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Signals in limb development: STOP, GO, STAY and POSITION

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Summary

Cell-to-cell interactions in early limb development are considered within the framework of the extracellular signals STOP, GO, STAY and POSITION, a classification which emphasises that the signals are elective rather than instructive, and that complexity arises from cells' response. Patterning in the limb is analysed in terms of signals that specify positional values along the anteroposterior axes, and retinoic acid is thought to be a positional morphogen. There is however, evidence for patterning which does not depend on a positional signal. In the early bud the mesenchyme gives POSITION signals to the apical ridge, which in turn provides a STAY signal to the mesenchyme in the progress zone. Non-ridge ectoderm produces a STOP signal with respect to cartilage differentiation. The pattern of cartilage differentiation is specified well before cartilage condensation. Growth factors affect both cartilage and muscle differentiation in culture. Pigment patterns result from feather germs providing STOP or GO signals to the melanoblasts which enter all feather germs. The pathways for the cell-to-cell signals are not known but may involve gap junctions.

Introduction

Limb development is both important in its own right and as a model system for studying pattern formation in development. Early development of the limb involves outgrowth of a bud from the flank, the loose mesenchyme of the bud giving rise to all the connective tissues of the limb except muscle. Muscle has a different origin, presumptive muscle cells migrating from the somites into the limb at a very early stage. Outgrowth of the limb bud is dependent on a distal thickening in the ectoderm, the apical ectodermal ridge. Beneath this ridge lies the progress zone containing mutliplying cells; as the cells leave the progress zone they begin to differentiate into cartilage and other connective tissues. The forelimb elements are laid down in a proximo-distal sequence – first the humerus, then the radius and ulna, and finally the wrist followed by the digits.

A number of cell-to-cell interactions have been identified in limb development and it may be instructive to consider them in terms of the effect they have on the cells. The extracellular signals controlling development may be classified in terms of their effect on cell development: STOP, GO, STAY and POSITION. This classification emphasizes that the factors operating on cells have at any one time a limited capacity for changing cell behaviour, and that the complexity of development lies within the cell and not in intercellular signalling. The complex tranducing mechanisms and second messenger systems offer further support for

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this view. For a factor to be classified in terms of one of these activities, its concentration must change either spatially or temporally and be correlated with the observed effect. Thus a distinction is drawn between the role of those factors which are necessary for development but which do not change and those which do. For example, if TGF-β is present throughout limb development at a constant concentration then it does not qualify as a control factor even if *in vitro* it stimulates chondrogenesis in a dose-dependent manner.

The four kinds of signal are defined with respect to their effect on cell development. However, it may not always be easy to assign unequivocally a signal to one of them. STOP is effectively an inhibitory signal and may be linked to a GO signal, inhibiting a cell proceeding along one pathway and directing it along another. GO signals may direct a cell along a maturation pathway or along one of several pathways and so it might best be represented by GO (x) where x represents the different pathways. At any one time the number of pathways, x, open to the cell is quite small, often being only two. STAY signals maintain the cells in their particular programme and the removal of such a signal results in the cell changing programmes. POSITION signals assign cells a positional value and this helps to distinguish them from GO signals.

Patterning

We have proposed a model to account for the spatial pattern of cell differentiation during limb development that is based on the concept of interpretation of positional information (Wolpert, 1981). Positional value, a cell parameter that is related to the cell's position, is thought to be specified in the progress zone. For the antero-posterior axis, position may be specified by a signal from the polarizing region at the posterior margin of the limb. When an additional polarizing region is grafted to the anterior margin of the limb bud a mirror image limb develops; the pattern of digits, for example, is now 432 234 compared to the 234 of a normal limb. From such experiments it has been concluded that the polarizing region provides a POSITION signal to the cells in the progress zone (Table 1,1). The signal from the polarizing region can be mimicked by a localized source of retinoic acid (Tickle *et al.* 1985) and retinoic acid is present in the bud with a higher concentration in the posterior half (Thaller and Eichele, 1987). Retinoic acid can alter positional values and is thus a very strong candidate for being the positional signal, but further evidence is required to establish that the cells respond to retinoic acid and not some other signal evoked by local application of retinoic acid. Even so the presence of both nuclear retinoic acid receptor (Dolle *et al.* 1989) and cytoplasmic binding protein (Maden *et al.* 1989) in the progress zone is very encouraging.

There are quite good reasons to think that these signals, other than those from the polarizing region, are involved in patterning along the anteroposterior axis. The only telling experiments involve disaggregation of the mesodermal cells, followed by reaggregation and placement of the aggregate in an ectodermal

Table 1. *Cell-to-cell interactions in early limb development*

Source	Target	Signal
1. Polarizing region	Mesenchyme in progress zone	POSITION
2. Mesenchyme progress zone	Mesenchyme progress zone	STOP/GO
3. Mesenchyme	Apical ridge	STAY/POSITION
4. Apical ectoderm	Mesenchyme in progress zone	STAY
5. Cartilage	Cartilage	GO
6. Ectoderm	Mesenchyme	STOP
7. Muscle connective tissue	Presumptive muscle cells	GO
8. Feather follicle	Melanoblast	GO/STOP
9. Mesenchyme	Ectoderm	POSITION
10. Feather germ	Feather germ	STOP

jacket. Without a discrete polarizing region, moderately good digits form (e.g., see Patou, 1973), and this suggests that another mechanism, such as one generating an isomorphic prepattern, is involved. Such mechanisms have, in fact, been put forward (see Wolpert and Stein, 1984).

The two types of mechanism make rather specific predictions with respect to the relationship between the number of elements along the anteroposterior axis and the width of the limb bud. We have tested these predictions with respect to the development of the humerus and found that some of the results do not conform with the predictions from a positional signal and suggest, rather, that a prepattern mechanism may be involved (Wolpert and Hornbruch, 1987). The nature of the prepattern mechanism is unknown but would involve interactions between mesenchyme cells. If this were based on a reaction diffusion mechanism (Murray, 1989) then it is not unreasonable to think of both inhibitory STOP signals and positive GO signals (Table 1,2).

The early bud

One of the earliest interactions in limb development is that involving the specification and maintenance of the apical ridge. The ridge is maintained by a signal from the underlying mesenchyme which provides a STAY signal. The polarizing region also plays a role in this process since both the length of the ridge and its position on the bud is specified by the polarizing region *via* the POSITION signal on the mesenchyme (Tickle *et al.* 1989). The polarizing region signal also specifies the position of that mesenchyme which specifies the position of the ridge (Table 1,3).

The effect of the apical ridge on the underlying mesenchyme can also be thought of as a STAY signal because it maintains the underlying mesenchyme at a high rate of proliferation and also prevents it differentiating into cartilage (see below) (Table 1,4). Indeed the high rate of proliferation in the early bud has its

origin in the reduction of proliferation in the adjacent flank rather than an increase in the bud region. In this sense, thinking of the ridge providing a STAY signal rather than promoting proliferation may be the most appropriate way of describing the factor's action.

When an additional polarizing region is grafted to the anterior margin the limb bud always widens and this requires additional cell proliferation. Aono and Ide (1988) have some rather indirect evidence that this may involve an FGF-like factor produced by the polarizing region which brings about the additional proliferation.

Cartilage differentiation

In the previous section emphasis was placed on POSITION with STOP/GO signals patterning the cartilage. There are however, additional signals involved because cartilage will not develop in the progress zone nor adjacent to the ectoderm. Moreover it seems that all the cells in the progress zone will differentiate into cartilage when all signals are removed. Thus one aspect of cartilage patterning is a STOP signal.

If progress zone cells are cultured in micromass culture, all the cells differentiate as cartilage giving rise to a sheet of cartilage (Cottrill *et al.* 1987*a*). The inference is that within the progress zone the cells are subject to a STAY signal from the apical ridge that keeps them proliferating and prevents them from differentiating (Table 1,4). When the cells begin to form cartilage it seems that they produce the GO factor which promotes cartilage differentiation. Evidence for such a GO factor comes from studies in micromass which suggest that mesenchymal cell aggregates must reach a threshold size before chondrogenesis can proceed (Cottrill *et al.* 1987*b*) (Table 1,5).

The ectoderm may provide an important component in patterning cartilage by providing a STOP signal (Table 1,6) which prevents cartilage differentiation. When ectoderm is placed on the surface of a micromass culture, cartilage is inhibited in the region both below and adjacent to the ectoderm (Solursh *et al.* 1981). We (Gregg *et al.* 1989) have found that the ectoderm completely inhibited the accumulation of cartilage specific type II collagen transcripts in the mesenchyme cells, and that the inhibition was not mediated by a change of cell shape as suggested by Zanetti and Solursh (1986). The ectoderm might also produce a GO signal directing the cell to a non-cartilage pathway.

Several authors have suggested that the cell contacts present at condensation and the associated cell-to-cell interactions are an important feature of cartilage differentiation. In one particular model of cartilage element patterning it is suggested that the condensation process itself and the associated mechanical changes are responsible for the patterning of the cartilaginous elements (Oster *et al.* 1985). A different interpretation of condensation is offered here which has important implications for interpreting the action of factors which promote or

block cartilage differentiation: condensation is seen as an early manifestation of cartilage differentiation and not a cause. If cells from wing buds at stage 22–23 are placed as single cells in a collagen gel, a significant number of the cells will form cartilage (Solursh *et al.* 1982). This shows that single cells can form cartilage without the necessity for any of the cell-to-cell interactions associated with condensation. Furthermore, when double anterior limbs are constructed before condensation occurs, two humeri or a thickened humerus develop in a substantial number of cases (Wolpert and Hornbruch, 1990). This shows that the humerus rudiment is specified well before condensation occurs, a result which is not consistent with the physico-mechanical model.

Taken together these results argue for an early specification of both cartilage and the spatial pattern of its differentiation. In these terms condensation should be seen as an early manifestation of cartilage differentiation, reflecting, most likely, a change in the nature of the extracellular matrix, causing the cells to come close together. More specifically, hyaluronic acid may be removed from the matrix and replaced by cartilage matrix (Singley and Solursh, 1981).

TGF-β is a potent stimulation of cartilage differentiation in micromass cultures (Kulyk *et al.* 1989) and we have recently shown that bFGF also stimulates chondrogenesis. Together their effects are additive. Of great interest is the suggestion by Lyons *et al.* (1989) that the TGF-β super family is required to control the progression of cell types through their differentiation pathway – a GO signal. Thus bone morphogenetic protein may stimulate early cartilage and TGF-β-2 would promote chondrogenesis; and at later stages similar molecules may be involved in maturation and hypertrophy.

Direct investigation of the ability of retinoic acid to control the differentiation of cartilage cells has been carried out in culture. It is well known that retinoic acid can inhibit chondrogenesis in culture and this is related to its teratogenic effect which is to inhibit cartilage differentiation. There is no reason to believe that this is in any way related to its effect on patterning. In serum-free culture, Paulsen *et al.* (1988) found that retinoic acid stimulates chondrogenesis in a dose-related manner at about $5\,\mathrm{mg\,ml^{-1}}$. The overall morphology of the cultures was unchanged. Ide and Aono (1988) found that retinoic acid at similar low concentrations promoted both proliferation and chondrogenesis of distal cells, while cells from proximal regions were unresponsive. It is far from clear whether these effects are related to the ability of retinoic acid to alter positional values in the limb.

There is a widespread view that molecules of the extracellular matrix play a controlling role in morphogenesis. The changes in these molecules in limb development have been catalogued by Solursh (1990), but there is little evidence for their role as signals. Newman (1988) has proposed a role for fibronectin, which is stimulated by TGF-β, in promoting aggregation which he considers important for cartilage differentiation. The role of such factors should be treated with caution since prevention of proteoglycan accumulation may inhibit chondrogenesis. A number of matrix components could act at this late stage of differentiation.

Muscle differentiation

In the limb, muscle cells have a lineage quite distinct from that of the connective tissue cells, since they are all derived from a small population of presumptive muscle cells that migrate into the limb at an early stage of development (Chevallier, 1979). The patterning of muscle results from the migrating muscle cells accumulating in specific regions. This patterning of muscle cells probably involves specific adhesive interactions with muscle connective tissue which may also provide a GO signal for muscle differentiation (Table 1,7).

Seed and Hauschka (1988) looked at the muscle colony-forming (MCF) potential of stage 23 chick myoblasts and concluded that two subclasses of MCF cells exist. The FGF-independent subclass showed a delay in differentiation in the presence of FGF, whilst the FGF-dependent subclass required FGF for terminal differentiation. In micromass culture we have found that TGF-β on its own has little effect on muscle cell differentiation while bFGF has a striking inhibitory action which has a clear dosage dependence. However, when TGF-β1 is added together with bFGF the inhibitory effect of bFGF is blocked (Schofield and Wolpert, 1990). Both the number and the morphology of differentiated muscle cells is restored to that seen in cultures grown in the absence of bFGF. Thus both TGF-β and bFGF could act together in the developing chick limb to control cell differentiation. bFGF is probably dispersed throughout the limb although not necessarily with a uniform distribution, whilst TGF-β is produced in the precartilaginous cellular condensation. We therefore hypothesise that TGF-β is able to affect both cells of the precartilaginous and the adjacent premuscle masses. If TGF-β and bFGF behave *in vivo* as they do in our culture system, their interaction could provide a possible mechanism for both enhancing cartilage differentiation and ending the inhibitory action of bFGF on myoblasts, thus allowing myogenic differentiation to precede.

Pigment patterns in the limb

The pigment patterns of birds are as beautiful as they are varied. How many factors might be required to specify such patterns? Only some of the patterns are based on the differentiation of pigment cells that migrate into the feather germs from the neural crest. Other patterns are structural and are due to properties of the feathers themselves.

We have investigated the cellular basis of local pigment patterns in the wings of the quail embryo. A local pigment pattern refers to a set of feathers which have a different pigmentation to those around them. There are also variations of pigment patterns within a feather and that is a separate issue. The now almost classical view of pigment patterning was put forward by Rawles (1948). On the basis of grafting together of melanoblasts between closely related strains she concluded that the pattern corresponded to that of the melanoblast donor. On this basis I proposed that the melanoblast cells must read the local positional values and interpret them according to its genetic constitution. However, almost all of

Rawles' experiments focussed on patterns within feathers rather than local patterns and our recent experiments lead to a quite different conclusion.

The dorsal feathers of the quail wing are all pigmented whereas there is a local pattern on the ventral surface, in which several rows of feathers are unpigmented. The unpigmented feathers lack pigmentation because of an inhibitory STOP signal exerted within the feather germ. The evidence for this is that if unpigmented germs are cultured and the melanoblasts allowed to migrate away from their ectoderm they differentiate and become pigmented (Richardson *et al.* 1990). Thus the pattern is determined by the character of the individual feather germs rather than the pigment cells. Consistent with such a mechanism, guinea fowl melanoblasts give a quail pattern on the ventral surface of the wing even though the guinea fowl wing is fully pigmented both ventrally and dorsally.

Local factors acting as STOP signals thus control pigment pattern formation. In addition there are GO signals that determine when pigment differentiation occurs, and thus control temporal expression (Table 1,8). The GO factors are clearly seen in an operation in which a quail wing bud is grafted onto a guinea fowl host before the neural crest cells have entered the bud. The bud now becomes populated with guinea fowl neural crest cells and pigment cell differentiation is visible in the quail wing many hours before differentiation occurs in the normal contralateral guinea fowl wing (Richardson *et al.* unpublished data).

There remains the key question of what specifies which feathers will inhibit melanocyte differentiation and which will not: that is, the specification of the basic pattern. The simplest view is that it reflects the basic pattern of positional information within the mesenchyme specifying POSITION in the ectoderm (Table 1, 9). In a mirror image duplication brought about by a graft of a polarizing region, the pigment pattern follows that of the underlying structures (Richardson *et al.* 1990).

While positional information may specify the character of the feather germs it is only indirectly involved in the patterning of the feather germs themselves. The feather germs are arranged in a highly ordered hexagonal pattern, and this spacing pattern is not directly linked to positional information, but probably involves an inhibitory STOP signal (Table 1, 10) during spacing of the feather germs. Positional information specifies which regions will have feather germs but the actual spacing of the feather germs within such regions is due to another mechanism. For example, if growth of the limb bud is reduced at the time when the feather spacing is being specified then fewer feathers are specified but their spacing remains the same as normal (McLachlan, 1986).

Discussion

The presence of growth factors in the early amphibian embryo and the evidence for their role in specifying the pattern in the mesoderm (Smith, J. C. *et al.* 1989; Slack, 1990) has given rise to the hope that they may be playing a similar role in limb morphogenesis, and be the crucial signals in cell-to-cell interactions. Thus

far only FGF has been shown to be present at the crucial early stages of pattern formation (Seed *et al.* 1988) while production of TGF-β is only detected in the limb itself at later stages (Heine *et al.* 1987). IGF-II mRNA has also been detected in the limb bud (Engstrom *et al.* 1987), and Ralphs *et al.* (1990) using antibody staining, have shown IGF to be present in the tip of the limb while it is absent in regions undergoing chondrogenesis. At later stages the peptides are present in chondrocytes. In more general terms we remain ignorant about the identity of the signals involved in early limb morphogenesis as listed in Table 1. Retinoic acid is a most promising candidate for specifying position.

Finally, we must consider the pathway the signals may take. Do they pass directly *via* cell-to-cell contact, *via* the matrix, through the extracellular space, or through gap junctions? The flow of blood through the limb suggests that extracellular diffusion is unlikely (Georgiello and Caplan, 1983). A lipid soluble molecule like retinoic acid could diffuse within cell membranes and pass from cell to cell at points of cell contact. Allen *et al.* (1990) have investigated the role of gap junctions in positional signalling by the polarizing region and have concluded that gap junctions may play a role in enabling the polarizing region cells to communicate with anterior mesenchyme cells.

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