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From soil mechanics to chick development

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ABSTRACT Here, I provide some recollections of my life, starting as a civil engineer in South Africa and how I gradually became interested in biology, particularly pattern formation. In retrospect, I think that my decision to work on chick embryos to study limb development back in 1966 turned out to be the right one. The principles discovered in these 50 years, both by my collaborators and by other colleagues, have established the principles of how the limb develops in higher vertebrates, including humans.

KEY WORDS: *positional information, Wolpert, autobiography, chick limb, theoretical biology, French flag model*

As a child, I became involved in topics related to science. I made aeroplanes out of balsa wood, and I had a Meccano set and built quite complex structures. I also fiddled with a chemistry set and played with electric trains and steam engines. At the age of about 14-15 I could build a radio, and more or less understood how it worked. One of the things I was obsessed with was wanting to be able to fly an aeroplane, so I would read books about how aeroplanes flew, and how one had to control them. At school the two subjects that I liked were science and mathematics, so I was very disappointed that I didn't get a distinction in science in my matriculation; however, I did get a First Class and a distinction in mathematics! Leaving school, I was faced with what to study for my career.

I chose to study civil engineering at university. I enjoyed the maths most and I really wanted to be a mathematician, but I really wasn't good enough. I nearly failed the final exam as I answered the exam questions wrongly, but I did spot two errors in the exam papers and so they had to pass me. Having qualified, I was offered a job, via a relative, as the personal assistant to the director, Jere Jennings, of the Building Research Institute in Pretoria. The research at the Institute was on constructing cheap housing for black Africans, but most of my research was on unsaturated soils. It was a big problem in South Africa at that time with certain housing, as you'd build something and then the soil would rise up and the whole building would crack badly. They were trying to understand what was going on.

So I became a soil mechanic and I was even the author of two papers on the behaviour of unsaturated soils. I worked in Pretoria for two years and then began to reassess my life. I was not happy in South Africa. I went to meetings to protest against apartheid and helped Nelson Mandela, but was frightened by the violence these activities might involve. I was not that interested in soil mechanics. So I decided to leave South Africa. In order to appease my parents, particularly my mother, I said I was going to Israel to work as an engineer. I also decided to start the journey by hitch-hiking up Africa with a friend. We hitched up to Nairobi and then took a dhow (sailing vessel) to Arabia and then boats to Israel, where I worked briefly as a soil mechanic on a dam.

As I prepared to leave Israel I kept thinking I wanted to get out of soil mechanics and to do something else, but I had no idea what I wanted to do. Should I become a doctor? Should I become a lawyer? Should I become a poet? I came back to London and did a course in soil mechanics at Imperial College; quite interesting, though medicine became my aim, but it was perhaps too long a course at my age. I decided instead to become a physiologist, which was related to medicine. I discovered that the Nuffield Foundation was offering scholarships for those trained in the physical sciences to change to biological sciences. Then a crucial letter arrived. A friend of mine in South Africa, Wilfred Stein, a biological scientist, knew I wanted to get out of soil mechanics. He came across a paper in which Michael Swann and Murdoch Mitchison, two distinguished biologists in Britain, were investigating how cells divided into two when they were multiplying. The cells double in size by growth and then divide due to a constriction, and they were investigating how that constriction developed by looking at the mechanical properties of the dividing cells. Wilfred thought that this was a problem I could work on with my background in mechanics, and said, *"Lewis, this is what you should do"*. He sent me to Prof. J.F.Danielli at Kings College in order to do a Ph.D in Zoology on the mechanics of cell

Abbreviations used in this paper: a-p, antero-posterior; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; RXR, retinoic acid receptor; Shh, sonic hedgehog; TLCCD, thin-layer counter-current distribution.

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division. I did my PhD on the mechanics of the membrane of dividing sea urchin eggs, and when I published my theory it received quite a lot of attention, but I was later proved to be wrong. Danielli gave me a lectureship in 1960.

Changing from soil mechanics to cell mechanics was really a very big change in my life but I would encourage others to make similar decisions. For my Ph.D, while working in the summers at a Swedish marine station, I met Trygve Gustafson, who was working on how sea urchin embryos actually developed by taking movies of them. I became involved in his project and together we published seven papers. I applied my engineering to explaining the changes in shapes as the sea urchin developed.

Danielli started a new Journal, the *Journal of Theoretical Biology* and I was involved with helping him, and would remain with the journal for a very long time. I was never very good in the laboratory nor enjoyed working in it, so my skill as a scientist has been to persuade other people to do the experiments. I am a theoretician, and although I care about the experiments and even design them, I'm not good at doing them myself.

I was always very impressed by the experiments of Hans Driesch over a hundred years ago. Working on sea urchins, he separated the two cells after the first division and observed their development. Each developed into a normal larva but half the normal size. He did not understand the mechanism, but proposed a mystical mechanism, the entelechy, whereby the cells still knew their position and developed a normal full size larva. Thinking a lot about it, I suddenly realized that the embryo was behaving like a flag whose pattern is the same no matter how big or small it is. This was the origin of my French Flag model with its blue, white and red regions, for pattern formation in development, and for positional information as the cells have their position specified (Wolpert, 1969). If the cells have their position specified with respect to boundaries, then the pattern will be the same for all sizes. To specify their position, a gradient of some kind was almost certain to be involved.

Fig. 1. Entrance to the Middlesex hospital c. 1966.

Quite simple experiments showed that cells have positional values. Regeneration experiments showed intercalation of missing regions of invertebrate limbs, and there seemed to be a set of positional values along the tibia of the cockroach leg, because if a portion of the tibia is removed, the missing region will be replaced through intercalary regeneration. More striking, when a proximal cut tibia is grafted onto a more distal site, making the tibia longer, intercalation makes the tibia even longer by intercalating the missing positional values. Another clear example of intercalation is from the salamander limb, when a distal regeneration blastema is grafted in place of a proximal one and the missing regions are intercalated to form a normal limb.

An important example providing evidence for positional information comes from the antenna and leg of *Drosophila* (Cummins *et al.,* 2003). If the Hox gene Antennapedia, which is normally expressed in parasegments 4 and 5 of the *Drosophila* embryo, is expressed in the head region, the antenna develops as a leg. Clones of Antennapedia cells in the antenna disc develop as leg cells, but the type of developed leg cells depends on their position along the proximodistal axis; for example, if they are at the tip, they will develop as a claw. This strongly suggests that the positional values along the leg and antenna are the same, but because of the Hox genes, the cells interpret their positional values differently. The molecular basis of the positional values is still not known, and the downstream action of the Hox genes that controls this process is also not understood.

Then in 1966 I accepted a chair at the Middlesex Hospital Medical School (Fig. 1), and had decided that we should work on the development of the chick embryo because this seemed to me more medically relevant. In this regard, see: *"Much more from the chicken's egg than breakfast – a wonderful model system"* (Wolpert, 2004): the eggs are easy to obtain and easy to open to manipulate the embryo. The limbs develop early and are also easily manipulated.

The basic idea of positional information is that pattern formation in the developing embryo may result from cells first having their positions specified with respect to boundary regions, as in a coordinate system, after which the cells interpret this positional information according to their genome and developmental history. The interactions involved in pattern formation may thus be quite simple, even universal, and need be involved only in specifying positional information. Cells with different positional values may undergo similar cytodifferentiation, but the difference in positional value makes them non-equivalent, a quality that can be used to specify other cellular properties. Simple gradients may provide positional information and a kinetic threshold model could provide the first step in interpretation leading to defining discrete cell states. Pattern formation in the chick wing may be viewed as a 3-dimensional coordinate system followed by cellular interpretation, leading to the ordered pattern of cartilage, muscle, and tendon development without further interaction. Hox-4 genes probably encode positional information (IzpisúaBelmonte *et al.,* 1991). We proposed rather different models for specifying positional information along the proximodistal and anteroposterior axes of the limb. For the former, the model is based on how long cells remain in the progress zone, and for the latter a signal from the polarizing

Fig. 2. Lewis Wolpert with Cheryll Tickle.

region at the posterior region was proposed (Wolpert, Lewis and Summerbell, 1975).

We focused on how position could be specified along the proximodistal axis of the embryonic limb, that is, from the humerus to the digits. Our model was novel and based on a timing mechanism (Summerbell and Lewis, 1975). It was already established that outgrowth of the limb depended on fibroblast growth factor (FGF) signals at the apical ridge at the tip of the limb bud, and when the ridge is removed, the limb is truncated. There was also a region of cell proliferation beneath the ridge which our model proposed was a progress zone, and cells measured how much time they spent in the zone and this determined their positional value along the proximodistal axis. As the cells in the zone were dividing, cells continuously moved out of the zone. Cells that were in it for a short time would have proximal positional values, while those in it for a long time would become more distal, like the digits. The paper came in for a lot of criticism (Galloway *et al.,* 2009), to which I responded (Wolpert, 2002). I even wrote a paper with a critic, Clifford Tabin (Tabin and Wolpert, 2007).

To test our theory we irradiated the early limb bud with X-rays, killing cells in the progress zone so very few cells moved out until it was repopulated (Wolpert *et al*., 1979). We predicted correctly that by doing this only distal structures like hands would develop. Our model might also explain the reason why thalidomide causes loss of proximal limb elements (Wolpert, 1999). Thalidomide is known to affect the development of the blood supply (D'Amato *et al*., 1994; Therapontos *et al.,* 2009). If thalidomide blocked the blood supply to the early limb, so that cells in the progress zone died, then one would expect to get similar results.

It had been discovered by Saunders and Gasseling (1968) that for the anteroposterior axis there is a signalling region at the posterior margin of the limb bud, and that if it were grafted to the anterior side, then extra digits formed. We proposed that positional information along the anteroposterior axis is specified by a signal from this polarizing region and that position may be specified by the concentration of a diffusible morphogen (Wolpert and Hornbruch 1981). While this model can account for a variety of results, it is now clear that a model based on intercalation by growth of positional values can do the same. The distinction between the two models lies in whether a grafted polarizing region can alter existing positional values and in the distance over which it exerts its influence. The two models make different predictions as to the effect of grafting two polarizing regions. The intercalation model predicts that this effect will be the sum of two single grafts, whereas the morphogen model predicts different results depending on how close together the two polarizing regions are placed. The pattern of digits following grafts of two polarizing regions show that it is sensitive to the distance between the grafts and consistent with a model based on long-range interaction, such as a morphogen gradient. The limb widens following a polarizing region graft to the anterior region and x-ray irradiation reduces the effect (Smith and Wolpert, 1981)

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. An article by Cheryll Tickle (Tickle, 2002) (see Fig. 2) along with her contribution to this Special Issue of the *Int. J. Dev. Biol.* (Davey *et al.,* 2018) trace some highlights in the history of the polarizing region from its beginning, 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. Tickle (2002) (see also Davey *et al.,* 2018) places 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 sonic hedgehog (Shh) and bone morphogentic proteins (BMPs) in mediating polarizing activity. *In situ* hybridization experiments showed that retinoic acid receptor (RXR)-α transcripts which can bind retinoic acid were present throughout the epithelium and mesenchyme of the chick wing bud at stages when retinoic acid can affect antero-posterior (a-p) patterning (Rowe *et al*., 1991).

We proposed that the signal from this region provided a gradient across the limb (Tickle *et al*., 1975). The wing has three digits, named 4, 3, and 2. If we grafted the polarizing region to the anterior margin, a 432234 pattern of digits developed, but grafting a small piece of the region resulted in 4322. In addition, leaving the graft in place for only a short time also resulted in a 4322 pattern of digits. This is consistent with the model that suggests that at high concentrations digit 4 is specified, whereas at low concentrations, digit 2 is specified. We found that retinoic acid induced the signalling region (Tickle *et al*., 1982).The signal is sonic hedgehog, and recent evidence suggests that sonic hedgehog does not diffuse but is carried on particles and transported by cell extensions, cytonemes (Sanders *et al.,* 2013).

In 3- to 4-day embryonic chick limb buds, the polarizing activity appears to be responsible for signalling positional information along the anteroposterior axis. However the result of grafting an additional polarizing region to different positions along the anteroposterior axis of the limb bud at stage 16 has remarkably little effect on the humerus that lies between the two polarizing regions and thus presents severe difficulties for the positional signal model (Wolpert and Hornbruch, 1987). This paper suggests a mechanism for patterning the humerus.

Experiments were designed to test which biosynthetic processes are required for polarizing activity (Honig *et al*., 1981). We treated polarizing regions with biochemical inhibitors, and then assayed their abilities to induce limb reduplications when grafted into anterior sites on the host limb, and also measured their capacities for protein, RNA, and DNA synthesis. DNA synthesis, and possibly

Fig. 3. Ideas flowing in the lab.

oxidative phosphorylation, do not seem to be required for polarizing activity. But glycolysis and protein and RNA synthesis are necessary, although not sufficient, for polarizing regional activity. Activity seems particularly sensitive to inhibitors (actinomycin D and alpha-amanitin) of RNA synthesis.

Positional signalling by mouse limb polarizing regions gives results similar to chick polarising region (Tickle *et al*., 1976). Hensen's node is at the anterior of the primitive streak which develops by epithelial cell intercalation (Voiculescu *et al*., 2007). From stage 4 to stage 10 the node shows polarizing activity when grafted to the anterior margin of the chick limb bud (Hornbruch, Summerbell and Wolpert, 1979; see Fig. 4). It can specify additional digits though its action is somewhat attenuated when compared with the effect of a grafted polarizing region. At stage 10 the activity disappears from the node and is found both posterior to the node and in the future wing region of the flank. The ability of Hensen's node to generate a positional signal suggests that the signal in the limb and early embryo may be similar. The results support the view of the polarizing region as a discrete signalling region. Quail grafts of Hensen's node were examined for their potential to induce somites in chick blastoderms (Hornbruch, Summerbell, and Wolpert, 1979). The origin of the structures induced depended on the distance of the graft from the host's midline. Nodes placed at the margin of the area pellucida resulted in structures differentiated from the cells of the graft, whereas medially the graft organized host cells to form rows of somites. Later studies suggested that the reason for the difference is due to BMP activity, which inhibits somite formation: BMP is low near the embryo's axis and high at the periphery (Tonegawa and Takahashi, 1998; Streit and Stern 1999; Dias *et al*., 2014).

Our evidence for gradients in the chick limb bud is discussed by Towers *et al.,* (2012). Only later did we propose that Alan Turing's reaction diffusion model (Kondo, 2017) could cause digits to develop, and the polarizing region gradient would specify their character. The recent development of a realistic two-dimensional simulation of digit patterning by Raspopovic *et al.,* (2014) is important.

The pattern of development of muscles and tendons is largely autonomous with respect to their position. Our earlier study of muscle development in the chick limb (Shellswell and Wolpert 1977) showed that the future muscle cells migrated in from the somites and were patterned by the limb mesenchyme, which determined where they adhered most strongly.

Hornbruch & Wolpert (1970) found that mitotic rates within the developing chick limb can vary over a factor of five. Fate maps for mesenchyme and apical ridge of a stage 20 chick wing bud (Vargesson *et al.,* 1997) show that most of the wing arises from the posterior half of the bud. Sub-apical mesenchyme gives rise to digits. Cell populations beneath the ridge in the mid apical region fan out into the anterior tip of the hand plate, while posterior cell populations extend right along the posterior margin. The absence of anterior bending of posterior cell populations has implications when considering models of vertebrate limb evolution. The fate maps of the apical ridge show that there is also a marked anterior expansion and cells that were in the anterior apical ridge later become incorporated into non-ridge ectoderm along the margin of the bud. Mesenchyme and apical ridge do not expand in concert - the apical ridge extends more anteriorly. Hoxd-13 and Fgf-4 are initially expressed posteriorly until about the mid-point of the early wing bud in mesenchyme and apical ridge, respectively. The apical ridge may be involved in determining limb bud shape. The cell density varies in a very regular manner, and is closely correlated with mitotic index: which is inversely proportional to cell density (Summerbell and Wolpert, 1972). Later in development, the genes come to be expressed throughout most of the hand plate and apical ridge respectively. At the proximal edge of the Hoxd-13 domain, cell populations stopped expressing the gene as development proceeded and there was no evidence that the changes in extent of the domains were due to initiation of gene expression in anterior cells. The changes in expression fit with the fate maps.

Fig. 4. Amata Hornbruch and Dennis Summerbell.

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 (Wolpert, 1990). Patterning in the limb can be analysed in terms of signals that specify positional values along the anteroposterior axes, but there is 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.

The onset of chondrogenesis in the embryonic chick is preceded by a pre-chondrogenic condensation of the prospective cartilage cells (Gould, Day, & Wolpert, 1972). Similarly, in culture, the chondrogenic phenotype is only expressed by limb mesoderm cells plated at densities above confluence. This has led to the proposal by Solursh and Reiter (1980) that chondrogenic differentiation requires immediate histogenic interactions prior to overt chondrogenesis. It is noticeable that in both the *in vitro* situations, cells secreting a cartilaginous matrix are rounded in shape. Later, Glowacki *et al.* (1983) showed a dependence of phenotypic expression by mature chondrocytes on cell shape. Cells maintained in a rounded configuration by culturing on a semi-adhesive substratum (polyHEMA) synthesised more sulphur-containing extracellular matrix than cells allowed to flatten on normal tissue culture plastic. We have investigated whether there is a similar shape-dependent relationship in the differentiation of chick embryonic mesoblasts into actively secreting chondroblasts. Our results show that a rounded cell shape is conductive to the synthesis of a sulphated matrix (Archer, Rooney and Wolpert, 1982).

The development of the avian fibula was studied both histologically and experimentally (Archer, Hornbruch and Wolpert, 1983). It was found that from the onset of chondrogenesis, the fibula possessed a smaller diameter than the neighbouring tibia. The truncated growth of the fibula was a result of the loss of its distal epiphysis between stages 27-31. This epiphysis subsequently became fused to the tibia and formed the fibulare of the tibiotarsus. It was concluded that there was no evidence for competitive interaction between the tibia and fibula. In addition, the differential growth in diameters between the tibia and fibula was largely a result of differential osteogenesis rather than chondrogenesis, as previously thought.

A technique which identifies cells differing in surface character, aqueous two-phase partition using thin-layer counter-current distribution (TLCCD), was used to study differentiation and pattern formation in the developing chick limb bud (Cottrill, Sharpe and Wolpert, 1986). The TLCCD profiles of cell populations, derived from various regions of morphologically undifferentiated mesenchyme from three different stages of limb development, have been compared. At no stage, or location, has the population been found to be homogeneous. Cells from progress zones and more proximal regions could all be resolved into several populations. The populations from progress zones at three different developmental stages were qualitatively similar but differed in the proportions of cells in each. The most striking differences in cell populations were those obtained from the most proximal region of the limb, closest to the flank, which represents the developmentally most advanced region.

During limb development, type I collagen disappears from the region where cartilage develops and synthesis of type II collagen, which is characteristic of cartilage, begins. *In situ* hybridization using antisense RNA probes was used to investigate the spatial localization of type I and type II collagen mRNAs (Devlin *et al.,* 1988). The distribution of the mRNA for type II collagen corresponded well with the pattern of type II collagen synthesis, suggesting control at the level of transcription and mRNA accumulation. In contrast, the pattern of mRNA for type I collagen remained more or less uniform and did not correspond with the synthesis of the protein, suggesting control primarily at the level of translation or of RNA processing.

BMPS are members of the transforming growth factor beta (TGF beta) superfamily which are involved in a range of developmental processes including modelling of the skeleton. We showed that Bmp-2 is expressed in mesenchyme surrounding early cartilage condensations in the developing chick limb, and that Bmp-4 is expressed in the perichondrium of developing cartilage elements (Duprez *et al*., 1986). To investigate their roles during cartilage development, BMP-2 and BMP-4 were expressed ectopically in developing chick limbs using retroviral vectors. Over-expression of BMP-2 or BMP-4 led to a dramatic increase in the volume of cartilage elements, altered their shapes and led to joint fusions. This increase in volume appeared to result from an increase in the amount of matrix and in the number of chondrocytes. The latter did not appear to be due to increased proliferation of chondrocytes, suggesting that it may result from increased recruitment of precursors. BMP-2 and BMP-4 also delayed hypertrophy of chondrocytes and formation of the osteogenic periosteum. These data provide insights into how BMP-2 and BMP-4 may model and control the growth of skeletal elements during normal embryonic development,

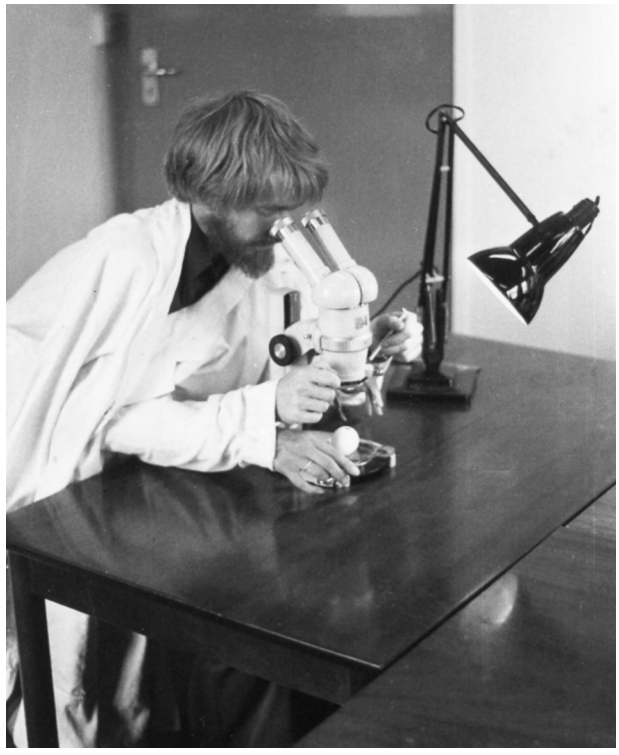

Fig. 5. "How to get limb duplications" as demonstrated by surgeons Cheryll Tickle and Dennis Summerbell.

suggesting roles for both molecules in recruiting non-chondrogenic precursors to a chondrogenic fate.

The chick heart tube develops from the fusion of the right and left areas of pre-cardiac mesoderm and in almost all cases loops to the embryo's right-hand side. Double-right sided embryos formed many more left-hand loops than double-left sided embryos (Hoyle, Brown and Wolpert, 1992). Now, the study of left-right asymmetry has become a huge field (see review by Monsoro-Burq and Levin, 2018 in this issue).

In retrospect I think that my decision to work on chick embryos to study limb development back in 1966 turned out to be the right one. The principles discovered in these 50 years, both by my collaborators and by other colleagues (see Wolpert 1999 and Tickle 2018 in this issue for reviews), have established the principles of how the limb develops in higher vertebrates, including human.

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