathways active in regen-eration (Monaghan & Maden 2012). The recent development of CRISPR/Cas9 technology (Fei et al. 2014; Flowers et al. 2014) to manipulate the expression of specific genes in axolotls will allow us to study the genetics and molecular mechanisms underlying blastema formation in a way that has not been possible until recently.

Although in vivo studies of limb amputations and ectopic blastema formation in the ALM have allowed us to identify many of the steps in regeneration, future progress eventually will require an in vitro model. Given that pathways involved in axolotl regeneration are shared with mammals, the key to inducing mammalian regeneration will require the discovery of the precise timing and dose−response parameters of the signals that regulate those pathways. The discovery that it is possible to induce blastema formation with purified mammalian growth factors is exciting, and reinforces the view that axolotl and human signaling pathways are highly conserved (Makanae et al. 2013, 2014). In the end, therapies to induce human regeneration will probably involve the delivery of molecules such as growth factors, which will depend on experimental work that identifies the appropriate concentrations of factors to be delivered as well as the timing of delivery.

Studies of regeneration in vitro historically have been limited because dissociated blastema cells quickly lose properties (e.g., positional information) associated with regeneration (Groell et al. 1993). Similarly, many of the cells in culture withdraw from the cell cycle and the rate of proliferation of dissociated blastema cells quickly decreases when the cells are cultured on a range of substrates and culture media (Albert et al. 1987; Kumar et al. 2007). Although some cells from salamanders have been expanded and passaged, they appear to be committed to the myogenic lineage (Ferretti & Brockes 1988; Tanaka et al. 1997). It therefore appears that the behavior of blastema cells in vivo is dependent on cell−cell and cell−matrix interactions established during blastema formation. In order to maintain the in vivo organization of blastema cells and matrix, we are working to optimize the technique of organotypic slice culture (OSC) that is commonly used in neurobiology. The technique uses a vibratome to make serial sections of unfixed blastemas that are of uniform thickness and that maintain the original tissue architecture. Assuming that salamander limb cells in OSC maintain their in vivo characteristics as occurs in OSC of neural tissues, it should be possible to quantify the response (e.g., proliferation, migration, and differentiation) of blastema cells to activation and/or inhibition of specific signaling pathways.

An alternative approach to experimentally regulating specific signaling pathways is to engineer the ECM in vitro so as to provide specific signals and then assay for the response of blastema cells in vivo. Advances in biomaterials and tissue engineering make it possible to design and manipulate artificial ECM. We are working to test the response of axolotl blastema cells to purified ECM components that are predicted to modify growth factor signaling (e.g., heparin sulfate proteoglycans). As noted above, this approach is encouraging in light of the ability of exogenously delivered human growth factors (FGF and BMP) to substitute for nerve signals and induce axolotl cells to form a blastema (Makanae et al. 2013, 2014).

Finally, aside from the prospect of learning how to induce regeneration, an understanding of the mechanisms underlying nerve−WE interactions combined with the ability to engineer pro-regenerative properties in an artificial ECM likely will allow for the therapeutic regulation of fibrosis. The inability to control the behavior of connective tissue fibroblasts leading to fibrosis is a major underlying cause of organ failure and thus a leading cause of damage and disease-associated death. In the end, discovering how to regenerate will necessitate learning how to talk to cells. In the case of fibroblasts, they are the cells in the salamander that make up the initial regeneration blastema and regenerate the positional information grid, whereas in mammals they make scars. The challenge is to understand the biology of these cells and to learn how to direct their responses away from scars and towards regeneration.

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