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A conceptual framework for analyzing axial patterning in regenerating urodele limbs

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ABSTRACT This review describes what we have learned about mechanisms of patterning in regenerating urodele limbs. Experimental evidence for three concepts is presented. First, the regeneration blastema is a self-organizing system based on positional memories inherited from parent limb cells. Second, the autonomous patterning mechanism involves local cell interactions that determine patterns of *Hox* gene activation. The effector molecules for positional identity reside in or on the cell surface, and can be altered by retinoic acid. Third, proximodistal patterning of the blastema is linked to blastemal growth, which in turn is dependent on a signal from the apical epidermal cap and on the non-uniformity of positional identity in the transverse axes. Lastly, the question of the degrees of similarity between the mechanisms of urodele limb regeneration, urodele limb embryogenesis, and the embryogenesis of other tetrapod limbs is discussed.

KEY WORDS: *pattern formation, regeneration, retinoic acid, Hox genes, sonic hedgehog genes*

Introduction

Larval and adult urodeles are the only vertebrates which can regenerate limbs, an ability which has fascinated biologists since it was first described by Spallanzani in 1768 (see Dinsmore in this issue). Limb regeneration is a post-embryonic form of epimorphic pattern regulation in which a blastema is formed by the controlled degradation of extracellular matrix (ECM) and the dedifferentiation of the liberated cells to mesenchyme-like blastema cells that accumulate and proliferate under the wound epidermis (reviewed by Stocum, 1995).

Immediately after amputation of a urodele limb a plasma clot forms over the wound surface. Within 6-9 h, epidermis has migrated through the clot from the cut edges of the skin to close the wound. The wound site is quickly invaded by neutrophils and macrophages, which are active in killing bacteria and phagocytizing debris. In a larval limb, degradation of extracellular matrix by metalloproteinases and acid hydrolases is visible in histological sections by two days post-amputation, and accelerates rapidly over the next 2-3 days. As the blastema cells accumulate the wound epidermis thickens at its apex into a structure called the apical epidermal cap (AEC). The ECM synthesized by the early blastema is similar to that of the embryonic limb bud, with high levels of hyaluronate, fibronectin and tenascin, and low levels of collagen and chondroitin sulfate (reviewed by Chernoff and Stocum, 1995). Cell marking experiments suggest that the dermis is the major contributor of cells to the blastema, with progressively smaller contributions made by muscle and cartilage (Muneoka *et al.*, 1986). The initial accumulation of blastema cells is avascular, but as the blastema enlarges into a cone (6-9 days post-amputation) new capillaries sprout into the mesenchyme. The nerves innervating the limb initially undergo Wallerian degeneration for a short distance proximal to the amputation plane and then regenerate rapidly to reinnervate the wound epidermis and mesenchymal cells of the blastema as it forms.

The blastema grows by cell mitosis through early and medium bud stages. As growth continues, the blastemal mesenchyme redifferentiates into the missing skeletal elements, soft connective tissue, and muscle of the amputated segments. Redifferentiation takes place in a proximal to distal sequence, as in the embryonic limb buds of all vertebrates. The urodele limb bud and regeneration blastema are unique, however, in that differentiation in the anteroposterior axis takes place in an anterior to posterior sequence, instead of the posterior to anterior sequence observed in the limb buds of other vertebrates.

The mechanism which organizes the cells of the regeneration blastema into the tissue patterns of the amputated limb parts is of great interest from several perspectives. First is the physical nature of the mechanism itself, whether it involves diffusable morphogens, local cell interactions, or both, and the identity and function of the signaling molecules. Second is how this mechanism is linked to growth of the blastema. Third is whether the patterning mechanism of the urodele limb bud and regeneration blastema is the same as

Abbreviations used in this paper: AEC, apical ectodermal cap; AP, anteroposterior; CRABP, cytoplasmic retinoic acid binding protein; DV, dorsoventral; ECM, extracellular matrix; HPLC, high performance liquid chromatography; PD, proximodistal; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; ROL, retinol; ZPA, zone of polarizing activity.

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that of other tetrapod limb buds. Finally, the mechanisms of blastema formation and patterning are of interest from the perspective of how tissue and organ regeneration might be evoked in adult mammals following injury or disease. This review will focus on the first three of these perspectives.

Concept 1: the regeneration blastema is a self-organizing system

Transplantation experiments carried out from the 1920s to the 1960s suggested that the blastema is a nullipotent and pluripotent mass of cells, and that its pattern of differentiation is imposed upon it by adjacent differentiated tissues (reviewed by Stocum, 1984). Undifferentiated blastemas of adult newts and salamanders exchanged between forelimbs and hindlimbs were reported to develop in accord with their new location, and limb and tail blastemas were reported to differentiate as lenses when grafted in place of the extirpated lens. Undifferentiated blastemas grafted to neutral (non-regenerating) locations failed to develop. These results were interpreted to mean that, during normal limb regeneration, the blastema receives limb type and level-specific instructional signals from adjacent differentiated tissues that dictate its development into the missing segments.

These experiments did not use cell or morphological markers to distinguish graft from host tissue, so an alternative explanation for these results was that the grafted blastemas resorbed, followed by regeneration from the host site. This explanation was supported by experiments showing that single limb blastemas grafted to the back disappeared, but several blastemas massed together to counter resorption developed into good regenerates (Polezhaev, 1937). These results suggested that the blastema is a highly regulative, self-organizing system, a view subsequently borne out by the results of experiments on the small, rapidly regenerating limbs of salamander larvae, the blastemas of which are less susceptible to resorption than adult blastemas (Fig. 1A,B). Undifferentiated blastemas of larval limbs differentiated *in vitro* and self-organized into complete and perfect regenerates when autografted to the dorsal fin (Stocum, 1968a,b). Blastemas grafted to a limb stump of different structure, or to a different proximodistal (PD) stump level, or rotated around the PD axis to disharmonize the anteroposterior (AP) or dorsoventral (DV) axes with those of the stump, always developed according to origin with respect to limb type, level of origin, and transverse axial polarity (Stocum, 1975, 1980a, 1982; Stocum and Melton, 1977; Pescitelli and Stocum, 1980). The blastema was thus shown to be a self-organizing system.

The self-organizational nature of the blastema suggests that its cells inherit level-specific memories of their axial positions from parent limb cells. Since the blastema is derived mainly from dermal fibroblasts around the circumference of the limb (Muneoka *et al.*, 1986), the "positional memories" (Carlson, 1983) carried by the blastema cells derived from the dermis would serve to specify the transverse axial poles of the regenerate. Blastema cells derived from any of the limb tissues at the amputation plane would carry the positional memory of PD origin. These positional memories would act as limiting boundaries for the autonomous pattern-forming mechanism (Stocum, 1980b, 1984). Blastema cells can change positional identity in circumferential and centripetal directions (Fig. 2). They can also change positional identity in a direction distal to, but not proximal to, their PD level of origin. These constraints insure that the blastema replaces only those structures that are lost. The extent to which cells derived from tissues internal to the dermis can change positional identity in a centrifugal direction is unknown, but it is likely that these cells are used as positional information boundaries when internal structural discontinuities occur.

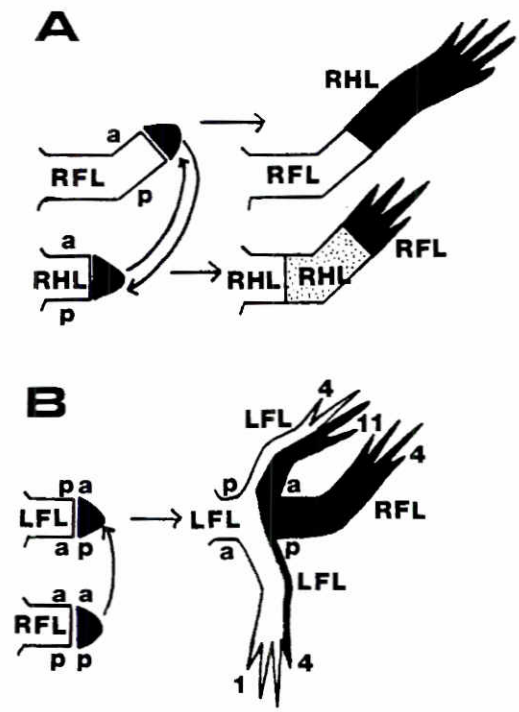

Fig. 1. Experiments demonstrating self-organization of the limb regeneration blastema. (A) *Wrist blastema of a right forelimb (RFL) autografted to the mid-thigh stump of a right hindlimb (RHL), and vice versa. The grafted blastemas (black) differentiate according to their limb type and PD level of origin. In the forelimb to hindlimb combination, a complete limb is regenerated, with the hindlimb host providing the cells for the intermediate segments (stippled) between the host amputation plane and graft. In the hindlimb to forelimb combination a hindlimb is regenerated distal to the host wrist stump.* **(B)** *Regenerates (black) formed after autografting a right forelimb (RFL) blastema to the contralateral left forelimb stump (LFL), reversing the AP axis of the blastema. The blastema maintains its AP polarity of origin and develops as a right limb. Two supernumeraries form by the interaction between anterior (a) and posterior (p) host and graft cells, one on either side of the host limb. One supernumerary is depicted as being derived equally from host and graft cells; the other is depicted as being derived predominantly from host cells. The illustrations are composites drawn from data acquired by the use of triploid-diploid cell markers, morphological and color markers, and chronic immunological rejection to distinguish graft and host tissues.*

Concept 2: the blastema is patterned by a local cell interaction mechanism

Missing ap positional identities are replaced by intercalary regeneration

How is axial pattern specified within the boundary positional memories carried by blastema cells? First, one of the boundary regions might act as an organizing center which is the source of a diffusable morphogen (s) that imposes pattern on an axis, while contributing little to the structure of the regenerate. Each threshold decrement in the concentration gradient of the morphogen would specify a positional identity. Alternatively, pattern might be speci-

fied by local interactions between cells. In this kind of mechanism, every cell is a boundary, or organizing center, that is constantly aware of the positional identities of its immediate neighbors and can sense discontinuities in the positional identity "map." When a discontinuity is sensed (i.e., when cells are confronted with non-neighbors), cells on all sides of the discontinuity can potentially react by proliferating and contributing to the structure of the regenerate. The daughter cells intercalated between their organizer parents are assigned positional identities that fill in the discontinuity, a process known as *intercalary regeneration.*

Studies on the origins of supernumerary limb structures induced by the confrontation of anterior and posterior tissues indicate that the blastema patterns itself by this kind of mechanism (Bryant *et al.,* 1981; Bryant and Gardiner, 1992). Reversal of either the AP or DV axes of a regeneration blastema by grafting it to a contralateral limb stump evokes the formation of two supernumerary regenerates, both of which have host handedness (Fig. 1B). The supernumeraries arise where anterior and posterior, or dorsal and ventral tissues of graft and host are confronted (Iten and Bryant, 1975; Tank, 1978). They can be derived equally from donor blastema and host stump tissues (Stocum, 1982; Muneoka and Bryant, 1984), or predominantly from graft or host tissue (Stocum, 1982; Papageorgiou and Holder, 1983; Maden and Mustafa, 1984). A dual contribution of cells to a supernumerary from both graft and host tissues implies that there is no special anterior or posterior organizing region which evokes supernumerary formation from adjacent tissue. Rather, cells of both host and graft act as organizers and react to the discontinuity created by triggering cell proliferation and intercalation of missing positional identities between the axial poles. By analogy, similar interactions would be involved in patterning the blastema during normal regeneration. The handedness of each supernumerary is predicted by the spatial relationships of the confronted anterior, posterior, dorsal and ventral poles of the graft and host tissues, and the direction of outgrowth of the blastema.

Two supernumeraries are also formed after ipsilateral APDV reversal of the blastema, but at more variable locations around the circumference of the limb stump (Iten and Bryant, 1975; Maden and Turner, 1978). Detailed analysis of skeletal and muscle patterns indicates that these supernumeraries fall into several anatomical classes: (1) normal limbs of stump handedness; (2) symmetrical (double dorsal and double ventral) limbs; (3) part normal/part symmetrical limbs; and (4) part normal/part inverted limbs (Papageorgiou and Holder, 1983; Holder and Weekes, 1984; Maden and Mustafa, 1984). When considered with the supernumeraries evoked by AP or DV reversal, it is clear that all anatomically normal supernumeraries have host limb handedness, regardless of the percentage contribution of graft and host to the supernumerary. Symmetrical or mixed handedness (normal plus inverted) supernumeraries arise only when discontinuities cannot be eliminated by intercalation. Under these circumstances discontinuities are tolerated, and the regions of tolerance have the polarities of origin of graft and host tissues.

The local cell interactions of limb regeneration follow a fundamental rule, the rule of shortest intercalation: given a choice of intercalating the longer or shorter number of positional identities that will restore structural continuity, interacting cells will always choose the shorter (French *et al.,* 1976; Bryant *et al.,* 1981). Intercalation might follow an averaging rule, in which the progeny of two interacting cells with different positional identities would adopt an identity that is midway between the two (Maden, 1977). Proliferation would continue until averaging restored all the missing positional identities.

One feature that has not been addressed by local cell interaction models is the anterior to posterior sequence of differentiation observed in the regeneration blastema. If differentiation is linked to intercalation of missing positional identities, the rate of elimination of discontinuity by intercalation should be graded from anterior to posterior. Alternatively, there may be a mechanism superimposed on a uniform intercalation rate that retards differentiation in a posterior to anterior direction. This question warrants further study.

What is the mechanism of distalization in the blastema?

The nature of the mechanism which re-establishes missing PD positional identities is not clear. Proximodistal intercalary regeneration is an appealing mechanism, since patterning in the transverse and PD axes would then use a common mechanism of boundary interactions. Proximodistal intercalation of missing positional identities does take place when a distal blastema is grafted to a more proximal level of the limb stump (Stocum, 1975, 1981; Pescitelli and Stocum, 1980). Thus the host amputation level and the base of the blastema could be acting as proximal and distal boundaries, respectively, for intercalary regeneration.

But what would be the distal boundary during normal regeneration? A logical candidate is the apical epidermal cap (AEC) (Maden, 1977), and there is some evidence that the AEC might function in this capacity. Factors supplied by the epidermis and the limb nerves are both essential for blastema cell proliferation (see Concept 3 below). A denervated cone stage blastema fails to increase in cell number and volume, but is nevertheless able to form a miniature, PD-complete regenerate; i.e., once the blastema has reached a critical size, its patterning and morphogenesis is independent of cell division (Singer and Craven, 1948). By contrast, an epidermis-free blastema implanted into a dorsal fin tunnel forms a truncated skeletal pattern (Stocum and Dearlove, 1972). This difference suggests that the epidermis is essential to patterning in a way that is distinct from its growth-promoting function. More precise experiments, however, are required to determine whether

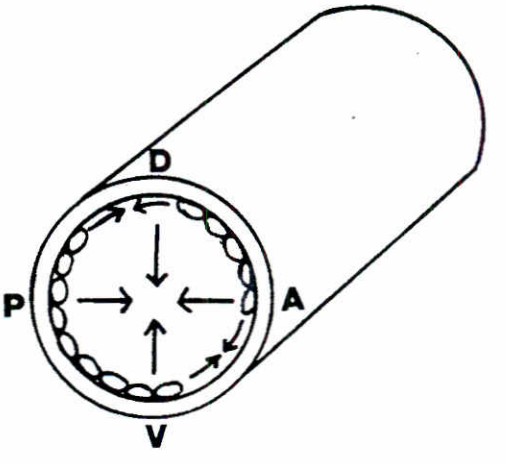

Fig. 2. Cross-section of amputated limb illustrating circumferential (shorter arrows) and centripetal (longer arrows) directions of intercalation to eliminate discontinuities in the transverse plane. *The directions are deduced from the results of experiments on supernumerary limb regeneration and deletion of limb tissues. A, anterior; P, posterior; D, dorsal; V, ventral.*

the AEC actually functions as a distal boundary during regeneration.

Alternatively, there may be no distal boundary involved in either PD intercalary regeneration or normal regeneration, and the epidermis or grafted blastema may function strictly in an outgrowth-promoting capacity. This possibility must be considered since, unlike supernumerary limb formation in the AP axis, where both blastema and stump can potentially contribute to the supernumerary, the cells forming the intermediate structures of the regenerate after a distal to proximal shift of a blastema are derived from the host amputation level only. One alternative mechanism that has been proposed, based on studies of the chick limb bud, is a counting mechanism linked to the number of blastema cell divisions (Summerbell *et al.*, 1973; Summerbell and Lewis, 1975). Another idea is that blastema cells adopt successively more distal positional identities as the result of successive waves of transverse intercalary regeneration (French *et al.*, 1976; Bryant *et al.*, 1981). Non-uniformity in transverse positional identities is essential for blastema formation and outgrowth (see Concept 3b below), but there is as yet no evidence that distalization of positional identity and transverse intercalation are mechanistically linked.

Retinoic acid and Hox genes in regenerate patterning

Retinoic acid (RA) treatment of larval and adult urodeles during the accumulation blastema stage alters the positional memory of blastema cells in all three axes of the limb in a dose-dependent way (Fig. 4). RA proximalizes the PD level of origin of the blastema; for example, blastema cells derived from the wrist level assume the positional identity of the shoulder girdle if treated with an appropriate dose of RA, so that the wrist blastema regenerates a complete limb instead of just a hand (Maden, 1982; Thoms and Stocum, 1984; Kim and Stocum, 1986a,c). At the same time, RA posteriorizes and ventralizes blastema cells, although this effect can only be observed in anterior and dorsal half or double half limb constructs (Stocum and Thoms, 1984; Kim and Stocum, 1986a,b; Wigmore, 1986; Ludolph *et al.*, 1990; Monkemeyer *et al.*, 1992). Two mirror-image, proximalized regenerates are produced by these constructs, suggesting that the regenerates formed by RA-treated limbs are supernumeraries evoked by interaction between posteriorized, ventralized and proximalized cells, and unaffected anterior cells of the limb stump (Stocum, 1995; Fig. 3). The effects of RA on positional identity are maximal when it is administered at the accumulation-early bud blastema stages. The first observable effect is a delay in blastema formation that is correlated with inhibition of mitosis (Maden, 1983; Kim and Stocum, 1986b). This delay period warrants a deeper cellular and molecular analysis, because it during this time that the positional identities of blastema cells are being reset.

Numerous *in vitro* and *in vivo* studies on mammalian and avian cells have shown that the biological effects of RA are mediated by the activation of nuclear receptors belonging to the steroid receptor superfamily of transcription factors (Evans, 1988), and that the amount of RA gaining access to the nuclear receptors can be regulated in the cytoplasm by cytoplasmic retinoic acid binding proteins (CRABPs) (Maden, 1992). Two sets of nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), bind to retinoic acid response elements (RAREs) in the regulatory regions of target genes when activated by RA. Each has three major isoforms, alpha, beta and gamma (Mendelsohn *et al.*, 1992; Manglesdorf *et al.*, 1994). The specific ligand for the RARs is all-trans RA, and the specific ligand for the RXRs is 9-cis RA. RARs and RXRs form both homodimers and heterodimers; the latter bind RA more efficiently than the former, and thus may be important in mediating the effects of RA on transcription.

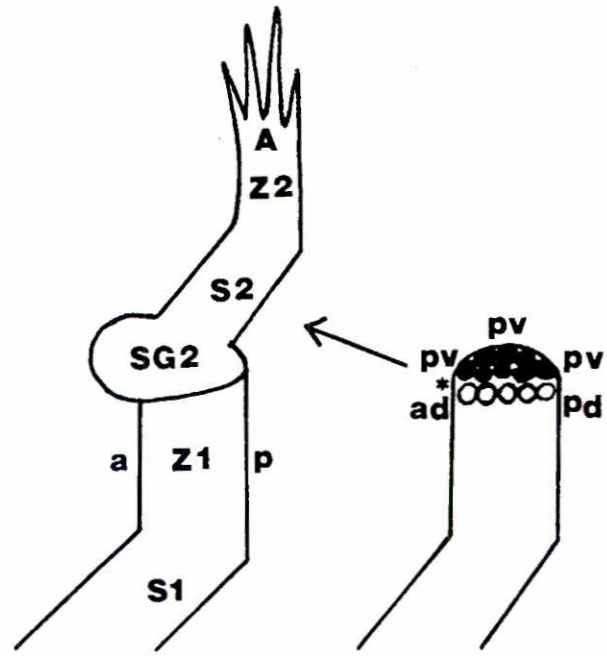

Fig. 3. RA-induced supernumerary limb formation. *Left, limb regenerate of a urodele treated with 100 ug RA/gm body wt by intraperitoneal injection during the accumulation blastema stage. The limb was amputated at the level of the wrist, but RA proximalizes the PD positional identity of the blastema cells so that a complete limb and partial shoulder girdle regenerate from the wrist level. Such regenerates appear to be derived from the anterodorsal quadrant of the limb. Right, how the regenerate probably forms. RA posteriorizes and ventralizes blastema cells at the same time it proximalizes them, but does not affect cells that do not dedifferentiate. Thus on the anterior side of the limb posteroventral cells (pv) are juxtaposed to more proximal anterodorsal cells (ad) that were not affected by RA. The confronted cells interact to intercalate a "supernumerary" transverse plane, which forms the base of the regenerate.*

Two forms of CRABP have been identified so far, both of which specifically bind all-trans RA. The amino acid sequence of CRABP I is conserved across species, but the sequence of CRABP II is species-variable. Data from overexpression studies on F-9 cells strongly suggest that the CRABPs act to titrate RA in the cytoplasm, thus modulating its access to the nuclear receptors (Boylan and Gudas, 1991).

Two isoforms of RAR alpha, one of beta, and two of gamma have been cloned from newt mRNA (Ragsdale *et al.*, 1989, 1992). The alpha and beta isoforms share significant homology with their mammalian counterparts, but the newt gamma is significantly different in its N and C-terminal sequences and has thus been named delta (Hill *et al.*, 1993; Ragsdale *et al.*, 1993). The alpha and beta RARs are uniformly expressed throughout the regeneration blastema (Giguere *et al.*, 1989). The spatial distribution of delta has not been reported, but Northern and RNAse protection analyses indicate that it is the most strongly expressed RAR in the blastema, suggesting that delta might play a central role in mediating the effects of RA on limb regeneration (Ragsdale *et al.*, 1993). The expression of RXRs in the blastema has not yet been reported.

Experiments have been conducted in which the RA-induced activation of RAR genes transfected into blastema cells is detected by the expression of a co-transfected reporter gene carrying a RARE. By correlating this activation with the up or down-regulation of a cellular activity or molecular marker affected by RA, the specific RAR mediating the regulation can be identified. Such experiments have shown that the alpha-1 receptor mediates the initial inhibition of blastema cell division induced by RA (Schilthuis *et al.*, 1993), and that the delta-1 receptor mediates the up-regulation of WE3 (Pecorino *et al.*, 1994), an antigen normally expressed in the wound epithelium of the blastema a week after amputation, but precociously expressed after administration of RA (Tassava, 1992).

The effects of exogenous RA on positional memory of blastema cells are undoubtedly mediated by the nuclear retinoid receptors, perhaps interacting with other transcription factors, and modulated by CRABPs. CRABP has been detected by binding assays in regenerating limbs of axolotls and newts (Keeble and Maden, 1986; McCormick *et al.*, 1988), but its spatial distribution has not been mapped. The concentration of unbound CRABP is highest at the accumulation blastema and early bud stages, precisely the time at which administration of exogenous RA has its maximal effect on positional identity. CRABP I transcripts have been detected in combined muscle and skin of unamputated limbs by Northern analysis, but no CRABP I transcripts have been detected in the regeneration blastema (Ludolph *et al.*, 1993). Collectively, these observations suggest that CRABP I expression (and perhaps CRABP II as well) is down-regulated in the blastema, allowing more free RA for interaction with nuclear receptors. This idea has yet to be tested directly.

The presence of RARs, and of RA-bound CRABP in the blastema (McCormick *et al.*, 1988) indicates that it contains RA. Measurements by high performance liquid chromatography (HPLC) indicate a much higher concentration of RA in the posterior half of the blastema than in the anterior half and about equivalent amounts of retinol (ROL) in each quadrant of the blastema (Scadding and Maden, 1994). Experiments in which a reporter gene coupled to a RARE was transfected into proximal or distal levels of the newt limb suggest that the concentration of free RA in the upper arm is three times higher than in the lower arm (Brockes, 1992). The reverse result was obtained with HPLC measurements (Scadding and Maden, 1994), but HPLC measures total RA, not the unbound (from CRABP) RA reflected by the activation of reporter genes. Thus there might well be more total RA at distal limb levels, but more free RA to bind to nuclear receptors at proximal levels.

The asymmetric distribution of RA suggests that retinoids might play a role in maintaining gradients of posteriorization, ventralization, and proximalization in limb cells, and that these gradients are preserved in the blastema. This idea is consistent with the fact that exogenous administration of RA results in posteriorization, ventralization, and proximalization of all blastema cells. These gradients would not be diffusion gradients of RA, but rather reflections of differences in the metabolism of retinol to RA within individual cells. This would push the question of the molecular basis of positional memory back another step, to the regulation of the enzymes that convert retinol to RA. Regardless, different concentrations of RA in different cells may be instrumental in specifying patterns of gene activity that contribute to the expression of positional memory.

One class of genes likely to have a role in regenerate patterning is the *Hox* family, of which the more 5' genes of the *A*, *C* and *D* clusters have been shown to be correlated with AP and PD patterning in chick and mouse limb buds (Tabin, 1991; Izpisua-Belmonte and Douboule, 1992). A number of urodele 5' *Hox A, C* and *D* genes have been cloned and are known to be active in regenerating limbs (Savard *et al.*, 1988; Brown and Brockes, 1991; Simon and Tabin, 1993; see Gardiner and Bryant in this issue). So far, *HoxD-10* and *HoxA-13* are the only genes that have been shown to exhibit changes in activity in response to RA. The level of expression of *HoxD-10* is 2-3 times higher in the mid-stylopodium than the mid-zeugopodium, and RA treatment elevates zeugopodial expression to the stylopodial level (Simon and Tabin, 1993). *Hox A-13* is down-regulated when blastemal cells are proximalized by RA (see Gardiner and Bryant in this issue). Interestingly, most of the urodele *HoxC* and *D* genes that have been cloned are active in unamputated limb tissues as well, leading to the speculation that expression of these genes in unamputated tissues is in some way correlated with regenerative capacity. It would be interesting to compare the spatial pattern of *Hox* gene expression in regenerating limbs to spatial variations in free RA content. It is not known whether these genes are affected directly or indirectly by RA.

Positional memory (identity) is related to differential adhesion

What are the effectors of positional memory by which cells interact? Assays *in vitro* and *in vivo* have shown that the adhesivity of cells at the base (origin) of the blastema exhibits level-specific differences along the PD axis in regenerating limbs. In the *in vitro* assay, blastemal mesenchymes from the wrist or tarsus, elbow or knee, and upper arm or leg, were pressed together at their base,

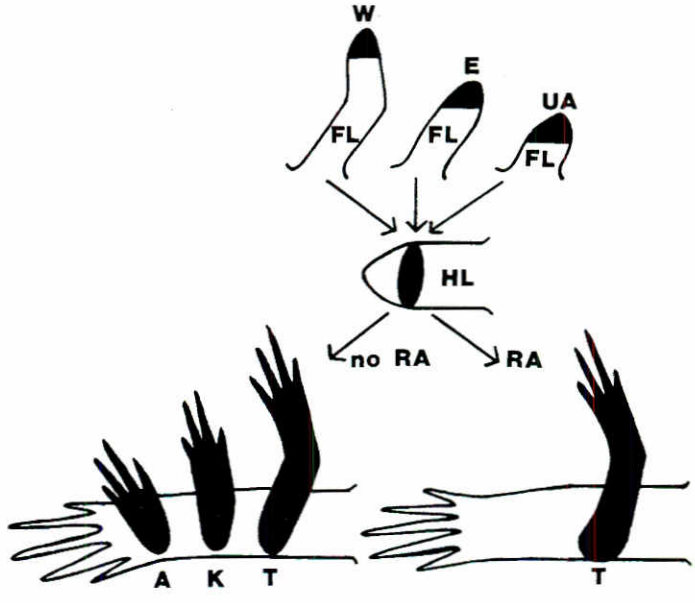

Fig. 4. Affinophoresis assay for blastema cell affinity. *Forelimb (FL) blastemas (black) derived from the level of the wrist (W), elbow (E) or mid-upper arm (UA) are grafted to the dorsal surface of the blastema-stump junction of a host hindlimb (HL) amputated through mid-thigh. In the control assay, the blastemas sort out along the PD axis of the host regenerate to articulate with their corresponding host level. Thus the wrist regenerate articulates with the host ankle (A), the elbow regenerate with the host knee (K) and the mid-upper arm regenerate remains at the mid-thigh graft site (T). When the donor blastemas are proximalized by RA, however, distal displacement is abolished, and wrist and elbow regenerates remain at the mid-thigh graft site.*

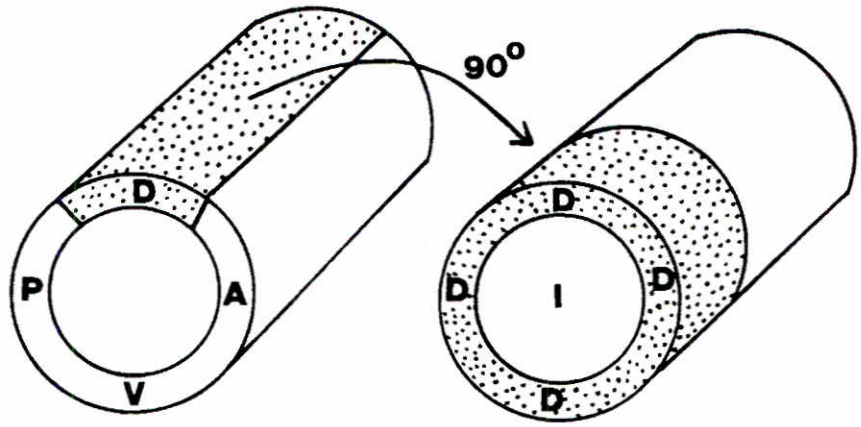

Fig. 5. Lheureux's (1975) experiment demonstrating that uniformity of positional identity in the transverse plane of an amputated limb inhibits blastema formation and regeneration. *A longitudinal strip of skin (stippled) bearing one set of positional identities (dorsal is illustrated here) is removed, rotated 90° and grafted around the circumference of an irradiated (I) host limb in place of the host skin. Regeneration does not take place after amputation through the grafted skin, whereas if strips of skin representing the dorsal (D) ventral (V), anterior (A) and posterior (P) faces of the limb are placed around the circumference of the irradiated limb, it regenerates after amputation.*

and cultured in hanging drops in all of the nine possible binary combinations between these three levels (Nardi and Stocum, 1983). One member of each pair was pre-labeled with ^{3}H-thymidine to distinguish it from the other member of the pair. Pairs of mesenchymes from the same level (wrist/wrist, etc.) fused to make a straight interface. By contrast, in pairs of mesenchymes derived from different levels, the proximal mesenchyme attempted to engulf the distal one. The engulfment behavior was hierarchical, with elbow and knee mesenchymes surrounding wrist and ankle mesenchymes, and in turn being surrounded by upper arm and leg mesenchymes.

These results are consistent with Steinberg's (1978) differential adhesion hypothesis, which predicts that two cell populations differing in adhesivity will adopt configurations that maximize adhesive bonds between like cells, thus minimizing the free energy of the system. This is achieved *in vitro* by the surrounding of the more adhesive cells by the less adhesive ones, regardless of whether the cells start from a disassociated state (in which case they sort out), or as juxtaposed tissue fragments (in which case one fragment spreads over the other). Hence the engulfment behavior of blastemal mesenchymes from different limb levels is indicative of position-dependent differences in the adhesivity of the cells at the base (proximal boundary) of the blastema, cells from more distal amputation levels being more adhesive.

A similar conclusion can be drawn from the results of an *in vivo* assay. Blastemas derived from the wrist, elbow or mid-upper arm levels of axolotl forelimbs were autografted or homografted to the dorsal surface of the blastema-stump junction of host hindlimbs regenerating from the mid-thigh (Fig. 4). The grafts moved distally to their corresponding levels on the PD axis of the growing host regenerate; i.e., they "sorted out" according to level (Crawford and Stocum, 1988a; Egar, 1993). Wrist regenerates were displaced to the ankle level of the host regenerate, and elbow regenerates to the level of the knee. Mid-upper arm regenerates did not displace, consistent with the fact that they were already at the corresponding level of the host leg. This displacement behavior has been termed "affinophoresis" (Crawford and Stocum, 1988a) and shows that blastema cells derived from different limb levels have level-specific adhesive properties that mediate their preferential association with cells from identical PD positions.

Proximalization of donor wrist or elbow blastemas by RA treatment prior to grafting abolishes their distal displacement behavior in the affinophoresis assay, indicating that level-specific blastema cell adhesion and positional memory are directly related. In another experiment, the ability of RA-treated wrist blastemas to evoke intercalary regeneration from the mid-thigh region of the hindlimb (or *vice versa*) was tested (Crawford and Stocum, 1988b). Whereas control blastemas always evoked intercalary regeneration of intermediate parts that were clearly derived from the host limb, RA-treated (proximalized) blastemas failed to evoke intercalary regeneration and formed complete limbs composed entirely of donor tissue. Collectively, these results indicate that positional identity resides ultimately in a cell surface organization that is able to recognize the disparity between non-neighboring cells, and initiate intercalary regeneration.

Retinoids have been shown to alter cell-cell and cell-substrate adhesiveness in a variety of normal and neoplastic cells, and these effects are correlated with qualitative and/or quantitative changes in cell and substrate adhesion molecules (Lotan, 1980). Tunicamycin, which inhibits N-linked glycosylation of proteins, prevents the proximalizing effect of RA in regenerating axolotl limbs (Johnson and Scadding, 1992). Hence it seems plausible that the molecular effectors defining positional memory are glycoprotein cell adhesion molecules. Changes in positional identity might be the result of a direct or indirect effect of RA on the activity of genes encoding the protein portion of these molecules, the enzymes that glycosylate or deglycosylate them, or both.

The target genes directly affected by RA in blastema cells are not known. Likewise, little is known about the molecular nature of the local signals that pass between cells carrying different positional memories, how they stimulate cells to divide, and how new positional identities are assigned to progeny cells. From what is known about other intercellular signaling systems, however, these signals could be transmitted through direct interaction of surface-embedded, complementary cell adhesion molecules, or by secreted short-range molecules that interact with their receptors on adjacent cell surfaces.

Concept 3: regenerate patterning is linked to pd outgrowth

The wound epidermis promotes entry of blastema cells into the cell cycle

The AEC and limb nerves are both essential for the proliferation of dedifferentiated cells (Stocum, 1995, for review). Some histolysis and cell dedifferentiation occur in amputated limbs that have

been denervated or covered with full-thickness skin, but a blastema fails to form because DNA synthesis in the dedifferentiated cells is much reduced and their mitosis is virtually non-existent (Mescher and Tassava, 1975; Mescher, 1976). Nerves are thought to be essential for both DNA synthesis and the G_2/M transition, possibly by providing the blastema with transferrin, which supplies cells with iron (Mescher, 1992). The AEC is believed to promote matrix remodeling (Stocum, 1995, for review) and the competence of dedifferentiated cells to exit G_0 into G_1 of the cell cycle (Tassava and Mescher, 1976). Consistent with this view, blastemal mesenchyme cultured *in vitro* without epidermis differentiates prematurely into cartilage, whereas mesenchyme cultured with epidermis does not (Globus *et al.*, 1980).

The competence signal(s) emitted by the AEC is effective only over a specific PD distance, termed the "progress zone" (Summerbell *et al.*, 1973; Smith *et al.*, 1974). At stages where Redifferentiation has not yet begun, the whole blastema constitutes the progress zone, and the mitotic index is essentially uniform along the PD length of the blastema (Stocum, 1980c). As the length of the blastema increases, cells at the proximal end of the progress zone are left out of range of the competence signal, withdraw from the cell cycle, and differentiate.

Several lines of evidence suggest that at least one AEC competence factor is a member of the FGF family. First, the mesenchyme and AEC of the axolotl limb regeneration blastema contain high levels of FGF-1 (Boilly *et al.*, 1991). Second, FGF-1 and 2 elevate the mitotic index of blastema cells cultured in the absence of epidermis (Albert *et al.*, 1987). Third, FGF-2 impregnated silastin blocks implanted into amputated axolotl limbs covered with full-thickness skin increase the mitotic index of cells at the amputation plane, although again, a normal blastema is not formed (Chew and Cameron, 1983). Fourth, the FGF receptors FGFR1 (which binds FGF-1, FGF-2 and FGF-4) and FGFR2 (which binds FGF-1 and KGF) have been cloned from newt mRNA and found to have different patterns of expression in regenerating limbs (Poulin *et al.*, 1993). FGFR1 is expressed throughout the growing blastemal mesenchyme from early to medium bud stages, suggesting that FGF-2 is important for stimulating mesenchymal outgrowth. FGFR2 is expressed in the basal layers of the wound epithelium, and later in the perichondrium of newly differentiating cartilage, suggesting that FGF-1 plays a role in chondrogenesis. Finally, both FGF-2 and 4 can substitute for the AER in promoting mesenchymal outgrowth and complete PD patterning in the chick limb bud, and are present in the AER, implicating them as natural proliferation signals (Niswander *et al.*, 1993; Savage *et al.*, 1993; Fallon *et al.*, 1994). It has not yet been shown, however, that a particular FGF can substitute for the AEC in regeneration.

Discontinuities in transverse positional identity are essential to blastemal outgrowth

Blastema cell proliferation is inhibited in the absence of discontinuities in transverse axial positional identity (Fig. 5). Lheureux (1975) found that irradiated limbs which received grafts of unirradiated skin containing only one transverse positional identity failed to form a blastema, whereas irradiated limbs that received grafts of unirradiated skin representing the poles of the AP and DV axes did regenerate. Blastema formation is also inhibited in amputated posterior and ventral half and double half limb constructs treated with RA (Kim and Stocum, 1986a; Ludolph *et al.*, 1990). These constructs undergo simple wound repair after amputation, prematurely forming a thick basement membrane and a pad of connective tissue under the wound epidermis (Kim and Stocum, 1986b). The failure of blastema formation is due to the fact that all discontinuities are eliminated by the posteriorization and ventralization of blastema cells, leaving no opportunity for the intercalary regeneration that creates the supernumeraries observed after treating normal or anterior and dorsal half or double half limbs with RA.

The relationship between the cell cycle, epidermal competence factors, and positional discontinuity is not well understood. The elimination of discontinuities might make cells unresponsive to competence factors, perhaps by down-regulating synthesis of receptors for these factors; reciprocally, the cell cycle-promoting activity of the wound epidermis may be dependent on factors provided by a proliferating mesenchyme. Perhaps most interesting of all is the mechanism whereby lack of discontinuity in transverse positional identity diverts the regenerative process into wound repair. The molecular mechanisms underlying these relationships have not been explored, and would be of great interest.

Question/Concept 4: do all tetrapod limb buds and the regeneration blastema use the same mechanism of patterning?

A special posterior signaling region is found in most vertebrate limb buds

Supernumerary limbs can be induced in embryonic chick limb buds by juxtaposing anterior and posterior tissue. Unlike the supernumerary regenerates of urodele limbs, however, a unique region of posterior cells called the zone of polarizing activity (ZPA) seems to organize the growth and patterning of supernumeraries in the chick limb bud. A single supernumerary is formed when the ZPA is transplanted to the anterior edge of the bud (Macabe *et al.*, 1973). Two supernumeraries with opposite handedness are evoked when wing bud tips are severed by a diagonal cut that bisects the ZPA and rotated 180° around the PD axis, so that ZPA tissue in the wing tip is juxtaposed to anterior stump tissue, and ZPA tissue in the posterior stump is juxtaposed to anterior tissue of the wing tip (Saunders and Gasseling, 1968; Javois and Iten, 1986). No supernumeraries are formed if the ZPA is removed before severing and rotating the wing tip (Fallon and Crosby, 1975; Dvorak and Fallon, 1987). Chick-quail, turtle-chick, and mammal-chick chimera experiments indicate that the ZPA is phylogenetically conserved, and that supernumeraries arising after wing tip rotation or ZPA transplantation are derived almost exclusively from the anterior tissue (Fallon and Crosby, 1977; Javois and Iten, 1986).

The evocation of supernumerary formation from anterior tissue by the ZPA has been taken to mean that in normal development the ZPA is the source of a diffusable signal which specifies the AP pattern of all tissue anterior to it. Further evidence for this idea comes from an experiment in which chick wing bud ZPA and a block of quail anterior leg bud tissue were grafted in tandem to the anterior border of a chick wing bud, with the quail tissue posterior to the ZPA. The quail tissue formed toes in most cases, but mirror-image chick wing digits were also formed posterior to the quail toes (Honig, 1981). This result suggests that a signal diffuses from the ZPA through the quail tissue (a distance of about 200 microns, or 20 cell diameters). The idea of a posterior to anterior gradient

of morphogen specifying AP pattern is consistent with the fact that AP axial differentiation in most vertebrate limb buds follows a posterior to anterior sequence.

The nature of the ZPA signal is still unknown, but one possible candidate is the hedgehog protein. The *hedgehog* gene is an important regulator of patterning in the *Drosophila* embryo (Basler and Struhl, 1994; Heemskerk and DiNardo, 1994; Tabata and Kornberg, 1994), in chick and mouse limb buds (Riddle *et al.*, 1993; Chang *et al.*, 1994), and in the neural tube (Roelink *et al.*, 1994). Both short-range and long-range effects of the *hh* signal have been observed. For example *hh* exerts short-range control over *wingless* expression in *Drosophila* embryo segments and floor plate induction in the vertebrate neural tube, but has long-range effects on the pattern of the dorsal cuticle in *Drosophila* and the induction of motor neurons in the vertebrate neural tube.

In the vertebrate limb bud, the *sonic* form of *hedgehog (shh)* is expressed by the posterior mesenchyme corresponding to the ZPA (Krauss *et al.*, 1993; Riddle *et al.*, 1993; Chang *et al.*, 1994). *shh* is expressed in the ZPA of the chick wing bud from stage 17 to 28, and is activated by RA (Riddle *et al.*, 1993). RA-impregnated beads induce supernumerary formation when implanted into anterior tissue (Tickle *et al.*, 1982; Summerbell, 1983), and the concentration of RA is 2-3 fold higher in the ZPA than in anterior tissue (Thaller and Eichele, 1987). It is likely that RA posteriorizes cells through *shh*, converting them to ZPA cells, but is not itself a diffusable morphogen. Grafts of anterior limb bud cells transfected with a constitutive *shh* construct induce the development of supernumerary structures, showing that sonic hedgehog can evoke supernumerary formation in the absence of RA (Riddle *et al.*, 1993; Chang *et al.*, 1994).

Four genes of the *HoxD* cluster (9, 10, 11, and 13) are asymmetrically expressed in overlapping and progressively more distoposterior domains along the PD axis of the developing chick limb bud. These genes are also expressed in anterior tissue forming supernumerary outgrowths induced by the ZPA or by RA, and are therefore thought to be involved in AP and PD patterning of the limb bud (Izpisua-Belmonte *et al.*, 1991; Nohno *et al.*, 1991). *shh*-transfected limb bud cells have been shown to activate the two distalmost of these genes, 11 and 13, in the anterior mesenchyme prior to the appearance of supernumerary digits (Riddle *et al.*, 1993). Whether *shh* affects *HoxD* expression directly or indirectly is not known.

Experiments with recombinant chick wing buds constructed of an epidermal jacket plus disassociated anterior mesenchyme suggest that the ZPA has both short-range and long-range effects on *HoxD* expression (Ros *et al.*, 1994). Such recombinants develop with symmetrical skeletal patterns and exhibit a symmetrical pattern of *HoxD* expression as well, instead of the normal asymmetric expression pattern. Implanting a piece of ZPA into the recombinant restores the asymmetry of skeletal development and *HoxD* expression. These results suggest that the ZPA is not required for *HoxD* expression per se, but is essential for producing the normal asymmetry of *HoxD* expression. Ros *et al.* (1994) postulate that the asymmetry is created by two ZPA signals, a short-range signal presumably responsible for the expression of the most 5' *HoxD* genes in ZPA cells, thus maintaining them as the most distoposterior, and a long-range signal that suppresses the expression of the most 5' genes anterior to the ZPA.

The hedgehog protein has a unique property that would enable it to function as both a short and long-range signal. The protein is autoproteolytic *in vitro* and *in vivo*, cleaving itself into 19 kDa N-terminal and 25 kDa C-terminal proteins (Lee *et al.*, 1994). *hh* genes with loss of function mutations in the autoproteolytic sequence have a subnormal effect on *wingless* expression in embryonic segments when introduced into the germ line of *Drosophila*, and are completely unable to respecify epidermal cell fates at a distance, indicating that the production of two proteins by autoproteolysis is important for both short and long-range effects on patterning genes. Additional *in vitro* experiments show that the N-terminal protein binds avidly to heparin and remains cell-associated (possibly through ECM), whereas the C-terminal protein diffuses readily into the culture medium. These observations suggest that the N-terminal protein of hedgehog is a short-range signal and the C-terminal protein is a long-range signal. Further experiments will tell if the C-terminal protein actually acts over a long distance and whether it acts by itself, or whether the N-terminal protein acts alone by inducing adjacent cells to produce yet a different signaling molecule. In the latter case, the C-terminal part of the original hedgehog protein would be required only to cleave off N, and would have no role in patterning (Lee *et al.*, 1994).

If the N-terminal protein acted by itself, it would be possible to interpret data on AP patterning in normal and supernumerary chick limb buds in terms of a local cell interaction model in which sonic hedgehog expression would be required only to maintain the posterior positional identity of ZPA cells (see also Bryant and Gardiner, 1992). *Shh* might trigger production of a cascade of short -range molecules across the AP axis, each one specifying a different part of the AP pattern, rather than acting as a long-range diffusable morphogen.

Urodele limb bud and regeneration blastema

Data on supernumerary limb formation argue that the urodele limb bud uses the same type of local cell interactions as the regeneration blastema. Two supernumeraries are produced after AP or APDV reversal of cone stage limb bud tips, one on the anterior side, the other on the posterior side. Like the supernumeraries evoked by axial reversal of regeneration blastemas, they can be derived from both host and graft tissue, or predominantly from one or the other (Thoms and Fallon, 1980). Furthermore, supernumerary limbs composed partly of graft tissue and partly of host tissue are evoked after exchanging the tips of undifferentiated limb buds with regeneration blastemas, suggesting that the cells of limb bud and blastema are using similar local signaling mechanisms to detect gaps in pattern, trigger mitosis, and intercalate missing positional identities (Muneoka and Bryant, 1982).

Other evidence suggests that peribrachial flank tissue may play a role in determination of the transverse axial pattern at the disc stage, prior to outgrowth of the limb bud (Stocum and Fallon, 1982). The prospective forelimb region fails to develop when transplanted to the dorsal or ventral midline (Nicholas, 1924; Finnegan, 1960), to the side of the head between eye and gill (Slack, 1977), or when cultured *in vitro* (Wilde, 1950), unless peribrachial flank tissue is included with the graft. This developmental failure is not due to nonspecific inhibitory effects related to the suitability of the transplant sites, since a limb disc grafted first to the head, then regrafted to the flank with a ring of head tissue around it fails to develop (Swett, 1945). Supernumerary limbs are evoked when posterior peribrachial flank tissue is grafted next to the anterior edge of the limb disc (Slack, 1976). These supernumeraries are derived from the limb disc tissue, like the supernumeraries induced by grafting a ZPA to

the anterior edge of the chick limb bud. It is possible that in the urodele embryo, AP and DV polarities (i.e., boundaries) are set up in the pre-limb mesoderm by special organizing regions in the peribrachial tissue, and that once this is done, local cell interactions within the boundaries are used for patterning as the limb bud mesoderm grows out (see Bryant and Gardiner, 1992; Stocum, 1995).

Studies of the *shh* gene have not yet been reported for either the urodele limb bud or regeneration blastema; in fact, there is no molecular data at all on the developing limb buds of urodeles. It is probable that *shh* is expressed in urodeles, and information on the patterns of expression of this gene and its products in developing and regenerating urodele limbs, as well as the expression patterns of other genes associated with patterning, will be of great interest. Expression of the same genes would suggest an essential similarity in the molecular mechanisms of patterning in developing tetrapod limb buds and regenerating urodele limbs. It is entirely possible that what currently appear to be differences in patterning mechanisms based on cellular contributions to supernumeraries will be shown by molecular data to be variations on the same theme.

Conclusion

Clearly, we have much yet to learn about the mechanisms of patterning in the urodele limb regeneration blastema. Unresolved questions are (1) are the AP and PD axes of the regenerate patterned by the same mechanism; (2) does the wound epidermis have any role in patterning other than the promotion of outgrowth; (3) what are the competence factors produced by the wound epidermis that keep mesenchymal cells in the cell cycle; (4) what is the relationship between the cell cycle, epidermal competence factors and transverse positional discontinuity in blastema formation, outgrowth, and distalization of PD positional identity; (5) what are the spatial patterns of expression of *Hox* and *shh* genes in the urodele limb bud and regenerating limb, how are they affected by retinoic acid, and are they direct targets for RA; (6) what is the role of endogenous retinoic acid in regeneration; (7) what are the cell surface ligand-receptor systems that define positional identity and mediate intercellular signaling; and (8) do tetrapod limb buds and the urodele limb regeneration blastema use the same fundamental mechanisms of patterning, albeit it with variations on a theme? With regard to the latter question, it may turn out that patterning in both limb buds and regeneration blastemas is based on local cell interactions mediated by short-range signaling molecules, rather than on long-range diffusable morphogens. However, there may be differences between limb buds and regenerating limbs (or even between axes in regenerating limbs) in how boundaries are used to establish the perimeters of patterns which would account for the differences in contribution to the structure of experimentally evoked supernumerary limbs. In this connection, it would be valuable to compare the expression of the *shh*, *HoxA, C* and *D* genes, and other patterning genes in urodele pre-limb discs, limb buds, and regeneration blastemas, with expression in the pre-limb regions and limb buds of other vertebrates. Finally, we know very little about patterning mechanisms in the dorsoventral axis of the developing or regenerating vertebrate limb, particularly in terms of gene expression, a void that will need to be filled if we are to have a comprehensive understanding of these processes.

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