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Novel Insights into the Flexibility of Cell and Positional Identity during Urodele Limb Regeneration

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The ability of diverse metazoans to regenerate whole-body structures was first described systematically by Spallanzani in 1768 and continues to fascinate biologists today. Given the current interest in stem cell biology and its therapeutic potential, examples of vertebrate regeneration garner strong interest. Among regeneration-competent vertebrates such as the fish, frog, and salamander, the salamander is particularly impressive because it can regenerate the entire limb and tail as well as various internal organs as an adult (Goss 1969). This spectacular natural phenomenon leads us to ask what cellular properties allow regeneration and what prevents this phenomenon in other vertebrates. From this perspective, it is imperative to know whether the stem cells in regenerating limbs harbor particularly special traits such as a higher plasticity in cell fate compared to tissue stem cells in other organisms. Flexibility in cell fate needs to be considered with respect not only to tissue identity, but also to patterning because limb amputation causes cells in a particular limb segment to form more distal limb elements. How positional identity is encoded in stem cells and how it is controlled to produce only the missing portion of the limb are also questions of fundamental importance.

Limb regeneration proceeds over the course of weeks and months and is a complex sequence of cellular events that unfold to produce the fully patterned and functional structure. We therefore discuss the problem of regeneration in four main parts: wound healing, blastema formation including the potency of cell fate, nerve dependence, and patterning.

EPITHELIAL WOUND HEALING

Reepithelialization during limb regeneration already shows distinctions to the mammalian situation. Whereas during mammalian wound healing, epithelial cell migration initiates only 48 hours after injury, during salamander limb regeneration, blood clotting is immediately followed by rapid migration of epithelial cells that close the wound within 8 hours. The cuboidal epithelial layer covering the end of the limb displays embryonic features where no basal lamina is formed between the epithelial and underlying layers. In subsequent days, this wound epidermis thickens to form the apical epidermal cap (AEC), which is molecularly and functionally similar to the apical ectodermal ridge (AER) of the developing limb bud (Saunders 1948; Christensen and Tassava 2000). For example, the AEC is essential for outgrowth of the regenerating limb because removal of the AEC or replacement of this specialized epithelium by mature skin blocks regeneration (Dearlove and Stocum 1974; Mescher 1976; Tassava and Garling 1979). Furthermore, the AEC expresses those factors found in the embryonic AER such as mito-

gens of the fibroblast growth factor (FGF) family, including FGF-1 and FGF-2 (Boilly et al. 1991; Mullen et al. 1996; Christensen et al. 2002; Dungan et al. 2002).

Lateral wounding does not induce regenerative events, whereas full amputation does. The AEC forms only after limb amputation and does not form at a wound on the side of the limb. In fact, lateral or internal wounds to the limb have more in common with mammalian wound healing than bona fide limb regeneration. For example, if a significant gap is made in the limb skeleton without amputating the limb (Fig. 1), the bone pieces do not grow together to replace the missing segment, whereas if the limb is amputated through the skeleton, that bone is regenerated (Weiss 1925; Bischler 1926). Thus, it is not even necessary for the bone to be present at the amputation site—removal of the bone before limb amputation still results in regeneration of a properly patterned skeletal element from the site of amputation, suggesting some plasticity in tissue cell fate, a topic that is addressed below.

Similar to the situation in bone, muscle regeneration exhibits distinct phenotypes when occurring along the side of the limb versus after full amputation. Carlson has extensively studied muscle regeneration in different vertebrate species and found that minced muscle preparations made along the limb are repaired in salamanders similar to repair in other vertebrates where the regenerated muscle is distinguishable from the original because of the central location of the nuclei in the regenerated fibers and the accumulation of fibrous tissue. In contrast, when the limb is amputated and the limb muscle regenerates in concert with other limb tissue, it is perfectly patterned and indistinguishable from the original (for review, see Carlson 2003).

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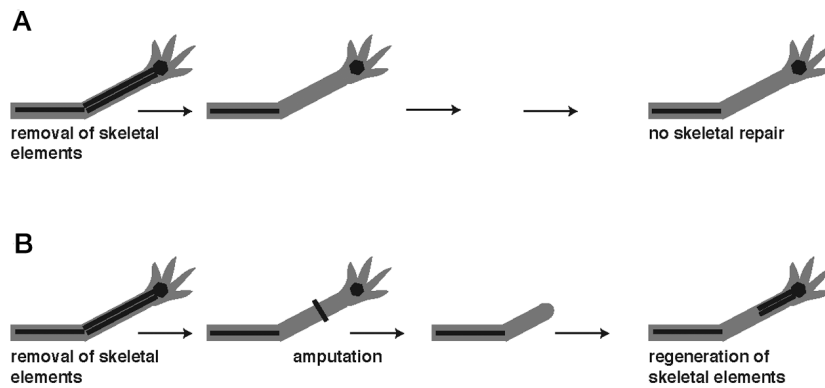


Figure 1. Plasticity during regeneration. (A) Skeletal elements do not grow back together when a gap is made, but the rest of the limb is kept intact. (B) If, however, a limb is amputated, even through a region lacking skeletal elements, the skeletal elements regenerate from the plane of amputation (Weiss 1925; Bischler 1926).

An exception to these cases is spinal cord injury where a gap lesion in the spinal cord is repaired. The ependymoglia lining the canal at both cut ends heal over to make terminal bulbs. The ends then grow toward each other, with the posterior-facing end growing faster than the anterior-facing edge (Butler and Ward 1967). Eventually, the two tubes grow together and intercalate to make a seamless tube. The unique ability of the spinal cord to repair gap lesions may be related to its powerful properties as an organizer for tail regeneration. In contrast to the situation in bone, removal of spinal cord before tail amputation prevents tail regeneration. Furthermore, if the spinal cord is rotated 180° along the dorsoventral (DV) axis and the tail is then amputated through the inverted region, all tail tissues subsequently regenerate with an inverted DV orientation according to the orientation of the spinal cord (Holtzer 1956). This indicates that the spinal cord harbors positional information that is transmitted to the surrounding tissues and furthermore shows that the spinal cord seems to autonomously harbor the requisite positional information and growth information for tail regeneration. A molecule involved in these processes is sonic hedgehog, which is expressed in cells of the ventral spinal cord. Treatment of amputated tails with the inhibitor cyclopamine blocked regeneration, indicating that it is one of the factors from the spinal cord required for regeneration of the surrounding tissues (Schnapp et al. 2005).

BLASTEMA FORMATION

What may distinguish the stem or progenitor cells produced after limb amputation from those used in normal wound healing? The defining cellular structure of the regenerating limb is the blastema—a zone of mesenchymal progenitor cells that accumulates underneath the wound epidermis in the first week(s) of regeneration and represents the progenitor cells that will form all of the various limb tissues.

Which mature tissues contribute to the blastema and what are the properties of blastema cells? Selective X-irradiation of the limb stump before limb amputation demonstrated that the cells for the blastema arise locally

from the tissues at the amputation plane (Butler 1931). Tracking of triploid or tritiated thymidine-labeled tissue showed that multiple tissues including dermis, muscle, cartilage, and Schwann cells likely contribute to the blastema and thus the regenerating limb (Steen 1968; Wallace 1973; Namenwirth 1974; Dunis and Namenwirth 1977; Lheureux 1983; Holder 1989).

In terms of how mature tissues give rise to blastema cells, a concept long discussed in regeneration is the dedifferentiation of mature, postmitotic cells to produce blastema cells. Detailed histological examination of regenerating tissue argued that differentiated cells at the amputation plane lose their mature features and return to the cell cycle to produce blastema cells (Chalkley 1954; Hay 1959; Hay and Fischman 1961). Muscle represents the only tissue type where concrete experimental evidence consistent with the possibility of dedifferentiation exists. Implantation of lineage-labeled myotubes, as well as injection of labeled endogenous muscle fibers, suggests that multinucleated, postmitotic muscle cells return to the cell cycle and resolve into mononucleated cells to produce cycling blastema cells (Lo et al. 1993; Kumar et al. 2000; Echeverri et al. 2001). The details of such dedifferentiation studies have been reviewed elsewhere (see, e.g., Straube and Tanaka 2006) and are not discussed here. At the moment, it is not yet clear what proportion of the blastema forms from bona fide dedifferentiation of cells versus recruitment of resident stem cells, an issue that is important to resolve in the future. Furthermore, it will be necessary to determine whether regeneration involves activation of rare cells that expand enormously to form the blastema or whether a large proportion of cells in mature tissue enter into the blastema. Muscle tissue might serve as a good model because it harbors satellite cells, a pool of myogenic stem cells that are activated in response to muscle injury and can be identified using molecular markers (for review, see Buckingham et al. 2003).

Beyond the issue of dedifferentiation versus stem cell activation, it was unknown whether the various tissues all contribute a similar generalized pluripotent stem cell to the blastema or whether the blastema is composed of tissue-specific stem cells that respect their developmental

origin. Indeed, the term blastema connotes a group of highly plastic progenitors, and it would be fair to say that the general expectation favored pluripotency of at least some blastema cells. For example, Wallace (1973) found that the implantation of healthy nerves into irradiated limbs was sufficient to rescue regeneration, implying that cells of neural origin could generate nonneural tissue. In a more recent study, satellite cells isolated from newt muscle tissue and labeled with bromodeoxyuridine (BrdU) were reimplanted into blastemas and contributed to cartilage and even epidermis of the regenerating limb, suggesting high plasticity of muscle-derived cells (Morrison et al. 2006). Such studies, however, were not definitive because cultivation and labeling of cells might have induced pluripotency, and the grafted cells may have been contaminated by cells from other tissues.

BLASTEMA CELLS RESPECT THEIR DEVELOPMENTAL ORIGIN

We wanted to determine if any tissue produces a highly pluripotent cell type in the blastema or whether blastema cells respect their developmental origin. To do this, we labeled every limb tissue separately in order to track its ultimate fate in regeneration (Fig. 2). Our labeling strategy was to graft the embryonic anlage of each limb tissue from a green fluorescent protein (GFP)-transgenic donor into a normal recipient. The donor embryos ubiquitously expressed GFP under the control of the CAGGS promoter (Sobkow et al. 2006). We implemented this method to successfully label limb muscle, dermis, cartilage, Schwann cells, and blood vessels. To achieve muscle and blood vessel labeling, we grafted mesoderm at somite level 3–4 at different DV locations; for dermis and cartilage, we transplanted lateral plate mesoderm. Because all lateral plate mesoderm transplants labeled both cartilage and dermis, we obtained specific cartilage or dermis labeling by performing a second graft of cartilage or dermis from the limbs of the labeled animals into unlabeled hosts. For Schwann cells, we obtained specific labeling by grafting the neural folds at embryonic stage 16. As expected, fluorescent cells migrated into the limb bud and contributed to tissues in the mature limb that developmentally derive from the respective grafted embryonic cells. Having confirmed integration into host tissue and specificity of each graft type, we amputated through the fluorescent region and tracked the fate of GFP⁺ cells during regeneration.

The results from our experiments show for the first time that blastema cells do not acquire pluripotency because no type of graft generated progeny that contributed to all limb tissues. The final fate of GFP⁺ blastema cells strongly reflected their tissue-specific origin. Labeled muscle fibers and satellite cells gave rise to muscle tissue in the regenerated limb but no cartilage; Schwann cells only generated new Schwann cells and blood vessels produced more blood vessels. Dermis-derived cells exhibited the highest flexibility, populating the regenerated skeleton and connective tissues at a significant frequency, but the lateral-plate-derived cells never contributed to muscle. The conversion between dermal and cartilage identity reflects

the close lineage relationship between these two tissue types, which both arise from lateral plate mesoderm.

Considering the overall lack of pluripotency that we observed, we were concerned enough to examine two potential confounding issues: (1) whether the transgene was silenced in cells that transited to another cell type and (2) whether cells are actually pluripotent but had been masked because during normal regeneration, cells stay close to their tissue of origin, and therefore most easily form their parent tissue type. To address these issues, we examined Wallace's paradigms to rescue irradiated limbs through implantation of nerve. In our experiments, the irradiated host was transgenically marked with constitutive nuclear-Cherry expression, and the nerve implant was derived from either the GFP–Schwann cell animals or the constitutive GFP–transgenic animal. When irradiated animals were implanted with GFP–Schwann-cell-labeled nerve, we observed GFP expression only in Schwann cells. Although these rescued appendages contained cartilage, the cartilage was unlabeled. In contrast, when nerve—where all cells were GFP⁺—was implanted, the rescued regenerate contained GFP⁺ cartilage, and all cells in the appendage were either nuclear Cherry or GFP positive. These experiments allowed us to conclude that (1) the transgene is not silenced during regeneration and (2) Schwann cells maintain their cell identity even under conditions when the nerve is asked to rescue regeneration. This further shows that the cartilage cells of the rescued regenerate come from contaminating non-Schwann cells in the nerve sheaths, presumably connective fibroblasts. We observed no muscle in the rescued regenerates, suggesting that cells from the nerve do not form muscle. Taken together, our results show that pluripotent cells do not appear to be implemented during limb regeneration, because cells respect lineage restrictions defined during development. Gargioli and Slack (2004) had observed evidence of tissue-specific restriction in muscle, notochord, and spinal cord during anuran tadpole tail regeneration, although their results left open the possibility that other tissue types such as dermis or blood derivatives could have produced a pluripotent cell type. Furthermore, it was unclear whether tadpole tail regeneration in the frog represented a truly representative regeneration system because the tail is lost during metamorphosis, and the regeneration of neural structures is deficient in the frog tadpole. Interestingly, cell tracking during axolotl tail regeneration that robustly regenerates all tail structures showed that spinal cord progenitors may have a more generalized potential during regeneration (Echeverri and Tanaka 2002).

ROLE OF NERVES DURING REGENERATION

Despite their limited plasticity, blastema cells are undifferentiated progenitors that repeatedly divide and then progressively differentiate until the missing portion of the limb has formed. What signals support the proliferation of these cells? The wound epidermis (AEC), as mentioned above, is required, but the regenerating nerve is also required to maintain proliferation of blastema cells because denervation of the limb causes blastema cells to

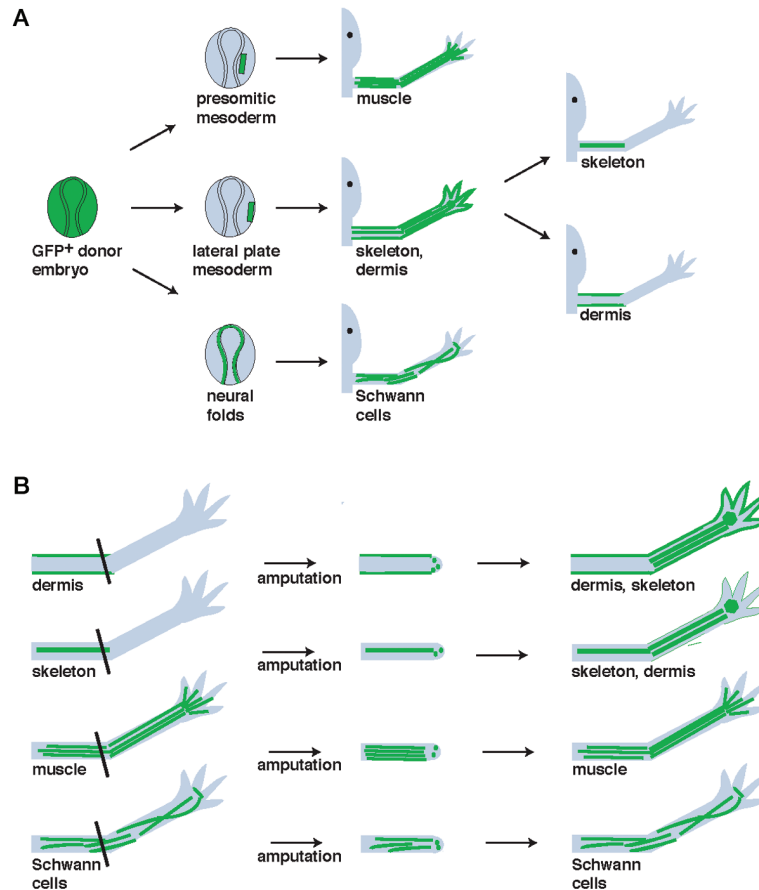


Figure 2. Blastema cells respect their developmental origin. (A) Specific labeling of different limb tissues was achieved by mapping and grafting the embryonic anlage of each limb tissue using GFP-transgenic donors. Lateral plate mesoderm ultimately contributed to dermis and skeletal elements, presomitic mesoderm to muscle tissue, and neural folds gave rise to Schwann cells. (B) These limbs were amputated through the fluorescent region, and GFP⁺ cells were tracked through the course of regeneration. Cells respected their developmental origin: Schwann cells only regenerated Schwann cells, muscle gave rise to muscle, and blood vessels formed new blood vessels. Dermis-derived cells gave rise to dermis and also contributed to skeletal elements. Likewise, skeleton regenerated new skeletal elements and also contributed to dermis. Both dermis and skeleton derive from the same embryonic anlage, the lateral plate mesoderm.

cease proliferating and thus prevents regeneration (Singer 1952). Interestingly, the nerve dependence of cell proliferation is specific to regeneration and is not found in the developing limb bud. Amazingly, when aneurogenic limbs are created by preventing innervation during development, these limbs can regenerate in the absence of nerve input, indicating that the limb becomes “addicted” to the nerve (Yntema 1959a,b). Singer (1952) demonstrated that either sensory or motor nerves can provide the crucial signal for blastema cell growth.

What factors may mediate the nerve dependence of regeneration? Transferrin, substance P, and rhGgf2 were suggested to be involved in this process because they were shown to be present in neural tissues and stimulated proliferation of blastema cells (Globus et al. 1983; Munaim and Mescher 1986; Globus 1988; Wang et al. 2000). However, FGFs and the recently identified nAG, a member of the anterior gradient family of secreted proteins, are of particular interest. Mullen and colleagues (1996) showed that FGF-2, like other members of the FGF family, is expressed in limb nerves and the AEC, and

its expression levels decrease after transection of the nerve. In denervated blastemas, FGF-2-soaked beads rescued blastema outgrowth. On a molecular level, FGF-2 was shown to be sufficient to restore the expression of the AEC-specific gene *dlx3* in denervated limbs. Although it remains to be resolved whether FGF-2 or other family members stimulate proliferation of blastema cells in vivo, these results strongly suggest that FGFs have an important role during nerve-dependent regeneration.

The recent discovery of nAG has provided new insight into the molecular cross-talk between nerve supply and blastema cell proliferation (Kumar et al. 2007). nAG is expressed in Schwann cells as soon as the first blastema cells divide and, later, in the specialized gland cells of the wound epidermis. Nerve transection causes loss of nAG expression in both nerve and wound epidermis, implying that the epidermal expression is nerve-dependent and may reflect a positive feedback between nerve and epidermis. To test the direct role of nAG in blastema cell proliferation, Kumar et al. (2007) showed that recombinant nAG induces the proliferation of cultured blastema cells.

Strikingly, when the *nAG* gene was ectopically expressed in denervated limb stumps 5 days postamputation, full limb regeneration occurred in these limbs. Therefore, this molecule appears to be an important contributing factor for nerve-dependent regeneration.

THE ALM DEFINES THE CONDITIONS REQUIRED FOR INDUCING ECTOPIC LIMB FORMATION

Although innervation is clearly an essential component of adult limb regeneration, nerve supply is clearly not sufficient to induce an entire regenerating limb. Deviation of nerves to a wound bed on the side of the limb induces an ectopic blastema-like structure to form but the outgrowth eventually regresses, indicating that the cut nerve endings are not the only component that directs formation of a blastema at the end of the amputated limb. If at the site of nerve deviation, a piece of full-thickness skin (including dermal fibroblasts) deriving from the opposite side of the contralateral limb were placed next to the wound, ectopic, fully patterned limbs would grow out (Lheureux 1977; Reynolds et al. 1983; Maden and Holder 1984; Egar 1988; Endo et al. 2004; Satoh et al. 2007, 2008). This showed that the combination of wound epidermis, nerve supply, and skin fibroblasts of different positional identities is sufficient to induce the formation of a fully patterned limb (Fig. 3). Endo et al. (2004) realized that this experimental system can be used to analyze the “minimal” essential conditions required to induce an ectopic limb on the lateral side of a limb. They developed the

accessory limb model (ALM) that describes limb regeneration as a sequence of events starting with wound healing, followed by the induction of a blastema by nerves, and finally outgrowth and patterning in response to the juxtaposition of dermal fibroblasts coming from different positional identities. One advantage of using this system is that processes related to wound healing, nerve dependence, and fibroblasts can be analyzed separately (Satoh et al. 2007, 2008). For example, Satoh and colleagues found that nerve deviation, but not contralateral skin grafts, was required for the expression of marker genes such as *msx2*, *tbx5*, and *hoxa13*.

The ALM experiments as well as earlier blastema rotation experiments showed that juxtaposition of anterior and posterior limb cells is a crucial cue for growing out a properly patterned limb (Iten and Bryant 1975). These results indicate that mature salamander limb tissue retains spatial coordinate information that is used during regeneration to define where a limb should grow out. The molecular nature of the anteroposterior (AP) information has not been defined, but it is likely to be a conserved molecular network used to define these axes during embryonic limb development. For example, the retention of spatial coordinate information has been better studied during spinal cord regeneration, where continued expression of the embryonic morphogen sonic hedgehog from the floorplate in the mature and regenerating spinal cord controls DV neural progenitor cell identity (Schnapp et al. 2005). In contrast, the adult mammalian spinal cord does not display expression of the embryonic patterning markers seen in the urodele (Yamamoto et al. 2001).

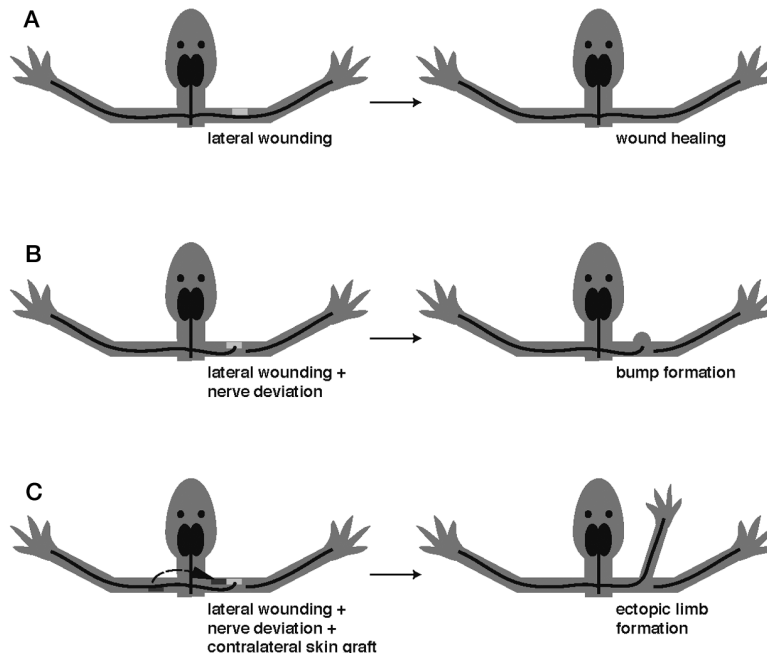


Figure 3. The accessory limb model (ALM). (A) A wound that is induced at the lateral site of the limb heals without scar formation. (B) If the limb nerve is transected and deviated to the wound, a bump forms that, however, does not possess the ability to regenerate an ectopic limb. (C) If, in addition, full-thickness skin from the opposite side of the contralateral limb is grafted next to a lateral wound with a deviated nerve, an ectopic limb forms (Endo et al. 2004).

PD INFORMATION EXISTS AS A GRADED PROPERTY

Another dimension of spatial patterning is reformation of the correct number of limb segments along the proximo-distal (PD) axis. Again, the results suggest that positional information for this axis is retained in adult salamander tissue. Under normal circumstances, salamander limb regeneration is unidirectional, meaning that cells at the amputation plane will always regenerate limb elements more distal to that location, a phenomenon called “the rule of distal transformation” (Rose 1962; Stocum 1975; Maden 1980). This was most clearly illustrated in an experiment where an “inverse” limb was created by suturing the wrist to the body (Fig. 4). After healing, amputation resulted in a limb where the wrist element lay in the proximal position and the upper arm was in the distal position. Such limbs regenerated lower-arm elements from the inverted upper arm, rather than upper-arm elements from the inverted lower arm, demonstrating that cells at the amputation plane have an identity associated with their position along the PD axis, a concept called positional memory (Wolpert 1969); however, amputation can then reprogram some cells or their progeny to a more distal identity. What cellular properties define positional memory and how is positional information manifested at the cellular level?

PD INFORMATION IS ENCODED BY CELL SURFACE PROPERTIES

A number of grafting experiments have implicated local cell–cell interactions in patterning the regenerating PD axis. When a wrist blastema is transplanted onto an upper-arm stump, a normal regenerate forms in which the positions between upper arm and hand derive primarily from the upper-arm stump (Iten and Bryant 1975; Stocum 1975; Pescitelli and Stocum 1980). This phenomenon has been termed intercalary regeneration, where the upper-

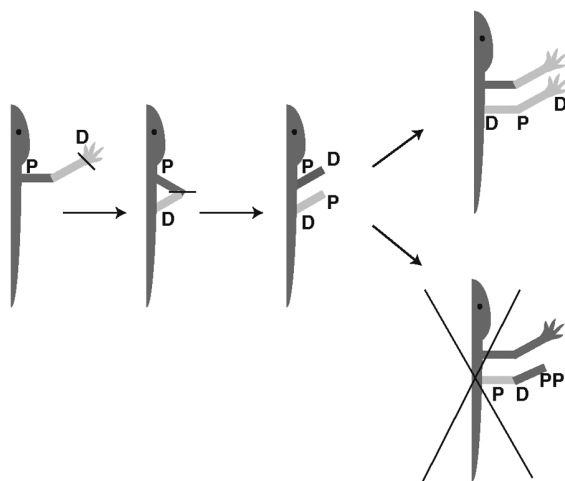


Figure 4. The rule of distal transformation. When an “inverse” limb is created, more distal (D) elements regenerate from the proximal (P) site. Regeneration of more proximal (PP) elements does not occur, demonstrating that distal-to-proximal changes of cell identity do not normally occur (Rose 1962).

arm stump intercalates the missing limb positions up to the transplanted distal blastema. Intercalary regeneration does not occur when a digit-stage distal regenerate or a mature hand is grafted to a proximal stump, implying that special conditions existing in the blastema are required to induce intercalary growth (Stocum 1975).

The ability of proximal and distal blastema tissue to respond to each other is also manifested in another manner that has been termed affinophoresis. When a distal blastema is grafted onto the side of a regenerating upper arm at the junction between mature and blastema tissue, the grafted distal blastema is carried along the regenerating proximal blastema so that it ultimately produces a second hand emanating from the wrist (Crawford and Stocum 1988), indicating that cells from the same PD region have a preferential affinity for like cells. This was further investigated by juxtaposing proximal and distal blastemas in hanging-drop cultures (Nardi and Stocum 1983). In such preparations, the proximal tissue engulfs the distal tissue, whereas distal-distal or proximal-proximal tissue confrontations maintain a straight border, indicating that distal and proximal cells have different adhesive properties.

It has been proposed that these observations reflect the cell surface recognition processes required for intercalary regeneration. Whether these recognition events are a cause or a consequence for PD patterning is one of the big challenges to address and mainly depends on elucidating the underlying molecules and genetic networks.

RETINOIC ACID RESPECIFIES CELLS TO MORE PROXIMAL IDENTITIES AND ALTERS CELL SURFACE PROPERTIES

A molecular handle for analyzing PD identity was provided by Niazi and Saxena’s (1978) report that treatment of toad tadpole wrist blastemas with retinyl palmitate caused an entire arm to grow from this wrist. Maden (1983) further showed that retinoic-acid (RA)-induced proximalization in the salamander is dose-dependent, so that at low concentrations, wrist blastemas duplicate only the distal-most elements of the lower arm, and progressively increasing concentrations generate more arm elements (Fig. 5). These results showed that RA could force a distal cell to acquire proximal identity, subverting the normal distalization process that occurs during regeneration. The intercalation and affinophoresis assays showed that RA-transformed blastemas acquire the cell surface properties of a proximal blastema because they did not travel down to the wrist level during regeneration, but rather the ectopic regenerate emerged from the upper arm. Furthermore, in the hanging-drop assay, RA-treated distal blastemas were not engulfed by proximal blastemas (Fig. 6) (Nardi and Stocum 1983; Crawford and Stocum 1988).

RA-RESPONSIVE GENES ARE INVOLVED IN PD IDENTITY

Considering the rule of distal transformation, it has remained unclear whether endogenous RA acts during normal limb regeneration to proximalize blastema cells. Nonetheless, RA has been used experimentally to identify

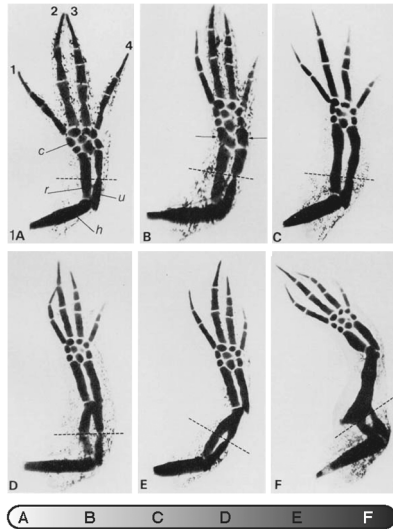


Figure 5. RA proximilizes the limb cell identity. RA respecifies cells from distal to proximal identity. Depending on the concentration (A–F), wrist blastemas produce limbs that contain duplications, with an entire arm growing out at the highest concentration (F). (Reprinted, with permission, from Maden 1983 [© Company of Biologists].)

two RA-responsive genes that are key regulators of PD identity: the GPI-linked protein Prod1 and the homeodomain protein Meis1/2. In a screen aiming to identify genes that are (1) up- or down-regulated by RA, (2) differentially expressed between proximal versus distal blastemas, and (3) coding for a cell surface molecule, da Silva et al. (2002) found that the only candidate meeting these three criteria was Prod1, a member of the Ly6 superfamily. Functional evidence supporting Prod1's role in PD recognition was first shown in the hanging-drop assay where phospholipase C and anti-Prod1 antibody treatment blocked proximal engulfment. Further evidence came from *in vivo* cell-labeling experiments where ectopic expression of Prod1 in distally fated blastema cells caused them to end up in a proximal limb location (Echeverri and Tanaka 2005). Still unresolved is whether Prod1 directly mediates proximal identity or whether its involvement in cell adhe-

sivity is a consequence of positional information. da Silva and colleagues (2002) proposed that Prod1 acts as a receptor that undergoes homophilic interactions that titrate the levels of Prod1 open to signaling via a heterotypic ligand. Thus, only the ligand-mediated interaction results in intracellular signaling to provoke proliferation and processes related to proximal identity. Because more Prod1 molecules are available on cells in the proximal domain, the putative ligand would bind and stimulate these cells. Interestingly, nAG was identified as a binding partner of Prod1 in a yeast two-hybrid screen, but whether an interaction between nAG and Prod1 mediates positional identity has not yet been resolved (Kumar et al. 2007). The nAG/Prod1 interaction brings up the exciting possibility that a link exists between nerve dependence and patterning, issues that were previously investigated separately.

Another class of RA-responsive molecules that dictate proximal identity are the Meis homeobox transcription factors (Mercader et al. 1999, 2005). During limb regeneration, Meis transcription factors are localized to the nucleus in the proximal domain of the blastema near the amputation plane, and overexpression of Meis1a or Meis2a plus their binding partner Pbx in distal blastema cells leads to their proximal translocation. Strikingly, electroporation of Meis antisense morpholinos into RA-treated wrist blastemas prevents limb duplications, demonstrating the requirement of the Meis proteins in RA-induced proximalization (Mercader et al. 2005). It is not yet known which genes become up-regulated by Meis transcription factors. Future experiments need to address whether Meis and Prod1 act in the same RA-induced pathway and, if yes, which one is upstream of the other.

TISSUE HETEROGENEITY OF PD IDENTITY

On the basis of our above studies tracking tissue cell fate during regeneration, it became clear that the blastema is a heterogeneous pool of progenitor cells. PD identity had so far been studied assuming that blastema cells behave homogeneously. Taking into account our recent results, we wanted to know if blastema cells deriving from the different tissues participate similarly in PD pat-

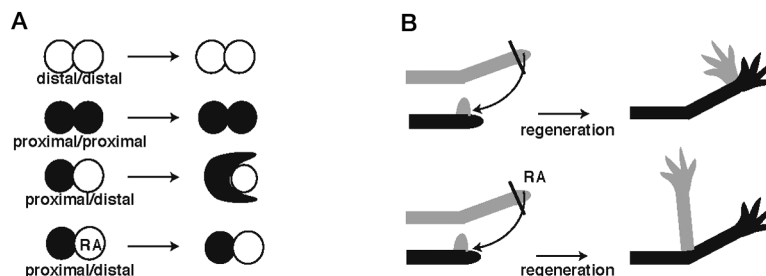


Figure 6. Two assays reveal that the cell surface properties of proximal and distal cells differ. (A) In the affinophoresis assay, a wrist blastema is transplanted onto an upper-arm blastema. The wrist blastema tracks along the regenerating limb and ultimately grows out at the wrist level (Crawford and Stocum 1988). An RA-treated wrist blastema behaves like an upper-arm blastema in the affinophoresis assay. (B) Hanging-drop cocultures show that of an upper-arm blastema engulfs the wrist blastema (Nardi and Stocum 1983). RA-treated wrist blastemas are no longer engulfed by an upper arm blastema, suggesting that it has acquired upper-arm properties.

terning or whether tissue-specific differences exist. We asked whether skeleton-, dermis-, muscle-, or Schwann-derived cells express markers of positional identity, namely, *Meis1/2* and *hoxa13*, similarly or whether differences exist. Second, we asked whether distal Schwann- or cartilage-derived blastema cells displayed affinophoresis capabilities by transplanting GFP⁺ Schwann or cartilage cells from the hand into the upper arm and asking if they ultimately came to reside in the hand or all along the limb after regeneration.

Our results revealed that cartilage- and Schwann-derived blastema cells display strikingly different behaviors with respect to PD identity assays. First, we found that 95% of bone-derived blastema cells harbored nuclear *Meis* in the proximal part of an upper-arm blastema, suggesting that these cells harbor a proximal identity. In contrast, no Schwann-derived blastema cells displayed nuclear *Meis* protein, suggesting that Schwann cells are neutral for PD positional identity. In addition, none of the Schwann-derived blastema cells expressed *hoxa13*, whereas some bone-derived cells expressed this marker. In concurrence with the molecular markers, we observed that cartilage and Schwann cells behaved differently in the affinophoresis assay. Hand-derived cartilage cells transplanted into the upper arm before limb amputation ultimately populated only the hand in the regenerate, indicating that they had maintained their distal identity. In contrast, hand-derived Schwann cells transplanted similarly spread throughout the PD axis of the regenerating limb, suggesting that they have no PD identity. These results clearly show that some blastema cells harbor positional identity whereas others do not. A second conclusion we can draw from this work is that hand-derived cartilage cells stably maintain their distal identity throughout the course of limb regeneration. Therefore, the proximal blastema is not able to efficiently convert distal tissue to a more proximal identity.

PERSPECTIVES

Our goal has been to understand which properties of the stem and progenitor cells of the regenerating limb blastema are important for regeneration and may distinguish them from mammalian cells that do not undergo regeneration. A surprising outcome of our cell-tracking experiments is that tissue-specific stem cells largely retain their tissue identity during the course of regeneration, and we see no evidence for the participation of a pluripotent stem cell. This means that with respect to tissue stem cell identity, the cells undertaking limb regeneration may not be astonishingly different from their mammalian counterparts. The ALM model has clarified that nerve signals and AP positional identity are important cues for initiating limb regeneration. Therefore, a key question is whether crucial differences exist between mammals and salamanders in the nerve/AEC growth stimulatory circuitry and/or maintenance of AP positional information in adult tissue required to induce the tissue-specific stem cells to undertake the complex program of regeneration. In addition to AP positional identity is information along the PD axis to

define the limb segments. Interestingly, Rinn et al. (2006) have surveyed adult human fibroblasts for anatomically related differences and do find that fibroblasts along the PD axis of human appendages express different developmental genes reflecting their PD position. So far, it appears that the positional identity of these human fibroblasts is stable even under multiple rounds of division and even when cells are confronted with cells from different locations (Rinn et al. 2008). Therefore, the ability to reprogram PD identity after limb amputation could be an important contributing trait for regenerative ability.

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