

The Early History of the Polarizing Region: from Classical Embryology to Molecular Biology

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ABSTRACT The polarizing region of the developing limb bud is one of the best known examples of a cell-cell signalling centre that mediates patterning in vertebrate embryos. This article traces some highlights in the history of the polarizing region from its discovery by John Saunders and early work that defined polarizing activity through a period in which modelling was pre-eminent, right up to the discovery of defined molecules with polarizing activity. There is a particular focus on the discovery that retinoic acid could mimic signalling of the polarizing activity and this finding is then set in the context of more recent work which implicates Shh and BMPs in mediating polarizing activity.

KEY WORDS: *chick embryo, limb development, Sonic hedgehog, Bone Morphogenetic Proteins, polarizing region*

The Discovery of the Polarizing Region and its Embryology

Saunders and Gasseling discovered the zone of polarizing activity (ZPA) or polarizing region in the late 60's. This region is central to limb patterning; it has also become one of the best understood examples of an "organizer" that mediates cell-cell interactions in vertebrate embryos. Saunders and his lab apparently came across the polarizing region while they were exploring how cell death was programmed in the developing chick wing bud (see for example (Saunders and Fallon, 1966)). When they transplanted the posterior necrotic zone (a zone at the posterior margin of the wing bud where programmed cell death occurs) to the anterior margin of another wing bud (Fig. 1A), this produced a remarkable and unexpected effect (Saunders and Gasseling, 1968). The normal chick wing has three digits, reading from anterior to posterior, digits 2, 3 and 4 (Fig. 1B). Following a graft of posterior wing bud margin, up to 6 digits developed in the wing giving the mirror-image symmetrical pattern 432234 (Fig. 1C), with the additional set of digits coming mainly from the host. When the polarizing region was placed at the apex of the bud, again digits were induced in anterior tissue, but this time the pattern was 234. (With apical polarizing region grafts, symmetrical digit patterns such 4334 also develop posterior to the graft). Because the pattern of digits is always polarized with respect to the graft -the posterior digit (digit 4) forming nearest and the anterior (digit 2) farthest away- Saunders called this posterior region of the wing bud, the zone of polarizing activity or polarizing region. In the last year or so,

we have suggested that the function of cell death in the posterior necrotic zone is to control the number of signalling cells in the polarizing region (Sanz-Ezquerro and Tickle, 2000).

One of the first jobs was to define the extent of the polarizing region at different stages during chick wing development. This had to be done by grafting experiments because cells of the polarizing region cannot be distinguished morphologically from other cells in the limb bud. Different regions of chick wing buds were systematically cut out and grafted to the anterior margin of other wing buds to test whether they could induce additional digits. From the results of these experiments, Saunders and his lab drew up "maps" of polarizing activity (MacCabe, *et al.*, 1973). These maps showed that highest polarizing activity is confined to wing bud posterior margin and remains near the tip as the bud grows out. The strong activity in wing buds persists until the hand plate is forming. Another detailed series of maps were made (Honig and Summerbell, 1985) and polarizing activity was detected both earlier and later in wing development. Polarizing activity has now been mapped extensively in pre-bud stages but, at these stages, it is the potential to produce a polarizing signal that is being monitored (Hornbruch and Wolpert, 1991; Tanaka *et al.*, 2000).

In the most commonly used polarizing assay, grafts are made to the anterior margin of wing buds, around stage 20 (Fig. 1A), and

Abbreviations used in this paper: BMP, Bone Morphogenetic protein; dpp, decapentaplegic; Hh, hedgehog; Ihh, Indian hedgehog; Shh, Sonic hedgehog; ZPA, zone of polarizing activity.

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duplications of the digits scored. When grafts were made to wing buds at earlier stages in development, duplications of the fore-arm can clearly be recognised. For example, sometimes three skeletal elements develop generating the pattern- ulna, radius, ulna- (Summerbell, 1974) but two separate humeri could only rarely be induced to form in the same limb (Wolpert and Hornbruch, 1987). Polarizing regions grafted to limb buds at later stages e.g. stage 24, produced digit branching rather than complete extra digits (Summerbell, 1974). Thus duplicated structures can be induced distal, but not proximal, to the elbow and polarizing regions grafted at later stages induce duplications starting at more distal levels than those grafted at earlier stages. This progressive restriction of duplication more distally during limb bud outgrowth fits with the proximal to distal sequence in which structures are laid down from an undifferentiated zone of cells beneath the apical ectodermal ridge. (The apical ectodermal ridge is the thickened rim of epithelium at the tip of the limb bud and maintains the region of undifferentiated cells, known as the progress zone (Summerbell *et al.*, 1973)). In order to obtain duplications, the polarizing region must be grafted in contact with the apical ectodermal ridge. The apical

ridge is much thicker over the posterior part of the bud than over the anterior part of the bud and it had been postulated for a long time that posterior mesenchyme produced an apical ridge maintenance factor (Zwilling, 1955). An important question was whether this factor is the same as polarizing activity.

Models for Polarizing Region Signalling

How does the polarizing region produce these spectacular mirror-image digit duplications? Lewis Wolpert (Wolpert, 1969) suggested that a model based on a long range signalling system could provide an explanation. According to his model, polarizing activity is the production of a diffusible morphogen that sets up a concentration gradient across the limb bud. Cells at different positions in the limb bud respond according to the local morphogen concentration to which they are exposed; cells near the source of polarizing activity will be exposed to high concentrations of morphogen and form posterior digits, while cells farther away will be exposed to lower concentrations of morphogen and form anterior digits. The results of a substantial series of experiments in which polarizing region grafts were systematically placed in different positions along the antero-posterior axis of the limb bud supported the model (Tickle *et al.*, 1975).

About the same time, another influential model, known as the clock face model, was formulated to explain regeneration and production of supernumerary structures in a number of different systems-cockroach legs, amphibian limbs, insect imaginal discs (French *et al.*, 1976). According to this model, when cells from different positions are opposed, this produces a discontinuity in positional values; this in turn, then leads to intercalation of the missing positional values by the shortest route. Grafting a polarizing region to the anterior margin of another bud opposes cells from different antero-posterior positions. Therefore, it was suggested that intercalation might explain the digit duplications produced by polarizing region grafts (see for example, Iten and Murphy, 1980).

The two competing models for the mechanism of action of the polarizing region generated a great deal of controversy -and discussion! An essential difference between an intercalation model and a morphogen gradient model is that local cell-cell interactions

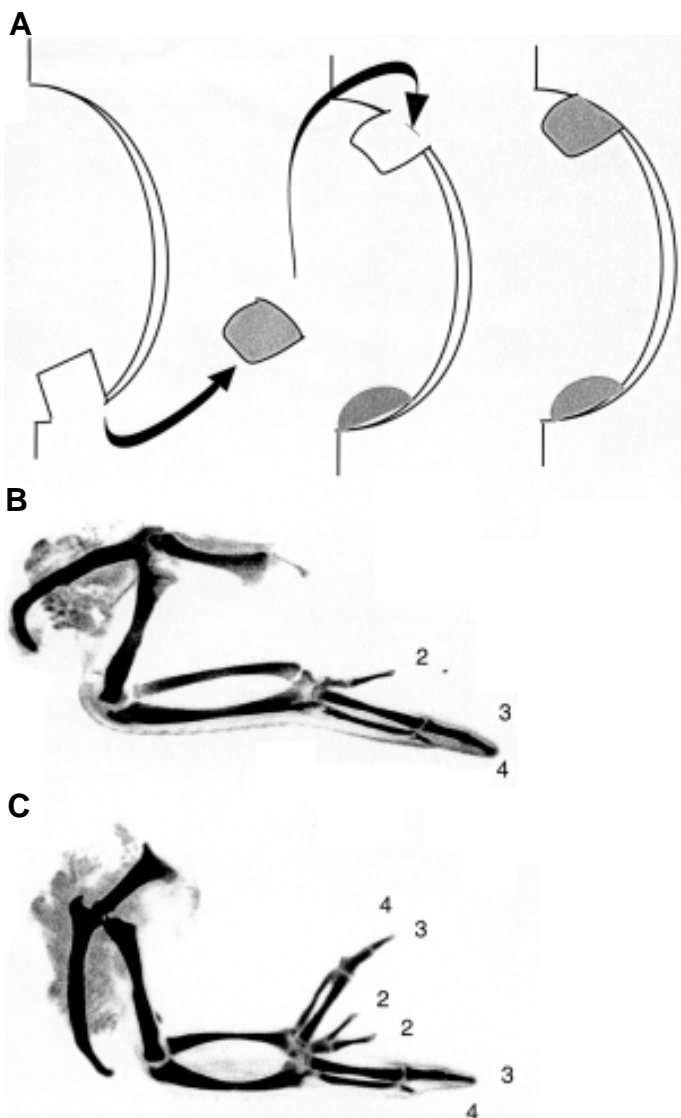


Fig. 1. The discovery of polarizing activity and the result of polarizing region grafts. (A) Diagram illustrating how polarizing activity was discovered. Cells from the posterior margin (stippled) of one wing bud were cut out and grafted to the anterior margin of a second limb bud. The wing buds are at stage 20 (Hamilton-Hamburger stages; around 3 days of incubation). The double line rimming the tip of the limb bud represents the apical ectodermal ridge and the graft is placed at the anterior edge of the ridge. In this diagram, anterior mesenchyme was cut out to make room for the graft; alternatively, grafts of tissue and/or beads soaked in retinoic acid or Sonic Hedgehog protein can be placed under a loop made by separating and lifting the anterior apical ectodermal ridge away from anterior mesenchyme. The posterior margin of the host limb is stippled to indicate polarizing activity in this location. Grafting of tissue and cells or application of factors on beads and other inert carriers to the anterior margin of a chick wing bud as shown here forms the basis of the assay for polarizing activity. (B,C) Whole mounts to show skeletal patterns of normal and manipulated chick wings. (B) The normal wing has three digits, 2 3 4 running anterior to posterior. (C) Following a polarizing region graft at the anterior margin, six digits develop in a mirror-image symmetrical pattern 432234. This wing is from a 10 day old chick embryo; the operation was carried out when the embryo was around 3 days old.

are involved in intercalation rather than long range signalling. A critical experiment that helped to distinguish between the two models involved grafting two additional polarizing regions, one to the anterior, and the other to the apex, of a host wing bud. This resulted in the development of digit patterns such as 4334 from anterior tissue between the two polarizing region grafts. This fits with the gradient model prediction that no anterior digits should form because the grafts are too close together; according to the intercalation model, a complete duplication should be obtained (Wolpert and Hornbruch, 1981). A more direct demonstration that polarizing region signalling is long range was obtained by placing a piece of anterior leg tissue between a polarizing region graft and responding anterior wing bud mesenchyme (Honig, 1981). Even with this arrangement, not only additional leg digits developed, but also extra wing digits. Thus the polarizing region must act at a distance. This, of course, need not necessarily be via a long range morphogen; there could be some sort of relay system.

A special feature of the gradient model is that polarizing region signalling should be dose-dependent. Indeed when a polarizing region graft was irradiated before grafting, signalling was attenuated (Smith, *et al.*, 1978). A more direct demonstration of dose-dependency was the finding that the extent of duplication depends on the number of polarizing region cells in the graft. When the polarizing region cells were diluted by anterior cells or, when small numbers of polarizing region cells plated on tiny pieces of plastic were implanted at the anterior margin of the limb bud, the extent of duplication was reduced and, for example, only an additional digit 2 formed instead of the full duplicate set of digits (Tickle, 1981).

A reduction in the duration of the polarizing signal was found to have the same effect as reducing signal strength (Smith, 1980). When a polarizing region graft was left in place for 14 hours, and then removed, it was found that an additional digit 2 formed, whereas when the graft was left in for longer, more complete duplications were obtained. This sequential specification of digits with time can be incorporated into the gradient model by assuming that there is progressive spread of a long range signal from the polarising region (Tickle, 1995). According to this idea, the final identity of a digit will be reached in a stepwise fashion, starting with an "anterior" identity that is then promoted towards a more "posterior" identity.

It was found early on that limb buds of other vertebrates had polarizing activity –the posterior margins of the limb buds of mouse, guinea pig, even human embryos were tested for polarizing activity by grafting the cells to the anterior margin of chick wing buds (see for example, Fallon and Crosby, 1977). In all cases, additional chick wing digits were produced. This showed that the same signals were produced in different vertebrate limb buds but the interpretation depended on the responding cells. More puzzling, however were the findings that other parts of the embryo had polarizing activity and, at the time, it was not clear what this meant.

Retinoic Acid - some Personal Notes

Retinoic acid was the first defined molecule to be found that could mimic the effects of the polarizing region (Tickle *et al.*, 1982). We had been interested for a long time in the idea that gap junctions might be involved in limb patterning and Lewis heard from John Pitts in Glasgow that retinoic acid, a vitamin A derivative, had

dramatic effects on cell-cell communication (Pitts *et al.*, 1981). At the time, Juliet Lee had just joined the lab for the summer and applying retinoic acid to chick wing buds seemed like a good project. Juliet was an undergraduate student who wanted to gain lab experience before starting her final year at Queen Elizabeth College in London. The digit duplications produced by retinoic acid were completely unexpected.

In the initial set of experiments, we used small pieces of paper to apply retinoic acid to wing buds. This approach was initiated by Bruce Alberts who spent a sabbatical at The Middlesex Hospital Medical School in 1975- the aim was to identify the polarizing signal(s)! We made extracts of polarizing region cells, soaked them up on small beads /and or pieces of paper and then grafted the impregnated beads/paper to the anterior margin of a chick wing bud. We also tried various molecules that we thought might be candidates for polarising activity; for example we pored through a book which catalogues the effects of numerous agents on chick embryos (Romanoff, 1972). Unfortunately none of these experiments were successful; all the wings were depressingly normal. We began to wonder whether the polarizing signal was even extracellular; perhaps it was passed from cell to cell via gap junctions. It was this thinking that ultimately lead us to retinoic acid. Once we had a defined compound with polarizing activity, we went back to the beads with Gregor Eichele, who had just started as a postdoc with Bruce. Juliet had joined me to study for a PhD. We identified those beads that were the best controlled release carriers for retinoic acid (Tickle, *et al.*, 1985). When we used a stable retinoic acid derivative and appropriate beads, we were able to obtain amazingly reproducible results.

Our detailed analysis showed that retinoic acid acted in a dose-dependent fashion. Moreover, it could diffuse into the limb bud from the beads and form a gradient (Tickle, *et al.*, 1985). All these were features consistent with the predictions of the gradient model. We were very aware, however, that this did not prove that retinoic acid was the morphogen. It could still be that only the highest concentration of retinoic acid close to the bead was important and we tried to resolve this by experiments in which we compared the effectiveness of retinoic acid as a gradient or distributed evenly across the bud (Eichele, *et al.*, 1985).

Towards the end of the 80's, new findings emerged that prompted the Nature News and Views "We have a morphogen" (Slack, 1987). Gregor and Christina Thaller showed in an heroic experiment (they had to dissect over 5,000 limb buds!) that there is endogenous retinoic acid in chick limb buds and furthermore that the posterior

TABLE 1

FEATURES OF THE POLARIZING REGION

Induces mirror-image duplications when grafted to anterior margin of host limb bud (additional digits arise mainly from host not graft)
Activity is dose-dependent (attenuate the polarizing region before grafting or graft fewer cells, anterior digits are specified but posterior digits are not)
Specifies additional digits sequentially, proceeding from anterior to posterior
Prevent host limb bud from widening after a polarizing graft or move grafted polarizing region closer to host polarizing region, anterior digits are lost before more posterior digits
Promotes anterior digits to posterior digits but not posterior digits to anterior digits
Digit order is always maintained in duplicated limbs
Found in limb buds of other vertebrates including humans
Other tissues have polarizing activity, such as neural tube, node etc.

part of the early limb bud where the polarizing region is located contains substantially more retinoic acid per cell than the anterior part (Thaller and Eichele, 1987). In addition, around this time, Chambon and Evans independently discovered retinoic acid receptors that provide the biochemical mechanism for the cellular response to retinoic acid (Petkovich *et al.*, 1987; Giguere *et al.*, 1987).

A few years later, there was another News and Views in Nature; this time the title was "We may not have a morphogen" (Brookes, 1987). Two groups, one led by Bryant and the other by Ide and Noji in Japan showed that retinoic acid caused digit duplications by inducing anterior wing tissue to form a new polarizing region (Wanek *et al.*, 1991; Noji *et al.*, 1991). But if retinoic acid was not the morphogen, what was the basis for polarizing activity?

The next candidate emerged a few years later. This was due to the growing realisation that the molecular basis of development is similar in vertebrates and flies. This led to a breakthrough when the "Hedgehog group" – Phil Ingham, Andy MacMahon and Cliff Tabin – discovered vertebrate *hedgehog* genes related to the *Drosophila hedgehog* gene, which encodes a signalling molecule. One of these, *Sonic Hedgehog (Shh)*, was found to be expressed in the polarizing region of the chick limb bud (Riddle *et al.*, 1993). The patterns of *Shh* expression matched almost exactly the maps of polarizing activity defined earlier by grafting experiments (Honig and Summerbell, 1985). Application of retinoic acid to the anterior margin of the wing bud induced *Shh* expression and furthermore, when *Shh* was misexpressed here, this led to mirror-image duplications (Riddle, *et al.*, 1993). At about the same time, Brigid Hogan's lab showed that Bone Morphogenetic Proteins (BMPs) are expressed in the mouse limb buds (Lyons *et al.*, 1990). In the chick limb, we showed that the polarizing region expresses *Bmp2* and that *Bmp2* expression can be induced in anterior cells by retinoic acid. However, when BMPs were placed anteriorly, they did not lead to digit duplications (Francis *et al.*, 1994). It later emerged that application of Shh protein on beads to the anterior margin of chick wing buds induces expression of *Bmp2* (Yang *et al.*, 1997). Interestingly, this mirrors a cell-cell signalling cascade involved in *Drosophila* early wing patterning in which Hh induces expression of *dpp*, which encodes a *Drosophila* signalling molecule closely related to the *Bmp2* and *Bmp4* (Nellen *et al.*, 1996).

TABLE 2

IMPORTANT LAND MARKS IN THE HISTORY OF THE POLARIZING REGION*

1968	Discovered by Saunders and Gasseling in chick limb buds
1969	Gradient model proposed for signalling by polarizing region
1975	Experimental evidence supporting a gradient model
1976	Polarizing region discovered in the limb buds of other vertebrates
1976	Clock face model proposed for limb regeneration –later applied to limb development
1981	Polarizing signalling shown to be dose-dependent in terms of cell number
1982	Retinoic acid is first defined chemical found to mimic the polarizing region
1987	Endogenous retinoids demonstrated in chick limbs
1991	Retinoic acid shown to induce a new polarizing region
1993	Sonic hedgehog emerges as the basis for polarizing activity
1997	Sonic hedgehog shown to specify additional digits in dose-dependent fashion
1999	Gremlin shown to be induced downstream of Shh and act as the apical ridge maintenance factor
2000-2001	Interactions between Shh and Bmps suggested to mediate digit patterning
2001	Long range diffusion of Shh demonstrated in limb buds

* These are only to date (Litingtung *et al.* 2002). It seems certain that other landmarks are still to come!

Molecular Biology of the Polarizing Region

It is nearly ten years since the discovery of Shh but it is fair to say that its role in digit patterning and its mechanisms of action are still not fully understood. It is generally agreed that Shh signalling is indeed synonymous with polarizing activity at least with respect to digit pattern. Thus only Shh protein or retinoic acid that can induce *Shh* have polarizing activity ie lead to mirror-image digit duplications when applied locally at the anterior margin of a chick wing bud; likewise, to date, all tissues with polarizing activity, either express *Shh* or have the potential to express (or induce expression of) *Shh* (or other hedgehog's eg *Indian hedgehog*, *lhh*) when implanted at the anterior margin. Furthermore in nearly all polydactylous mutants examined, ectopic *Shh* (or *lhh*) expression at the anterior margin can be detected.

Even though Shh signalling is the basis of polarizing activity in the limb, this does not necessarily mean that Shh protein itself fulfills all downstream functions of polarizing activity. Indeed apical ridge maintenance factor appears to be Gremlin, a BMP antagonist (Zuniga *et al.*, 1999; Capdevila *et al.*, 1999). *Gremlin* expression is induced in response to Shh signalling (via a number of steps including both *Formin* and *Bmps*); Gremlin then antagonizes BMP signalling in the apical ridge which results in maintenance of expression of *Fgf4* in posterior ridge (and probably also of the other *Fgfs* that are expressed in the same region of the ridge). The FGFs produced in the posterior part of the ridge in turn maintain *Shh* expression in the polarizing region. This explains why the polarizing region has to be grafted in contact with the apical ridge in order to induce digit duplications. Furthermore, the fact that this series of signalling interactions between mesenchyme and epithelium depends on Shh signalling explains why in the limb buds of *Shh* -/- mouse embryos, outgrowth is severely compromised (Chiang *et al.*, 1996).

But is Shh the polarizing region morphogen? A longstanding problem with this idea was whether Shh could indeed act long range in the limb bud. In just the last year, it has been directly demonstrated that Shh can diffuse across the limb bud (Zeng *et al.*, 2001; Gritti-Linde *et al.*, 2001). It is also striking that two genes that are early immediate genes expressed in response to vertebrate Hedgehog signalling, *Patched* and *Hedgehog Interacting Protein*, encode molecules that bind vertebrate Hedgehog proteins and could serve to limit rather precisely the range of Shh signalling in the limb bud. We have suggested that this long range action of Shh is concerned with specifying the width of the limb bud and thus number of digits ((Drossopoulou *et al.*, 2000). This would explain why in *Shh* -/- mutant limb buds, the handplate collapses and structures distal to the elbow are represented by a very reduced series of cartilage rudiments (Chiang *et al.*, 2001; Kraus *et al.*, 2001).

An attractive possibility is that it is the BMP signalling that is regulated by Shh signalling, specifically BMP2, that conveys positional information about digit identity and leads to progressive promotion of digit identity ((Drossopoulou *et al.*, 2000) or that some combination of Shh and BMP signals are involved (Lewis *et al.*, 2001). This would fit with findings in other systems such as teeth, in which BMPs also appear to be responsible for specifying tooth type (Tucker *et al.*, 1998).

Coming full circle back to retinoic acid, recent work has indeed confirmed that retinoic acid signalling does play a role in normal development of the limb bud. Not only is retinoic acid required for

establishing the polarizing region, *Meis*, which is a gene regulated by retinoic acid, has also been shown to be responsible for patterning the proximal part of the limb (Mercader *et al.*, 1999; Capdevila *et al.*, 1999). Skeletal development in this part of the limb could again be achieved via BMP2 signalling downstream of retinoic acid signalling. Indeed in *Shh*^{-/-} mutant limb buds lingering BMP signalling initiated by retinoic acid might be sufficient to allow specification of distal anterior limb structures although these could never be promoted to form posterior structures.

Finally it should be borne in mind that there is emerging evidence that antero-posterior patterning in the limb bud is not only mediated by signals emanating from the polarizing region at the posterior margin of the limb bud but that anteriorly produced signals play a role. BMP4, for example, produced anteriorly may negatively regulate limb bud width and be antagonised by Shh (Pizette and Niswander, 1999); Tumpel *et al.* 2002). Indeed, this antagonism between Bmp and Shh signalling is reminiscent of dorso-ventral patterning of the neural tube. Furthermore the fact that anterior limb cells are not entirely passive gives an additional twist to understanding how digit duplications are induced.

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