

# A model for chondrogenic condensations in the developing limb: the role of extracellular matrix and cell tractions

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## SUMMARY

The hyaluronate component of the extracellular matrix is a powerfully hydrophilic polymer, capable of osmotically swelling and deswelling by a volume factor of 5 or more. At the time of cartilage condensation in the limb bud the chondrocytes start to produce hyaluronidase, an enzyme which degrades hyaluronate. The consequent deswelling brings the chondrocytes closer together – close enough for intercellular cell tractions to become effective and intercellular junctions to form. By analysing the physicochemical situation we show how these processes, principally the coupling of the osmotic deswelling with cellular traction forces, can produce cartilage condensation patterns resembling those in the early limb bud. In distinction from our earlier model for chondrogenic condensations this mechanism does not depend on cell motions other than convective transport by contraction.

## INTRODUCTION

In a previous paper (Oster, Murray & Harris, 1983; hereafter referred to as OMH) we demonstrated how cell tractions could create spatial patterns of cell aggregation. There we proposed models for the morphogenesis of feather germ patterns and the patterns of condensation of chondroblasts that presage bone formation. These models were built on earlier experimental work by Harris and his coworkers on the role of cell traction in the aggregation process of mesenchymal cells (Harris, Stopak & Wild, 1981; Harris, Stopak & Warner, 1984).

The cell traction model as presented in OMH depended on cell motion; that is, the aggregation patterns formed by the migration of motile cells which were guided by haptotactic cues set up by the cell tractions. We also briefly mentioned the possibility that condensation patterns could occur without any cell motion other than passive 'convection' (i.e. dragging of cells by the traction of other cells).

Furthermore, the extracellular matrix (ECM) played a passive mechanical role in OMH: it served only as an elastic substrate which could deform under the tractions of cells and transmit stresses from one point to another.

In this paper we show how the ECM may be an active participant in the formation of spatial patterns (Toole, 1972). We present a physical mechanism that can generate spatial patterns of cell aggregation in the absence of active cell motility. Of course, if cell migration is present, the pattern formation possibilities are enhanced; however, our point here is that patterning can be generated without cell locomotion.

Intuitively, the mechanism works like this. The ECM is composed of a polyelectrolyte gel which is osmotically swollen. If the cells commence secreting an enzyme which digests the osmotically active component of the ECM, the gel will deswell, and bring the cells closer together. When the cells are brought close enough together by the collapsing ECM, intercellular contacts can form (e.g. via filopodia) and cell tractions between cells become more effective. By analysing the physics of this process it turns out that the aggregations of cells produced by the gel deswelling of the ECM, coupled with the cell tractions, exhibit the same spatial regularities as described in OMH for aggregations produced by cell traction and migration. We note that the model we present here is based on entirely different physical assumptions than hitherto proposed models (e.g. Ede & Law, 1969; Newman & Frisch, 1979; Wolpert, 1971).

#### THE MODEL

Our model of the developing limb bud is illustrated in Fig. 1. Cells proliferate at the distal tip and emerge from the progress zone secreting extracellular matrix. It is the hydration of the ECM which keeps the limb bud 'inflated', and maintains the spacing between cells. There is also a 'sleeve' of high hyaluronate concentration encasing the limb bud (Feinberg & Beebe, 1983). This tends to keep the limb bud osmotically inflated independently of the internal cell-matrix constitution. This sleeve will provide a 'boundary condition' for the model. The formation of condensation patterns will emerge from the interaction of forces generated by the ECM and by the cells. In order to understand this we discuss each force separately.

#### *The ECM is osmotically swollen*

The extracellular matrix is a complex of crosslinked polymer molecules whose composition and properties change during development (Stern, 1984; Hay, 1981; Alberts *et al.* 1983; Trinkaus, 1984). The principle components of the ECM are collagen, and various glycosaminoglycans (GAGS). The physical chemistry of the ECM is quite complex; however, for our purposes we shall focus on one particular property: the proteoglycans comprise a polyelectrolyte gel which can generate a powerful osmotic swelling pressure. The mechanical effect of the swelling



pressure is to distend the ECM so that it fills a much larger volume than it would otherwise occupy. The term 'swelling pressure' refers to the pressure that would have to be applied to the gel to prevent it from expanding; that is, it is the total force tending to dilate the gel.

The swelling pressure can be understood by referring to Fig. 2 (c.f. Oster, 1984; Tanaka, 1983; Hill, 1960). Here a piece of ECM has been placed in contact with solvent (extracellular fluid) through a porous piston. Solvent will flow down the chemical potential gradient into the gel, causing it to swell and exert pressure on the piston.

The swelling pressure, which we denote by  $P_S$ , is composed of an osmotic component,  $P_{OSM}$ , which tends to dilate the gel and an elastic component,  $P_{ELAS}$ , which tends to contract it:

$$P_S = P_{OSM} + P_{ELAS} \quad [1]$$

That is, if a piece of dry gel is placed in a solvent bath it will imbibe fluid and swell. If the gel were not crosslinked, this swelling would proceed indefinitely, or until the gel expanded to fill the container. However, because they are crosslinked, the polymer strands can generate elastic forces. These forces arise from the thermal motion of the polymer fibres as they writhe about under the impact of solvent molecules. At large strains there is a small contribution from the deformation of the intermolecular bonds. Note that in equation [1] we must count the elastic pressure as negative since it tends to contract the gel.

The osmotic pressure,  $P_{OSM}$ , in turn has contributions from three effects: (1) the osmotic pressure arising from the mixing of the polymer with the solvent, (2) the intermolecular interactions between the polymer molecules, (3) the osmotic contribution of the counterions in the solvent (Tanaka, 1983; Hill, 1960; Flory, 1956). Of these, the dominant force is the ionic contribution; therefore, the effects of ionic strength and pH are likely to be important in regulating the hydration state of the ECM. However, for our purposes here we need only deal with the total osmotic pressure,  $P_{OSM}$ .

#### *Cells can control the hydration state of the ECM*

The swelling pressures in the ECM derive mostly from negatively charged polymer constituents, principally hyaluronic acid (HA) and chondroitin sulphate (Grodzinsky, 1983; Alberts *et al.* 1983; Stern, 1985). These negative charges attract positive counterions; the effect of these counterions is to create an 'ion pressure' which is the major contribution to the osmotic pressure in the ECM (Tanaka, 1983). The HA macromolecule can be degraded by the enzyme hyaluronidase (HAase); the term 'hyaluronidase' actually refers to a class of enzymes capable of cleaving the glycosidic bonds of hyaluronic acid (c.f. Stern, 1984). The hyaluronate/hyaluronidase system admits the capacity for cells to swell and shrink their extracellular milieu: secreting HA osmotically swells the ECM, while secretion of HAase deswells it. Indeed, the hydration state of the ECM is

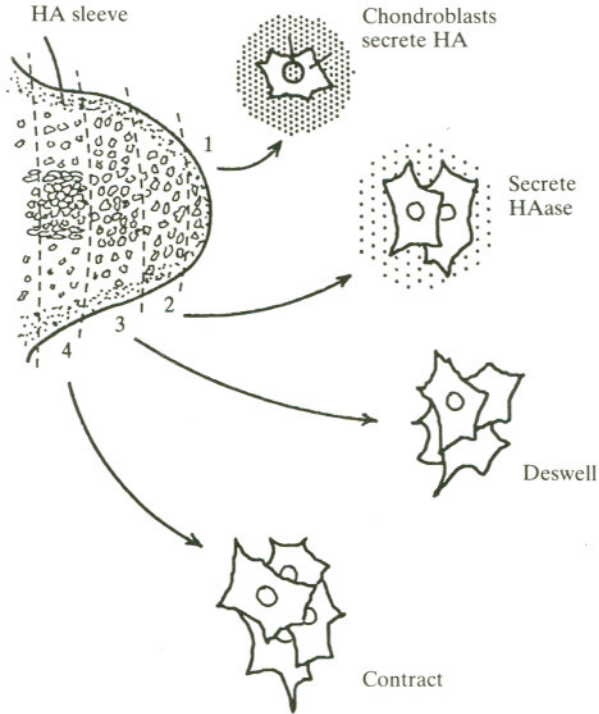


Fig. 1. The scenario for chondrogenesis described by the model equations. (i) Chondroblasts emerge from the progress zone secreting hyaluronate (HA). The hydration of the HA inflates the tissue, keeping the cells separated and preventing them from interacting strongly. (ii) After leaving the progress zone, the high level of HA triggers the cells to secrete HAase. The balance between HA and hyaluronidase (HAase) production begins to shift towards HAase. (iii) The increase in HAase causes the extracellular matrix (ECM) to osmotically deswell. As the intercellular distances decrease the chondroblasts commence to interact strongly and the cell density increases. (iv) As strong intercellular contacts are made the cell tractions pull the chondroblasts into close apposition and the cell density rises precipitously to form the chondrogenic condensation.

probably determined by an equilibrium between the rates of secretion of HA and HAase.

The hydration of the ECM may exert some control over cell motility. Swelling may open sufficient intercellular space to permit cell migration. Too much hydration, however, may inhibit motion by confining the cells within a hydrated 'coating'. Since cell migration will not play a major role in our discussion, we shall ignore this potentially major effect, although it can easily be included in the model, and will enhance its pattern-generating potential (c.f. OMH).

The major role of ECM hydration from our viewpoint is to keep the tissue inflated, and to keep the cell density low enough so that intercellular interactions are inhibited.

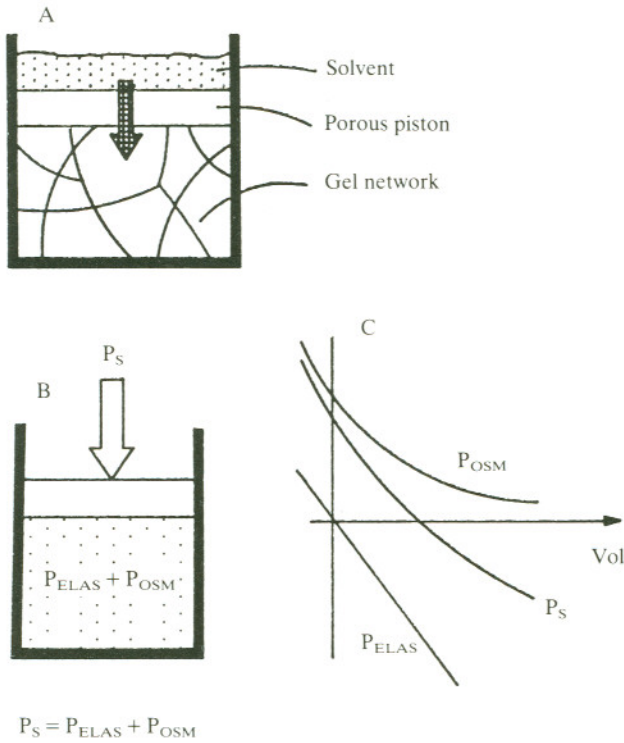


Fig. 2. The swelling pressure of the ECM. (A) A piece of hyaluronate-rich gel is confined by a piston and placed in contact with solvent via a porous piston. Solvent will flow down its chemical potential gradient into the gel. The solvent will tend to swell the gel and the elastic forces generated by the gel fibres will tend to contract it. (B) The swelling pressure,  $P_S$ , of the gel is that pressure which is required to prevent the gel from swelling. It is the sum of the osmotic pressure,  $P_{OSM}$  (counted as positive), and the elastic pressure,  $P_{ELAS}$  (counted as negative). (C) As the volume of the cylinder increases, the osmotic pressure falls and the elastic pressure becomes more negative (i.e. more strongly contracting). The swelling pressure,  $P_S = P_{ELAS} + P_{OSM}$  falls to zero at the point where the expansive osmotic pressure is just balanced by the compressive elastic pressure.

#### *Cell tractions can become effective at high cell densities*

As discussed in OMH, cells can generate substantial traction forces on one another and on the ECM. They accomplish this by extending motile appendages such as lamellipodia and filopodia, attaching to adhesive sites and contracting (c.f. Oster, 1984). However, in this model we shall assume that cells are inhibited from protrusive activity by a superabundance of ECM. That is, when surrounded by a hydrated coat of HA-rich ECM, they are effectively isolated mechanically from their neighbours and can exert little tractions. However, if the HA coat is degraded substantially so that cells are brought into close proximity to one another, then intercellular tractions can become effective. Thus the actual cell



density depends on the balance between the swelling pressure generated by the ECM and the contraction pressure produced by the cells.

It is worth noting that, as the ECM collapses and cells are brought into closer apposition, the number of intercellular contacts will not increase linearly, but will rise in a sigmoidal fashion (Perelson & Oster, 1979). That is, a decrease in tissue volume will initially increase the intercellular contacts slowly; however at some specific volume the number of contacts will suddenly jump to near its final value. This type of behaviour is characteristic of 'phase changes' such as the condensation of a vapour to a fluid. This phenomenon, which is purely geometrical in nature, will facilitate the sudden onset of the condensation process.

#### *Cell density depends on the relative effects of swelling and traction*

Consider a small volume of tissue in the limb bud just proximal to the progress zone. We can characterize the tendency of cells to condense by a 'condensing pressure',  $P_{\text{COND}}$ , which is the difference between the swelling pressure of the ECM and the traction pressure of the cells,  $P_{\text{TRACT}}$ :

$$\begin{aligned} P_{\text{COND}} &= P_s + P_{\text{TRACT}} \\ &= P_{\text{OSM}} + P_{\text{ELAS}} + P_{\text{TRACT}} \end{aligned} \quad [2]$$

The system is in mechanical equilibrium when the condensation pressure is zero (i.e. no tendency for the volume element to change size). The formation of condensations comes about when the condensation pressure becomes negative for a time; that is, when the forces of matrix elasticity and cell tractions overpower the dilating osmotic pressure.

#### *The mathematical model*

In the Appendix we formulate the mathematical equations that govern the condensation process. In this section we present a heuristic description of those equations so as to highlight the important physical parameters.

The model is built around the following variables which describe the condition of the limb bud in a typical volume element located at position  $x$  at time  $t$  (c.f. Fig. 3):

$n(x,t)$  = the density of cells (chondroblasts).

$m(x,t)$  = the density of matrix components other than hyaluronate.

$h(x,t)$  = the density of hyaluronate.

$a(x,t)$  = the density of hyaluronidase.

$\sigma(x,t)$  = the stress (force per unit area) due to the expansive osmotic forces and the compressive elastic and traction forces.

The equations that govern these five quantities, while complicated in appearance, are simply conservation, or balance laws, which bookkeep the mass flows into and out of the volume element, and the balance of forces which

maintains the volume element in mechanical equilibrium. The form of these equations are, for each volume element:

$$\begin{aligned} \text{[Rate of change of cell density } (\partial n/\partial t)] = \\ \text{[Convection into and out of the volume element]} \end{aligned} \quad [3a]$$

$$\begin{aligned} \text{[Rate of change of matrix material } (\partial m/\partial t)] = \\ \text{[Convection into and out of the volume element]} \end{aligned} \quad [3b]$$

$$\begin{aligned} \text{[Rate of change of HA concentration } (\partial h/\partial t)] = \\ \text{[Convection into and out of the volume element]} + \\ \text{[Production by cells]} - \\ \text{[Degradation by hyaluronidase]} \end{aligned} \quad [3c]$$

$$\begin{aligned} \text{[Rate of change of HAase concentration } (\partial a/\partial t)] = \\ \text{[Diffusion of a]} + \text{[Production of a by cells]} - \\ \text{[Degradation of a]} \end{aligned} \quad [3d]$$

The model equations [3a–d] do not include the effects of matrix secretion or cell proliferation. It is a simple matter to incorporate these effects; however, they do not alter the qualitative aspects of the model, and so we omit them for conceptual clarity. Notice also that in equations [3a,b] the cells and matrix material move only

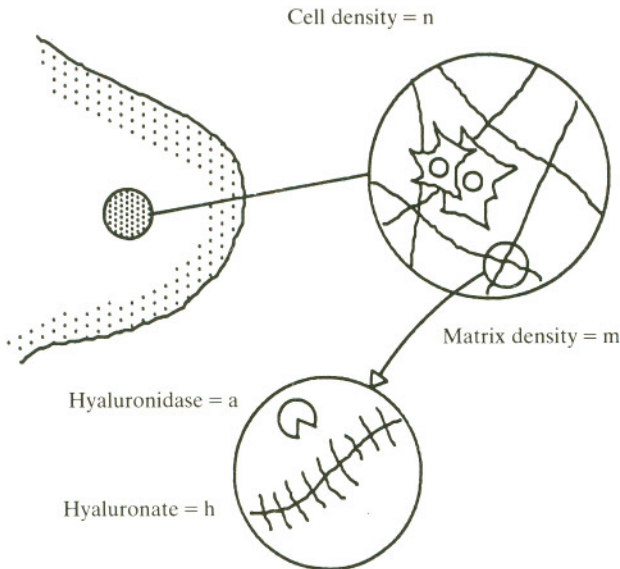


Fig. 3. The model consists of five balance equations: mass balances for the cells ( $n$ ), matrix ( $m$ ), hyaluronate ( $h$ ) and hyaluronidase ( $a$ ), and a force balance between the viscous, osmotic, elastic and cell traction forces, which describes the distribution of strains in the tissue. The boundary conditions include the hyaluronate 'sleeve' which encases the limb bud.



by convection; that is, by being passively dragged along by deformations of the matrix. These deformations are governed by the force balance equation:

$$0 = \Sigma \text{ Forces} = [\text{Viscous drag forces between the solid and fluid components of the tissue}] + [\text{Passive elastic forces of the cells and matrix}] + [\text{Osmotic swelling pressure}] + [\text{Active cell traction forces}] \quad [3e]$$

An important aspect of the elastic and cell traction forces is that they are 'long range': because the packing density in the condensing regions is so high cells can extend filopodia and interact mechanically with cells beyond their nearest neighbours. The conservation equations for the cells and matrix material are mathematically identical. This, together with the long-range effects associated with the high cell-matrix densities permits certain simplifying approximations which reduce the model to an even more transparent form involving only two quantities: the force balance and the hyaluronidase concentration. These equations have the same formal structure as diffusion-reaction equations familiar from morphogen-based models (e.g. Murray, 1977, 1981; Meinhardt, 1982; Segel, 1984):

$$[\text{Rate of change of strain } (\partial\epsilon/\partial t)] = \frac{\delta\partial^2\epsilon/\partial x^2}{\text{DISPERSION OF STRAIN}} + \frac{F(\epsilon,a)}{\text{PRODUCTION OF STRAIN}} \quad [4a]$$

$$[\text{Rate of change of HAase } (\partial a/\partial t)] = \frac{D\partial^2 a/\partial x^2}{\text{DIFFUSION OF HAase}} + \frac{G(\epsilon,a)}{\text{REACTION OF HAase}} \quad [4b]$$

where  $\epsilon$  is the mechanical strain (i.e. the fractional deformation) and  $\delta$  is a 'diffusion coefficient' which contains the elastic moduli of the extracellular matrix material.  $G(\epsilon,a)$  is the 'reaction' which accounts for production and degradation of hyaluronidase, but  $F(\epsilon,a)$  is a 'reaction' in formal terms only: it contains the osmotic, elastic and cell traction forces. Thus it has a form that has no obvious analog in chemical kinetics.

It should be emphasized that there is only a formal mathematical similarity between equations [4] and those of reaction-diffusion models based on morphogens. The motivation and mechanisms underlying both models are quite different. Furthermore, in our model the variables (cells, matrix, HA, HAase, and strain) are all quantities whose properties are readily measurable with conventional assays.

In the Appendix we show how these equations conspire to produce spatial patterns from an initially homogeneous distribution of cells and matrix. That is, the equations mimic the scenario described in Fig. 1:

- (a) Cells emerge from the progress zone secreting hyaluronate. The hydration of the hyaluronate inflates the tissue, and inhibits intercellular contacts.
- (b) After leaving the progress zone the balance between the cells' production of



hyaluronate and hyaluronidase shifts toward hyaluronidase. This initiates an osmotic deswelling of the tissue which brings the cells closer together.

- (c) When the collapsing matrix increases the cell density so that intercellular contacts commence to increase significantly, the intercellular traction forces come into play.
- (d) At this stage, the deswelling is sufficient so that the interplay between the osmotic swelling, tissue elasticity, and cell traction conspire to cause a condensation of cells into the dense aggregation which presages chondrogenesis.

#### *Bifurcations of spatial patterns*

What is not apparent from the verbal description of the model equations is why any particular spatial pattern of condensations should emerge. In particular, if condensation does occur, why should more than one appear, and why in any particular spatial arrangement?

The reason for this phenomenon is not easy to explain qualitatively. Mathematically, the evolution of the homogeneous state (i.e. uniform cell and matrix density) to a non-homogeneous state occurs for very much the same reasons familiar from chemical systems: at some set of conditions the uniform state becomes unstable and 'bifurcates' into a spatially non-uniform state (c.f. OMH; Segel, 1984). Roughly, the reason is as follows.

At the onset of condensation, each volume element is in a state of mechanical balance between the expansive osmotic pressure and the contractive cell tractions. As the cell tractions come to dominate, and condensation commences, it does so about certain foci. The nature of the contraction focus is autocatalytic: once a focus of contraction nucleates, it recruits to itself cells and matrix from the surrounding tissue. However, the 'range of influence', or mechanical domain, of a contraction focus is limited by the elastic nature of the cell-matrix medium. Therefore, some distance away from a contraction focus, another focus can form. The distance between foci depends on the properties of the tissue, such as its elastic properties and the scale and geometry of the system. Foci need not be points; line condensations will form in a cylindrical domain. Subsequently, such line condensations can break up into smaller condensations. Indeed, the nature of the bifurcation process dictates that only certain patterns of chondrogenic condensations are admissible (Alberch *et al.* 1985).

#### *The admissible types of bifurcations*

According to the model, in a domain of cylindrical or elliptical cross section, there are three basic types of bifurcation, as illustrated in Fig. 4 (c.f. fig. 10 of OMH). We shall call these axial (A), transverse (T), and longitudinal (L) bifurcations. Type A bifurcations produce the first axial condensations (femur, humerus) from an initially homogeneous tissue. The splitting of the femur into the

tibia and fibula is an example of a type T bifurcation, and the formation of the phalanges exemplifies a type L bifurcation.

Successive condensations can start in one of three ways. A type A condensation may appear *de novo* in a previously homogeneous region. A type T condensation may branch off from an existing condensation, forming a Y-shaped juncture joining the branches. This junctional region may later disappear as the cells are recruited into the main condensations. Alternatively, the junctional region may be devoid of cells (c.f. OMH). A type L condensation may also appear in two ways: as a splitting of an existing columnar condensation, or a new condensation appearing as an extension of an existing one. The model generally precludes 'trifurcations', i.e. triple splittings of an existing condensation. However, because the model equation [4a] is a tensor equation it is possible – albeit unlikely – that trifurcations could exist. More likely, an L bifurcation that follows a T which appears as a trifurcation may be resolved into binary processes.

As the limb bud grows, it is reasonable to suppose that the model parameters vary smoothly from the progress zone proximally. If so, one expects that the condensations will form sequentially in proximal to distal order. Therefore, the progression of condensations will appear as a sequence of T and L bifurcations. In general, we expect that at a level where two or more condensations coexist (e.g. radius and ulna) when one of the elements bifurcates (either A or T), the bifurcation of the other element will be delayed distally. This is because a condensation has a 'domain of influence' wherein it recruits cells into itself. An

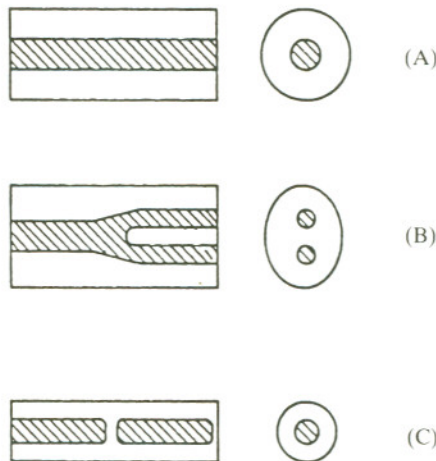


Fig. 4. The three types of bifurcations generated by the model. (A) Axial (type A) bifurcation initiates an axial condensation in a cylindrical domain from an initially homogeneous cell distribution. (B) A transverse bifurcation (type T) splits an axial condensation into a Y-shaped pattern, producing a doubling of the original condensation. The two arms of the Y may be unequally proportioned and/or eccentrically located according to the shape of the domain and the tissue parameters. (C) A longitudinal (type L) bifurcation divides an axial condensation perpendicular to the long axis of the cylinder. The size and proportions of the segments depends on the tissue geometry and the parameter values.



initiating condensation will tend to coopt recruitment by creating a focus of compressive stress. This will tend to inhibit nearby condensations until they grow past the stress focus and can commence their own bifurcation centre. That is, condensation distorts the stress field near the bifurcation site that inhibits the surrounding foci from bifurcating.

In a broad, flat tissue expanse, such as the distal 'palm' area of the forelimb, it is possible for several isolated condensations to arise independently. That is, there is enough space and tissue for separate foci to appear, and there need not be a strict proximal to distal order (Alberch, Murray & Oster, 1986). If this happens, then each focus can initiate its own progression of T and L bifurcation structures. These structures will grow outward from each centre, and there is some ambiguity as to what happens when the two islands eventually merge. We will discuss how the physics of the condensation process may constrain the possible limb morphologies in a subsequent publication. Here we shall only point out that the model puts severe constraints on the developmental process.

*The bifurcations are controlled by dimensionless parameter ratios*

Each of these bifurcations can be triggered by variations in material parameters, geometry, and/or tissue size. As discussed in OMH, the quantities that control the bifurcations are dimensionless ratios of the physical parameters. Thus a bifurcation may not be attributable to a unitary cause, but may be brought about by the interaction of several effects. One of the contributions of the model is to delineate the balance of effects which may precipitate a condensation or a splitting of an existing condensation.

For example, in most species, as the limb grows distally it generally flattens into a paddle shape. In OMH we attributed this phenomenon to the tractions generated by the condensing chondroblasts. In the present model the deswelling of the ECM is a force tending to flatten the limb from its cylindrical proximal geometry into its elliptical distal cross section. At some degree of ellipticity a bifurcation can be triggered (e.g. femur to tibia-fibula). However, the bifurcation may be deferred or promoted by variations in other parameters, according to how they enter into the dimensionless ratios.

*Reswelling of the ECM and the formation of the perichondrion*

Following the formation of a condensation, the perichondrial membrane forms around the cell aggregate. If this constraining envelope is prevented from forming, the condensation frequently disappears. A clue to the possible origin of this structure lies in the orientation of the chondrocytes within the condensation.

Cells at the centre of the condensation tend to become rounded, while peripheral cells are flattened circumferentially (Rooney, Archer & Wolpert, 1984). The model suggests a mechanism for this. If, after condensation, the cells recommence secreting hyaluronate (and/or other hydrophilic matrix components) then the centre of the condensation will tend to reswell. When this happens, a pattern

of strains will be set up in the ECM. This pattern of strains can be computed from elementary considerations, as described in the Appendix.

It turns out that the stress pattern in a swelling cylinder is such that the circumferential ('hoop') stress is twice the longitudinal (axial) stress (this is why a boiled hot dog always splits lengthwise, not transversely). Thus the reexpanding matrix in the centre of the condensation will cause fibres to align circumferentially at the periphery of the condensation. Since cells embedded in a fibrous matrix are known to align along strain directions, the reexpansion will create the observed pattern of cell geometries. If there is some mechanism that causes the flattened peripheral cells to differentiate into perichondrial tissue, then the condensation will be consolidated and will not disperse. While it is tempting to speculate on the role of cell shape in triggering such differentiations (c.f. Zanetti & Solursh, 1984), the scope of this model is limited to generating the appropriate geometrical configurations of cell aggregations.

#### DISCUSSION

We have described the phenomenon of matrix deswelling coupled with cell tractions as a mechanism for generating the patterns of cell aggregation that accompany chondrogenesis. The model does not depend on active cell crawling, as does the previous model discussed in OMH. However, there is no difficulty in adding cell motility to the model, and as expected, the pattern-forming capabilities are enlarged. The point of the present model is to demonstrate that cell crawling is not strictly necessary for producing regular aggregations of mesenchymal cells.

There are other instances of morphogenetic processes which commence as aggregations of mesenchymal cells. In OMH we discussed the formation of feather germs by the cell traction mechanism which depended on active cell crawling. However, the same deswelling/traction mechanism discussed here can accomplish the formation of the feather germ papillae. Indeed, it is easy to see that a combination of the two processes would be an even more effective pattern generator. Theory can only point out the physical possibilities; experiments are required to distinguish between them. If the osmotic swelling of the ECM is a crucial factor in creating cell aggregations, then experiments which modulate this force should have a profound morphogenetic effect. In this context the polyelectrolyte nature of the hyaluronate may permit experiments which modify the extracellular ionic strength and/or pH without unduly disrupting normal cell function. Antibodies to hyaluronidase are also a possible mode of intervention. We note that cytoskeletal agents such as cytochalasin which disrupt the contractile abilities of cells should be distinguishable from agents which affect the swelling pressure of the ECM. Indeed, recent experiments suggest that the profound morphogenetic aberrations produced by retinoic acid may derive from its influence on cellular production of hyaluronate (Kochhar, Penner & Hickey, 1984). From the viewpoint of the present model it is easy to see how disrupting the hyaluronate/hyaluronidase system can alter morphogenetic patterns.



Finally, the model does not address the issue of what cues the switch in the hyaluronate/hyaluronidase system which initiates condensation near the progress zone. This may well be a proximodistal gradient of some sort, or a cell lineage and/or ageing effect.

P. Alberch first called our attention to the relevant experiments on the role of ECM in chondrogenesis, and suggested modifying the original cell traction model to include the effects of matrix deswelling. Conversations with Julian Lewis were crucial to the development of the model, as were conversations with Nigel Holder, Claudio Stern and Lewis Wolpert. Albert Harris provided valuable advice and criticism. Support for this work was provided by grants from N.S.F. (MCS-8110557) to GFO and from the Science and Engineering Research Council of Great Britain (GR/c/63595). PKM would like to acknowledge the research studentship support from the Department of Education of Northern Ireland. This work was performed at the Centre for Mathematical Biology, at the University of Oxford.

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## APPENDIX

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## THE MODEL EQUATIONS

The model is built on the following field variables:

$n(x,t)$  = the density of chondrocytes at position  $x$  and time  $t$ .

$m(x,t)$  = the density of the non-osmotic components of the extracellular matrix at position  $x$  and time  $t$ .

$h(x,t)$  = the density of hyaluronate at position  $x$  and time  $t$ .

$a(x,t)$  = the density of hyaluronidase at position  $x$  and time  $t$ .

$u(x,t)$  = the displacement of a material point initially at  $x$  at time  $t$ .

A.1 *The stress equation:*

$$\nabla \cdot \sigma = 0 \quad [\text{A1a}]$$

where the stress tensor per unit mass of matrix,  $\sigma$ , is given by

$$\begin{aligned} \sigma &= E[\epsilon - L_1 \nabla^2 \epsilon] && = \text{Elastic stress} \\ &+ \mu \partial \epsilon / \partial t && = \text{Viscous stress} \\ &- \Pi(h, \epsilon) \mathbf{I} && = \text{Osmotic pressure} \\ &+ \tau(n)[n + L_2 \nabla^2 n] \mathbf{I} && = \text{Cell tractions} \end{aligned} \quad [\text{A1b}]$$

where  $\epsilon = \nabla u + \nabla u^T$  is the (linear) strain,  $E$  the Young's modulus,  $\mu$  is the viscosity,  $\mathbf{I}$  is the unit tensor, and the coefficients  $L_1$  and  $L_2$  govern the magnitude



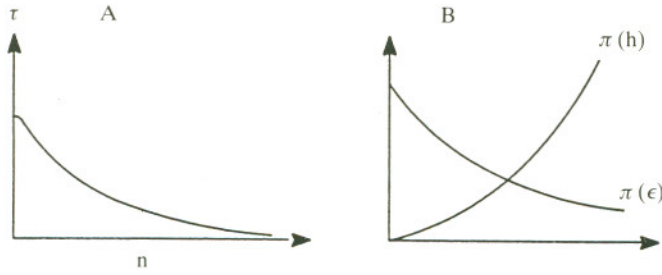


Fig. 5. The constitutive relations employed in the model equations [A1c,d]. (A) The cell traction decreases with cell density, since at high cell densities the cells are inhibited and only cells at the periphery of the condensation are effective. (B) The osmotic pressure decreases as the matrix dilates, and increases faster than linearly as the hyaluronate concentration increases.

of the second order strains, which arise from long range interactions (c.f. Oster *et al.* 1983). The qualitative shapes of the functions  $\tau(\cdot)$  and  $\Pi(\cdot, \cdot)$  are shown in Fig. 5. For computational purposes we choose the following convenient parametrizations

$$\tau(n) = \tau / (K + n^2) \quad [\text{A1c}]$$

$$\Pi(h, \epsilon) = \Pi h^2 / (1 + \epsilon) \quad [\text{A1d}]$$

where  $\tau$ ,  $K$  and  $\Pi$  are constant parameters – physical properties of the medium which are measurable by mechanical means.

#### A.2 Conservation equations for cells and matrix

Here we assume that mitosis and matrix secretion are not major effects during the aggregation stage.

*The equation for cell density:*

$$\partial n / \partial t = -\nabla \cdot (n \partial u / \partial t) \quad [\text{A2}]$$

*The non-osmotic component of the ECM*

$$\partial m / \partial t = -\nabla \cdot (m \partial u / \partial t) \quad [\text{A3}]$$

*The hyaluronate component of the ECM*

$$\partial h / \partial t = -\nabla \cdot (h \partial u / \partial t) + S_h - D_h \quad [\text{A4a}]$$

where  $S_h$  is the secretion rate of HA per cell and  $D_h$  is the rate of degradation of HA by HAase. The qualitative shapes of these functions are shown in Fig. 6A. For computational purposes we have parametrized these functions as follows:

$$S_h - D_h \equiv F_1(n, h, a) = B_0 h n / (K_0 + h^2)(K_1 + n) - B_1 h a / (K_2 + h) \quad [\text{A4b}]$$

where  $B_0$ ,  $K_0$ ,  $K_1$ ,  $B_1$  and  $K_2$  are constants.

*Hyaluronidase (HAase)*

$$\partial a / \partial t = D \nabla^2 a - \nabla \cdot (a \partial u / \partial t) + S_a - D_a \quad [A5a]$$

where  $S_a$  and  $D_a$  are the rates of secretion and degradation of HAase, respectively, and  $D$  is the diffusion coefficient. The shapes of these functions are shown in Fig. 6B.  $S_a$  is sigmoidally increasing, which embodies our assumption that HAase

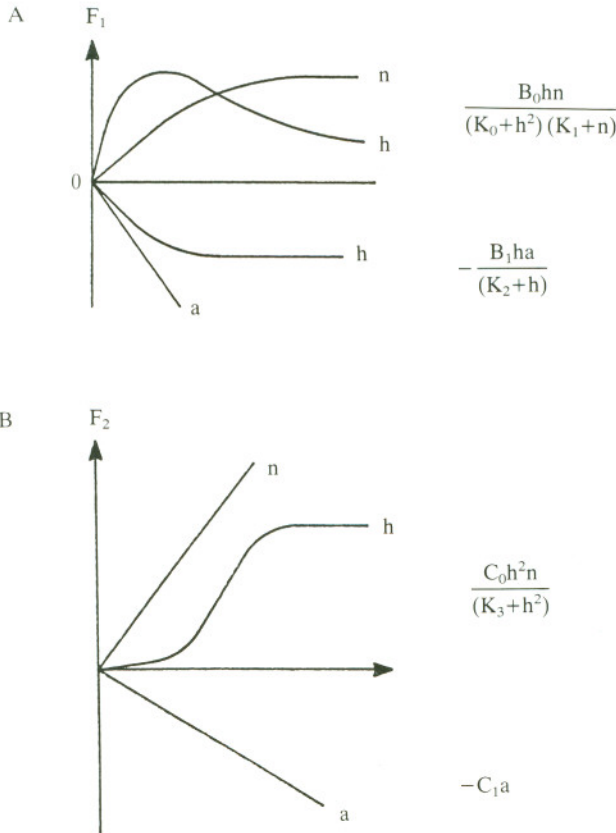


Fig. 6. The constitutive relations employed in the model equations [A4b] and [A5a]. (A) The rate at which cells secrete HA increases monotonically with the number of cells; however, it must saturate at some point. We assume that the secretion rate of HA is self-inhibiting: if too much HA is produced, resulting in hyper-hydration of the ECM, the cells decrease their production rate of HA. The rate at which HA is degraded by HAase increases linearly with the amount of the enzyme,  $a$ , but for a fixed enzyme concentration the degradation follows the usual Michaelis-Menten saturation kinetics. (B) Hyaluronidase production increases linearly with the cell density. For a given cell density, the onset of production is assumed sigmoidal in  $h$ : this models a 'trigger' mechanism that turns on HAase production when the HA concentration rises too high. Degradation follows first order kinetics.



production commences when the HA component of the ECM reaches a threshold value. The parametrizations we have employed are:

$$S_a - D_a \equiv F_2(n, h, a) = C_0 h^2 n / (K_3 + h^2) - C_1 a \quad [A5b]$$

where  $C_0$ ,  $C_1$ , and  $K_3$  are constants. The quantities  $\tau$  (cell traction) and  $C_0$  (HAase production) are the principle parameters controlling the emergence of spatial patterns. Therefore, we shall investigate the model's behaviour as these parameters are varied.

### B.1 One-dimensional equations

Linear analysis can be carried out for the full system; however, for illustrative purposes we shall consider the case with only one spatial dimension. The above equations, with subscripts  $t$  and  $x$  referring to differentiating in time and space, become respectively:

$$\text{Cells:} \quad n_t + (n u_t)_x = 0 \quad [B1]$$

$$\text{Matrix:} \quad m_t + (m u_t)_x = 0 \quad [B2]$$

$$\begin{aligned} \text{Hyaluronate:} \quad h_t + (h u_t)_x &= F_1(n, h, a) \\ &= B_0 h n / (K_0 + h^2) (K_1 + n) - B_1 h a / (K_2 + h) \end{aligned} \quad [B3]$$

$$\begin{aligned} \text{Hyaluronidase:} \quad a_t + (a u_t)_x &= D a_{xx} + F_2(n, h, a) \\ &= D a_{xx} + C_0 h^2 n / (K_3 + h^2) - C_1 a \end{aligned} \quad [B4]$$

$$\begin{aligned} \text{Stress:} \quad 0 = \partial \sigma / \partial x &= \partial / \partial x \{ \mu \epsilon_t + E(\epsilon - L_1 \epsilon_{xx}) + \\ &\quad \tau n / (K + n^2) - \Pi h^2 / (1 + \epsilon) \} \end{aligned} \quad [B5]$$

In [B2] we have set  $L_2 = 0$ ; this will not qualitatively affect our results.

#### *A simplified version of the model*

During the aggregation process, we believe that only small strains are generated. Therefore, we can make the following approximations obtained by integrating the linearized forms of [B1] and [B2]:

$$n \approx N(1 - \epsilon), \quad [B6a]$$

$$m \approx M(1 - \epsilon), \quad [B6b]$$

where  $N$  and  $M$  are positive constants.

We make a further simplification of the system by relating the HA-generated osmotic term in [B5] to the concentration of HAase. Since the presence of HA stimulates the cells to make HAase we can make the following replacement

$$\Pi h^2 / (1 + \epsilon) = P_0 / (1 + \epsilon) - \alpha a \quad [B6c]$$

Where  $P_0$  is a constant. The rationale is as follows: (a) the presence of  $h$  invokes a production of  $a$  which in turn degrades  $h$ ; this is equivalent in effect to the term  $-\alpha a$  in [B6c]. (b) The inverse relationship between strain and osmotic pressure is contained in the term  $P_0 / (1 + \epsilon)$ .

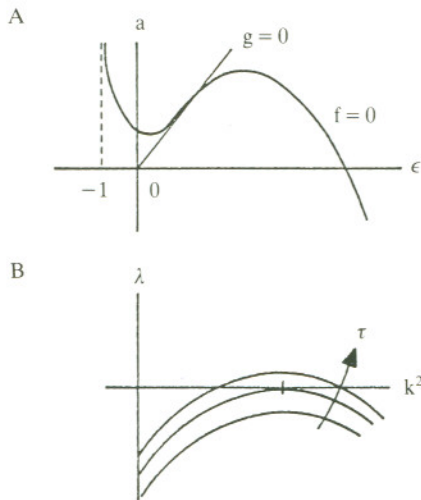


Fig. 7. (A) The nullclines for the system [B11,12]. (B) The dispersion relation  $\lambda(k^2)$  for the simplified model obtained by linearizing equations [B11,12] and substituting trial solutions  $\sim \exp(\lambda t + i k x)$ . The curve exhibits the classical shape characteristic of spatial instabilities: i.e. there is a finite range of unstable wavelengths.

Substituting these approximations into [B1-4] and the integrated form of [B5] uncouples the system to yield the following equations of motion which, although caricatures of the original system, nonetheless capture the essential physics:

$$\mu \epsilon_t = E L_1 \epsilon_{xx} + \left[ \sigma_0 - E \epsilon - \frac{\tau N (1 - \epsilon)}{K + [N(1 - \epsilon)]^2} + \frac{P_0}{1 + \epsilon} - \alpha a \right] \quad [\text{B7a}]$$

$$= E L_1 \epsilon_{xx} + F(\epsilon, a) \quad [\text{B7b}]$$

where  $\sigma_0$  is the constant stress at the boundary; we discuss this below.

The equation of HAase can be simplified as well. Since the production of  $a$  is high when the cells are less densely packed, the production term in [A5b] increases with strain,  $\epsilon$  (i.e. higher strains accompany dense cell packing). Thus we can write the equation for  $a$  as:

$$a_t = D a_{xx} + v \epsilon - \omega a \quad [\text{B8}]$$

where  $v$  is the production rate constant for  $a$  and  $\omega$  is just  $C_1$  from [A5b].

Thus we have reduced the model to a pair of equations for the strain,  $\epsilon$ , and the hyaluronidase,  $a$ . Note that these equations have the structure of a diffusion reaction system; however, the 'reaction' term in the mechanical equation [B7] is not one that would arise obviously from chemical kinetics.

A crucial issue is what boundary conditions to impose on the model equations. We base our assumptions concerning the boundary conditions on the observation that the limb bud is encased in a 'sleeve' of hyaluronate that appears to be secreted by the epithelium, or by dermal cells near the outer surface. This sleeve is never



degraded by hyaluronidase, and so keeps the limb bud 'inflated' even while the chondrogenic condensations are proceeding within the limb. Thus the boundary condition on the stress is:

$$\sigma(x = 0, t) = \sigma(x = l, t) = \sigma_0 \quad [B9]$$

*Dimensionless equations*

As always, we can reduce the parameter count, as well as illuminate the physics by rendering the equations [B7,8] dimensionless. Therefore, we scale the quantities as follows (where \* denotes dimensionless variables)

$$\begin{aligned} t^* &= t/T, \quad x^* = x/L, \quad a^* = a/a_0, \quad \sigma_0^* = T\sigma_0/\mu, \quad \delta^* = L_1TE/L^2\mu, \quad E^* = ET/\mu, \\ \tau^* &= \tau T/N\mu, \quad K^* = K/N^2, \quad P_0^* = P_0\tau/\mu, \quad \alpha^* = \alpha Ta_0/\mu \end{aligned} \quad [B10]$$

where L, T and  $a_0$  set the scales for size, time and HAase concentration.

The dimensionless equations of motion become (dropping the asterisk for simplicity of notation)

$$\begin{aligned} \epsilon_t &= \delta\epsilon_{xx} + \left[ \sigma_0 - E\epsilon - \frac{\tau(1-\epsilon)}{K + (1-\epsilon)^2} + \frac{P_0}{(1+\epsilon)} - \alpha a \right] \\ &= \delta\epsilon_{xx} + f(\epsilon, a) \end{aligned} \quad [B11]$$

$$\begin{aligned} a_t &= Da_{xx} + v\epsilon - \omega a \\ &= Da_{xx} + g(\epsilon, a) \end{aligned} \quad [B12]$$

We can reduce the number of parameters still further by selecting appropriate time (T) and length (L) scales. For example, if we are interested in the process on a time scale associated with the elastic response time, we choose  $T = \mu/E$ ; then  $E^* \equiv 1$  in [B10].

*B.2 Spatial patterns*

The possibility for generating spatial patterns by the model equations [B11–12] can be appreciated by examining the nullclines of the spatially independent system, as shown in Fig. 7A. The mathematics which demonstrates the existence of spatially structured solutions to equations [B11–12] is quite standard (see, for example, Murray, 1981). These equations are capable of generating a wide variety of spatial patterns; indeed, all of the results obtained by the previous model (c.f. the Appendix of OMH) emerge from the present model, although they are based on different physical assumptions.

Note that the spatial patterns generated by [B11,12] are described by the strain (i.e. the spatial displacement of material points) and the concentrations of HAase. From [B6] the cell density may be computed directly. The scenario for chondrogenesis which emerges from this model is identical to that described in figures 8–10 in OMH.

### B.3 Stress patterns during formation of the perichondrium

Following the condensation of chondroblasts into the dense aggregations that presage cartilage secretion, the perichondrium forms. This is accompanied by a striking sequence of cell shape changes. The cells in the centre of the aggregation round up, and those around the periphery flatten circumferentially. These peripheral cells differentiate into the perichondrium – a membranous container for the cartilage capsule. Indeed, if the perichondrium is prevented from forming, the aggregation frequently disperses, and chondrogenesis fails.

The shapes of the cells at this stage may be a clue as to the physical forces the cells experience. For example, it is known that cells in a fibrous matrix will tend to align themselves along strain directions. A hypothesis that is consistent with the observed distribution of cell shapes following condensation is that following aggregation the central cells recommence secreting hyaluronate (or, what amounts to the same thing, the balance between HA and HAase secretion swings back to HA).

If this happens, the aggregation will swell osmotically, and set up a particular stress distribution in the cell-matrix medium. This stress distribution will be such that the longitudinal stresses will be about half of the circumferential stresses. That is, the 'hoop' stresses are twice the axial stresses (see, for example, Wainwright, Biggs, Currey & Gosline, 1976, pp. 293–294). This is true in any cylinder under internal pressure, which is why a boiled hot-dog always bursts lengthwise, rather than circumferentially.

To see this, consider a hollow cylinder of length  $L$ , radius  $R$ , with a wall thickness of  $t$ , that is under an internal pressure,  $p$ . The circumferential stress is

$$\sigma_{\theta} = \text{'Hoop' Force/Area} = p \cdot 2RL / 2tL = pR/t$$

The longitudinal stress is

$$\sigma_L = \text{Axial Force/Area} = p \cdot \pi R^2 / 2\pi Rt = pR/2t$$

Thus  $\sigma_{\theta} = 2\sigma_L$ .

The consequence of this stress distribution is that cells and ECM at the periphery of the condensation will be under larger hoop stresses than longitudinal stresses. Thus alignment in the circumferential direction is expected for the same reason that cells embedded in a fibrous material will align along the direction the material is stretched. Conversely, cells in the middle of the condensation will experience a more or less isotropic stress environment, and so rehydration of the ECM will lead them to simply round up.

That the perichondrium is a tension-induced structure has been suggested previously (e.g. Wolpert, pers. comm.); we simply point out that, in the context of the present model, the cell orientations accompanying perichondrium formation can be understood in terms of the stress distribution accompanying rehydration of the chondrogenic condensation. This should be a testable hypothesis.