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Classic limb patterning models and the work of Dennis Summerbell

Jenna L. Galloway and Clifford J. Tabin*

Dennis Summerbell was a leading contributor to our understanding of limb patterning prior to the advent of molecular biology. He published several groundbreaking papers, including one that developed a key model for patterning the limb from the shoulder to the fingertips and another that presented the co-discovery of the effect of retinoids on limb morphogenesis. He brought detailed quantitative analyses to bear on these studies, as highlighted in two of his insightful papers published in the *Journal of Embryology and Experimental Morphology*, in which he provided elegant models that, today, remain relevant to limb patterning, as well as to many disciplines of developmental biology.

Introduction

Because the developing limb bud is easily accessible to manipulation and is not required for the survival of the embryo, it has long served as an ideal system in which to study patterning mechanisms. It begins as a relatively undifferentiated mass of cells that becomes sculpted into an adult limb, which contains asymmetries along the three axes: proximodistal (PD), anteroposterior (AP) and dorsoventral. When Dennis Summerbell's two papers were first published in the *Journal of Embryology and Experimental Morphology* (*JEEM*), separate signaling centers were known to be present in the limb and to influence each of these axes. In particular, the apical ectodermal ridge (AER) was known to be essential for proper PD outgrowth and the zone of polarizing activity (ZPA) had been identified as being important for AP patterning. Although the molecular identity of the signals that emanate from these centers was unknown at the time, based on the effects of experimental manipulations, Summerbell and others proposed elegant models that conceptualized limb-patterning ideas. These models shaped our thinking about developmental patterning, not just in the limb but throughout the embryo.

Limb development in the pre-molecular era

The AER is a thickened ectoderm that is located at the distal end of the limb (see Fig. 1). It overlies the early limb bud mesenchyme and is essential for the outgrowth of all three PD limb segments: the stylopod (humerus/upper arm), the zeugopod (radius and ulna/forearm) and the autopod (digits/hand). AER extirpation experiments, which were published by John Saunders in 1948 (Saunders, 1948), were the first to show that chick limbs in which the AER was removed at early stages completely lacked distal structures, whereas the removal of the AER at later stages resulted in limbs that had increasingly more intact distal structures, At this time, it had already been established that there was a progressive proximal-to-distal order of limb skeletal differentiation. It was also recognized that there was no change in the influence of the AER on PD axis specification over time, suggesting that the AER provided only a permissive signal that allowed for limb outgrowth. This conclusion was based on transplant studies in which older and younger AERs were transplanted onto differently aged chick limb mesenchyme, and in which no effect on PD patterning was observed (Rubin and Saunders, 1972).

Based on these findings, Summerbell, Julian Lewis and Lewis Wolpert proposed the progress zone model to explain patterning along the PD axis of the limb in a landmark paper in 1973 (Summerbell et al., 1973) (Fig. 1A). This model stated that the PD positional identity of a cell is intrinsically determined by the amount of time it spends in the progress zone, an area of mesenchymal cells approximately 300 μm thick that is located just beneath the AER. This model provided a twist on other types of limb patterning models that previously had been proposed by adding a new dimension, time, as having an influence on limb patterning. Central to this hypothesis was the idea that progress zone cells possess an autonomous clock that records the time they spend in the labile region. The authors rejected a morphogen-based model system because of experiments that had shown that grafts of limb bud pieces transplanted to hosts retained their presumptive fates.

By contrast, the idea of a morphogen was central to the way in which Summerbell and others conceptualized the establishment of the AP axis of the limb. Saunders and Mary Gasseling were the first to show that cells located at the posterior lateral edge of a chick limb bud had the unique ability to cause mirror-image duplications when transplanted to an anterior location (Saunders and Gasseling, 1968). Because of its potency in re-patterning the limb along the AP axis, this region was termed the zone of polarizing activity (ZPA). Based on this result, the idea that different cell fates could be specified at different concentration thresholds of a diffusible signal, or morphogen gradient, was first proposed by Lewis Wolpert in 1969 (Wolpert, 1969). In the context of the developing AP limb axis, Wolpert hypothesized that tissue closest to the ZPA would experience the highest levels of a morphogen and would develop into posterior structures (ulna, digit 4), whereas anterior tissues would receive lower levels of morphogen and would develop into anterior skeletal elements (radius, digit 2). This model was strongly supported by two experiments by Cheryll Tickle. The first experiment showed that a direct relationship exists between the number of ZPA cells that are grafted into a chick limb and the identity of the digit(s) induced by the graft (Tickle, 1981). The second showed that grafting ZPA tissue to different locations in the limb bud gave rise to digit patterns that were consistent with the transplanted ZPAs releasing a diffusible morphogen that was capable of re-patterning the host tissue (Tickle et al., 1975).

A potential candidate for the ZPA morphogen came out of studies of limb regeneration. Iqbal Niazi and Saroj Saxena first demonstrated that ectopically provided vitamin A (retinoic acid, RA) would result in the reorganization of the PD axis of regenerating amphibian limbs (Niazi and Saxena, 1978). Building on this work, both Cheryll Tickle and Dennis Summerbell independently showed that, in the context of developing chick limb buds, ectopic RA served to mimic the ZPA-induced mirror-image duplications (Summerbell and Harvey, 1983; Tickle et al., 1982).

Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.

*Author for correspondence (e-mail: tabin@genetics.med.harvard.edu)

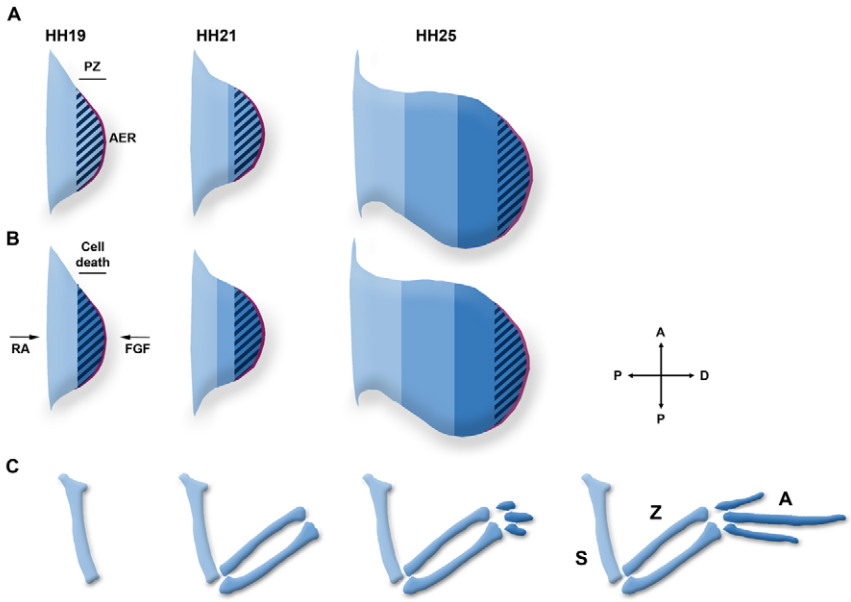

Fig. 1. Models of proximodistal limb patterning. (**A**) AER removal results in the loss of distal structures as explained by the progress zone model. According to Summerbell, AER removal serves as an assay for measuring the timing of specification of the progress zone cells. When the AER (purple) is removed at HH stage 19, the progress zone (diagonal lines) has just specified the most proximal segment, the stylopod (light blue). When it is removed at later stages, at HH21, it has specified the zeugopod (medium blue), and, at HH25, the distal autopod (dark blue). Summerbell notes that specification of the wrist elements requires a relatively long period of time spent in the progress zone (HH21-HH24) and suggests that this is due to the complexity of the structure. (**B**) More recently, studies have shown that the absence of distal structures following AER removal in chick is caused by cell death in the region that underlies the AER (diagonal lines) (Dudley et al., 2002). The two-signal model suggests that progenitors for proximal (light blue) and distal (dark blue) segments are specified by opposing RA and FGF signals (Capdevila et al., 1999; Mercader et al., 1999; Mercader et al., 2000; Sun et al., 2002; Mariani et al., 2008). The cell death and decreased proliferation that occurs following AER removal results in the loss of distal segments: the zeugopod and autopod at HH19, the autopod at HH21, and the distal phalanges of the autopod at HH25. The more proximal regions that have exited the progress zone remain intact, suggesting that AER removal could be used as an assay for the specification of cells just proximal to the progress zone. (**C**) The skeletal elements that form following AER removal at the stages indicated. AER, apical ectodermal ridge; AP, anteroposterior axis; A, autopod; FGF, fibroblast growth factor; HH, Hamburger Hamilton; PD, proximodistal axis; PZ, progress zone; RA, retinoic acid; S, stylopod; Z, zeugopod.

The removal of the AER and its effect on PD patterning, and the application of RA and its effect on AP patterning, were important results that provided assays for exploring and understanding limb patterning in the pre-molecular era of developmental biology. In the two extremely influential papers published by Summerbell in *JEEM*, each of these findings was readdressed on a quantitative level, providing new insights and, indeed, new ways of looking at these problems.

A quantitative analysis of AER extirpation

The progress zone model (see Fig. 1A) stated that PD identity is continuously changing in the distal limb bud and does not become specified until cells are displaced away from the distal domain. In particular, the positional identity of a cell is determined by the amount of time it spends in the progress zone, which lies beneath the AER. When the AER is removed, the cells of the progress zone cease to alter their PD fate, just as if they had exited the distal progress zone in an unaltered limb bud. Thus, distal-most fates are never specified following AER removal, resulting in the observed truncations in distal limb pattern.

To gain further insight into this phenomenon, Summerbell took a careful, quantitative approach to the AER removal experiments (Summerbell, 1974), in which he removed the AER from chick embryo right forelimb buds at Hamburger Hamilton (HH) stages 18-28. At 10 days of development, the embryos were harvested and stained with Alcian Green to visualize the cartilage elements. The length of each element (humerus, ulna, radius and digit III) was measured and compared between control and operated limbs. Similar to the previous studies, he found that AER removal at later stages resulted in truncations that were progressively more distal. Moreover, he found that elements could be partially lost: skeletal elements were normal on the proximal end, but truncated at the distal end. This led to the important suggestion that limb segments did not undergo regulative patterning (which would have led to the formation of a whole segment of smaller size). Thus, rather than being patterned segment-by-segment, the limb bud appeared to be patterned continuously along the PD axis. Perhaps the key insight in this paper is that, if the progress zone model is correct, the pattern that forms after AER removal directly reflects the extent of PD specification present in the distal limb at the time of AER extirpation (Fig. 1A,C). Thus, by comparing the pattern of the limb that results from the removal of the AER at different time points, one can assay the rate of change of a cell's positional value within the progress zone.

We now have an improved molecular understanding of the signals that emanate from the AER and of the cellular events that occur following its removal. Fibroblast growth factors (FGFs) are known to be the key signals generated by the AER that promote limb outgrowth (Fallon et al., 1994; Niswander et al., 1993). In addition, the AER FGFs are now known to be crucial for the survival of the limb progenitors located within the progress zone (Dudley et al., 2002; Rowe et al., 1982). This knowledge has brought about a reinterpretation of the AER extirpation experiments. If the resultant truncations were due to distal cells being frozen in an inappropriately proximal positional identity, they should contribute to proximal structures. Instead, as highlighted by cell labeling experiments, distal cells fail to be maintained in any skeletal elements following AER removal, owing to cell death (Dudley et al., 2002). Thus, the AER extirpation experiments do not give a reliable estimate of when PD segments are specified in the progress zone during limb development. Nonetheless, the logic presented by Summerbell (Summerbell, 1974) still holds, although the parameter being assessed is not the rate of change of positional value in the progress zone, but rather the process of differentiation at the proximal edge of the progress zone, where cells cease to require the AER for survival (Fig. 1B,C).

Interestingly, Summerbell considered but then discounted cell death. This is because an earlier study of cell death following AER removal had erroneously reported that the wave of cell loss extends from the distal tip to the base of the limb bud (Janners and Searls, 1971). Summerbell correctly reasoned that, were this to be true, and if its effect was indeed significant, cell death would result in the loss of proximal, as well as distal, structures (which was not observed). It was not until eight years after Summerbell published his analysis that it was realized that the cell death that follows AER removal is actually confined to the distal 200-300 μm of the limb bud (Rowe et al., 1982).

As AER extirpation causes distal cell death, the timing of PD specification in the distal limb at the time of AER removal is, therefore, not a valid test of the progress zone model. In the decades following Summerbell's work, other models have been proposed to explain limb PD patterning. Based on the analysis of cell death following AER removal and other experiments, it has been proposed that cell fates for all three segments, stylopod, zeugopod and autopod, are established within the early limb bud and are subsequently expanded before differentiation into particular skeletal elements (Dudley et al., 2002). However, the current lack of molecular expression data supporting either the early specification model or the progress zone model has forced a re-examination of both models (Tabin and Wolpert, 2007). Instead, molecular evidence suggests that PD specification may be based on a system that involves two opposing signals, which operate distally and proximally to coordinate gene expression along the PD axis. In particular, the distal signaling molecule is thought to be FGF, while the proximal signal has been suggested to be RA (Fig. 1B) (Capdevila et al., 1999; Mercader et al., 1999; Mercader et al., 2000). Studies in mice have supported this two-signal model, and have proposed that FGFs from the AER have dual functions: specifying the initial progenitor size of each segment, and maintaining and expanding proper progenitor cell numbers prior to condensation (Mariani, 2008; Sun et al., 2002). Integrating the dynamic changes in target gene expression that occur in response to these signals as the limb bud grows out with the proximal-to-distal wave of differentiation (Tabin and Wolpert, 2007) can in principle provide a context for understanding, in modern terms, the process of PD specification that was so elegantly analyzed by Summerbell in his 1974 *JEEM* paper.

The ZPA and the role of RA

At the time of Summerbell's 1983 paper, many in the field believed that a morphogen gradient produced by the ZPA specified positional information along the AP axis of the limb. The finding that RA could, like the ZPA, induce mirror-image duplications (Summerbell and Harvey, 1983; Tickle, 1983; Tickle et al., 1982) raised the intriguing possibility that a retinoid might, in fact, be the endogenous morphogen released by the ZPA. Summerbell undertook a quantitative analysis of this paradigm, by placing newspaper soaked in various concentrations of RA into slits cut into chick limb buds from HH stages 17-22. Summerbell discovered that the addition of RA at intermediate stages of chick development (HH19-HH20) gave mirror-image duplications similar to those caused by the ZPA grafts. He also found that the extent of mirror-image duplications was dependent upon the concentration of RA and the stage at which it was applied. This provided important additional evidence that AP patterning is laid down as a series of threshold responses. However, the introduction of very high doses of RA at early stages of limb development caused severe reductions in all skeletal elements. As such truncations are never seen in grafts that contain large numbers of ZPA cells when they are transplanted at early stages, Summerbell was led to re-evaluate the presumption that ectopically applied RA reflects the activity of an endogenous retinoid morphogen.

In his paper, Summerbell very thoughtfully describes the possible models that could explain all of his results, particularly the reduction in skeletal elements. He first considered RA to be the ZPA morphogen. For this to be the case, the reduction in digits that occurs in response to high doses of RA would result from an enhancement of the signal, such that the concentration of morphogen was too high to specify the most anterior digits and only the most posterior digits would form. However, this does not easily explain the complete loss of skeletal elements at some doses. Alternatively, Summerbell proposed that RA might have dual functions in instructing patterning, as well as in causing cell death. Still, this explanation seemed unsatisfactory as Summerbell found it difficult to generate dose-response curves that incorporated both duplication and reduction phenotypes. Ultimately, Summerbell proposed that the phenotypical effect of RA could be more fully explained in terms of RA being an ectopic agent that acts on an endogenous patterning system, which he put in the context of a reaction-diffusion model for generating a concentration gradient of a morphogen in the limb bud.

The general reaction-diffusion model was proposed by Alan Turing in 1952 to describe how patterns could form from two interacting substances with different diffusion rates (Turing, 1952). Alfred Gierer and Hans Meinhardt added to this model by demonstrating that an important aspect of pattern formation is self-activation and long-range inhibition (Gierer and Meinhardt, 1972). In this model, an activator is produced that diffuses slowly promoting its own production and that of a more rapidly diffusing inhibitor. In effect, the concentration ratio of activator to inhibitor near the source is higher than the ratio far from the source. Based on this model, Summerbell proposed that rather than being the ZPA morphogen, RA alters the activator to inhibitor ratio, thereby causing the activator to be released from inhibition. At low to moderate RA concentrations, a stable anterior peak of activator forms, resulting in mirror-image duplications of the skeletal elements. This model also explains the loss of structures that is observed when high RA concentrations are applied to the limb as resulting from there being an increased amount of activator across the whole of the limb field. Anterior skeletal elements that are normally specified at lower activator concentrations are progressively lost at increasing doses of RA.

Summerbell was correct in interpreting the effect of RA as a pharmacological influence on an unrelated endogenous morphogen released by the ZPA. We now know that morphogen to be sonic hedegehog (SHH) (Riddle et al., 1993). In causing limb duplications, RA does not exactly mimic the ZPA, but instead acts to convert anterior cells into ZPA cells, causing SHH to be expressed in the anterior limb bud (Fig. 2A) (Noji et al., 1991; Riddle et al., 1993; Wanek et al., 1991).

Although the spatial gradient of SHH activity across the limb bud is not established by a true reaction-diffusion mechanism, many elements incorporated into Summerbell's model have held true. Intrinsic to the reaction-diffusion mechanism is an activator that positively influences its own activity and that also induces the formation of an inhibitor. Indeed, through an FGF-feedback loop, SHH indirectly promotes its own expression (Fig. 2B) (Laufer et al., 1994; Niswander et al., 1994), and it also activates the expression of inhibitors, such as patched (Chen and Struhl, 1998; Goodrich et al., 1996; Marigo et al., 1996) and hedgehog-interacting protein (HIP) that act to limit SHH activity (Chuang and McMahon, 1999). Currently, SHH is believed to control digit identity by acting as a

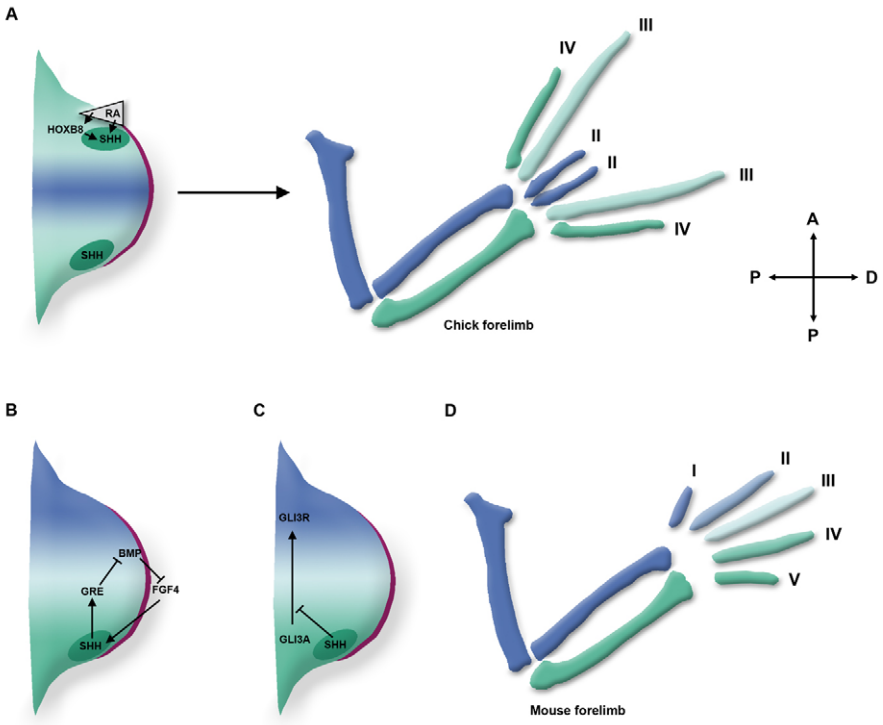

Fig. 2. Anteroposterior patterning in the limb bud. Our current understanding of the ZPA signal SHH. (**A**) Summerbell placed newspaper soaked in RA in the anterior region of an early stage limb bud. We now know that RA can induce in the forelimb the expression of genes that are not induced by SHH (green) or ZPA grafts, such as HOXB8 (Lu et al., 1997; Stratford et al., 1997). HOXB8 is sufficient to induce SHH expression in the anterior limb bud, but is not required for endogenous SHH expression (Charite et al., 1994; van den Akker et al., 1999). The application of RA to the anterior limb bud results in the induction of an anterior ZPA and mirror image duplications. In chick, the digits are labeled II-IV in the anterior to posterior direction. (**B**) The SHH-FGF feedback loop serves to maintain SHH signaling: SHH induces the expression of Gremlin (GRE) in non-SHH-expressing cells and GRE antagonizes BMP signaling, allowing maintained FGF signaling in the posterior AER, which then maintains SHH expression (Zuniga et al., 1999). (**C**) SHH signaling represses the processing of full-length GLI3 into a shorter repressor fragment (GLI3R, blue), resulting in a gradient of GLI3A (GLI3 activator) to GLI3R across the AP axis of the limb (Litingtung et al., 2002; te Welscher et al., 2002). The levels of GLI3R possibly coordinate digit identity across the AP axis. (**D**) The mouse limb has the digits labeled (I-V) from anterior to posterior. Based, in part, on fate mapping of SHH-descendents and SHH-responsive cells (Ahn and Joyner, 2004; Harfe et al., 2004), a model of digit patterning has emerged: digit I is SHH independent; digit II depends on SHH concentration; digit III depends on SHH concentration and the duration of SHH exposure; and digit IV and V depend on the duration of high levels of SHH signaling. AP, anteroposterior axis; BMP, bone morphogenic protein; FGF, fibroblast growth factor; PD, proximodistal axis; RA, retinoic acid; SHH, sonic hedgehog.

morphogen both spatially and temporally. From studies in mice that have examined the fate of SHH descendants and SHH-responsive cells, and from experiments in chick in which the time period over which the limb bud is exposed to SHH has been altered, it is thought that the digits are differentially patterned by the length of time and the concentration of SHH (Fig. 2D) (Scherz, 2007; Ahn and Joyner, 2004; Harfe et al., 2004).

The interplay between activators and repressors in establishing the AP limb axis also comes into play at the level of the downstream transcription factor GLI3, which is modulated in response to SHH signaling. Full-length GLI3 is cleaved into a transcriptional repressor (GLI3R) in the absence of SHH signaling. This cleavage is prevented after SHH signaling occurs, and GLI3 is maintained as a transcriptional activator (Wang et al., 2000). Based on mouse knockout data, it is thought that SHH signaling in the limb alters the activator-to-repressor ratio of GLI3, which ultimately determines digit number and identity (Fig. 2C) (Litingtung et al., 2002; te Welscher et al., 2002). Although Summerbell could not have known these molecular details at the time, his quantitative analysis provided profound insights into the mechanisms patterning the developing limb.

Conclusions

The two papers by Dennis Summerbell that we have discussed in this essay illustrate how early conceptual models of patterning events can shape our thinking about developmental processes for decades. Although the progress zone model is still contested today in the limb field, its implications – that time can be an important factor in patterning mechanisms – has been broadly felt. The concept of a cell-autonomous clock has been validated in other processes, such as somitogenesis (Hirata et al., 2002; Jouve et al., 2000; Palmeirim et al., 1997). Moreover, the reaction-diffusion mechanism that Summerbell applied to AP patterning in the limb can be found in other developmental contexts, such as in the initial stages of left-right body axis determination and in skin pattern formation (Asai et al., 1999; Jung et al., 1998; Nakamura et al., 2006). The fact that Summerbell pushed beyond the initial conclusion that RA is the ZPA morphogen and drew upon a model that, to him, better satisfied all the data is a testament to his commitment towards gaining a fuller understanding of limb patterning. Ultimately, what Summerbell's work truly exemplifies is how a careful and thorough approach to generating, interpreting and modeling the data can have a profound impact on our understanding of developmental patterning mechanisms.

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