Supporting Text

Model Parameters and Sensitivity

Cell Cultures. Cells were isolated and cultured as described (1). Confluent multipotential vascular mesenchymal cells (VMCs) were trypsinized, strained, and plated in tissue culture dishes (2.5×10^4 cells per cm^2) to achieve ≈75% confluency. Cells were cytochemically stained for alkaline phosphatase or for calcium mineral deposits by using the von Kossa silver nitrate method (1). In the von Kossa method, only the mineralized central portion of the ridge is stained black, thus the black regions in Figs. 1*d* and 4 *a*, *c*, *e*, and *g* do not reflect the full thickness of the ridges. The present studies were performed by using the more rapidly mineralizing type of VMC (2, 3). Our findings may well also apply to the slow-rapid populations to the extent that similar patterns emerge in both types of VMC, differing only in rate of formation. Warfarin (4 nM) was added at day 2 of culture and with each medium change (approximately every 3 days) through the time of culture, another 27 days. Matrix carboxyglutamic acid protein (MGP) (40 nM) was also added at the time of plating and with each medium change. Treatment was applied from day 1 through the entire culture period.

Mathematical Model. We modeled the cell culture as a 2D medium, using Eqs. **1** and **2**, assuming that the small "pile-ups" of cells at the aggregation points do not essentially change the biology. Our 2D spatial domain was discretized with a uniform mesh (100 × 100; the results remained stable when finer meshes were used). The diffusion terms were discretized by using explicit second-order finite differencing; we updated the concentrations of U and V by explicit time marching using a second order Runge-Kutta time integration scheme. In all simulations, we use no-flux boundary conditions and initial concentrations of U and V that are uniformly distributed near their equilibrium values, with a 2% random fluctuation.

Movies. We include two movies made from the simulations. Both show the evolution of the initial pattern (i.e., Fig. 2). Movie 1 is from the simulation for Fig. 3, and Movie 2 is from the simulation for Fig. 4.

Parameter Selection and Sensitivity. We follow the approach of a recent report by Eldar *et al*. (4), which provides a good discussion of parameter selection and sensitivity questions for modeling cell migration patterns in a model of embryonic pattern formation.

Diffusion coefficients. To estimate the ratio of activator/inhibitor diffusivity, parameter D, we first estimated the diffusivity of each molecule from experimental data. Diffusion of the activator, bone morphogenetic protein-2 (BMP-2), was estimated from diffusivity of its homologue, decapentaplegic (Dpp), which was directly measured by Entchev *et al.* (5) Using GFP labeling and imaging, they found a rate in their assay corresponding to $\approx 1 \times 10^{-8}$ cm^2/sec. Because Dpp can rescue BMP-2 deficiency *in vivo*, these proteins are likely to have similar structure and diffusion behavior.

We also took into consideration the nonlinear slowing of diffusion of large molecules by extracellular matrix. Papadopoulos *et al.* (6) found that diffusivity of larger molecules (50-200 kDa) is reduced as much as 10- to 20-fold in muscle tissue. Similarly, Baxter *et al.* (7) reported that the diffusion coefficient for 70-kDa dextran in cheek pouch tissue was reduced to 2×10^{-9} cm^2/sec. BMP-2 diffusion also may be reduced by its tendency to dimerize (8) and to unfold in acid pH (9). These considerations would reduce the estimated diffusivity of BMP-2 to $\approx 0.1 \times 10^{-8}$ cm^2/sec. Because tissue culture may present less resistance to diffusion than muscle tissue, we estimated a final value of 0.15×10^{-8} cm^2/sec for BMP-2 diffusivity. Thus we choose $D_U = 0.15 \times 10^{-8}$ cm^2/sec.

Diffusivity of the inhibitor, MGP, has not been directly measured. A theoretical approximation can be made based on MGP's 10-kDa molecular mass (10). Using empirical formulas (11-14), the diffusivity of MGP can be estimated at $\approx 30 \times 10^{-8}$ cm^2/sec. This is realistic when compared with the known diffusion coefficient of 50 ×

10^{-8} cm^2/sec for amyloid B, which has a molecular mass of 4 kDa (15). The small size of MGP makes it less subject to the nonlinear slowing described by Papadopoulos *et al.* (6). Thus we choose $D_V = 30 \times 10^{-8}$ cm^2/sec. This yields a value of ≈1/200 for the ratio of BMP-2 to MGP diffusivity. This is the value of $D = D_U/D_V$ we used in the simulation.

Length scale. The length of the domain is based on actual well sizes used in tissue culture, with a diameter range of 1-4 cm. Thus we took $L = 4$ cm.

Autocatalysis. Ghosh-Choudhury *et al.* (16) previously showed that BMP-2 autoregulates in a saturating manner. The value of k sets the saturating value for specific values of V; however, because the level of V (i.e., MGP) was not known in those experiments, it is difficult to define its precise value.

Degradation and production rates. The production rates for MGP and BMP-2 were estimated experimentally to provide a time scale for the equation for the scaling factor, γ. In unpublished work, we transfected a cell line of embryonic kidney cells (HEK-293) with expression vectors for BMP-2 with a cytomegalovirus (CMV) promoter. By ELISA, these cells release 20-125 ng/ml of BMP-2 into 7-8 ml of supernatant in 48 h, i.e., ≈3-20 ng/hr. This would be an upper limit of the production, because the CMV promoter is known to have a relatively high transcription rate. In additional unpublished findings, we assessed the production of BMP-2 in calcifying vascular cells and in endothelial cells. Rates were ≈0.06-0.12 ng/hr for both cell types.

Using biological inactivation of BMP-2 by MGP, we are able to estimate by bioassay that the production rates of BMP-2 and MGP are similar (17). These estimations have been verified by using a FLAG-tagged MGP (18) and a newly developed ELISA (K.B., unpublished results). Altogether, these data yield production rate estimates ranging from 0.06 to 20 ng/hr for both BMP-2 and MGP. This production rate was used to establish a value for the scaling parameter, γ

Although it was less successful, we also attempted to assess production using a promoter-reporter construct. We transfected the U2-OS bone cell line with a construct consisting of the MGP promoter and a luciferase reporter. Luciferase activity was measured at ≈3,000 activity units per day. However, the conversion from activity units to molecules of luciferase could not be reliably determined due to reagent variation, and the results vary excessively with transfection efficiency.

Degradation. Entchev *et al.* (5) estimated the upper limit of proteolytic degradation of the BMP-2 homologue, Dpp, as 5% of production rate. Therefore, we conservatively estimated BMP-2 degradation as 1% of production rate (thus we took $c = 0.01$). The primary form of "degradation" expected for both BMP-2 and MGP is sequestration into extracellular matrix (18). Based on unpublished work, we have found that MGP is taken up more avidly by matrix than is BMP-2, with an approximate ratio of 2:1. Therefore, we estimated MGP degradation to be 2% of production rate ($e = 0.02$).

Source term. The value of the source term, S, was chosen as follows. A typical concentration of MGP in the supernatant of CVC is 120 ng/ml. This corresponds to 12 nM (10 kDa = 10,000 gm/mol). Exogenous MGP was added at 40 nM, thus approximately tripling the concentration. For the model, the initial MGP concentration was scaled as 2. The source term, S**,** was chosen as 0.006 per time step. This was applied for 1,000 time steps, yielding a tripling of the concentration to 6. This tripling corresponds to the actual tripling of the MGP concentration in the cell culture.

Scaling parameter (γ). The parameter γ is equal to $(L^2/D_V)(1/T_C)$, where T_C is the characteristic time scale of the biological kinetics, based on the time required for BMP-2 synthesis, which we take to be ≈1 ng/hr (3.6×10^3 sec). Thus we estimated γ by calculating

$$\gamma = \left(\frac{L^2}{D_V}\right)\left(\frac{1}{T_C}\right) = \left(\frac{16\ cm^2}{3\times10^{-7}\ cm^2/\sec}\right)\left(\frac{1}{3.6\times10^3 \sec}\right) \approx 15{,}000$$

which is the value of γ we used in the simulation.

Initial conditions. The model was run from initial conditions of $U = U_0$ and $V = V_o$, where U_0 and V_o are the equilibrium values of the pure kinetics. To test the robustness of the model to initial conditions, we also varied these initial conditions over a 10-fold range.

Validity of the Model. In this particular reaction-diffusion model, features of the patterns are sensitive to changes in some of the parameters, such as k and γ, but not to others, such as the diffusion coefficients (19). Significant changes (e.g., doublings) in γ generate the mode-doubling as described, and significant changes in S or k can alter the pattern from stripes to spots. For some of the parameters, the values do not influence the ultimate predominant pattern, only the transitional stages leading to it. In the case of the diffusion coefficients, the values may vary substantially without influencing the results, as long as the inhibitor diffuses more rapidly. Within this inequality, the ratio can change by orders of magnitude, and pattern formation is preserved in a robust manner. The significance of the sensitivity of certain parameters is an interesting issue; one possibility is that it represents a mechanism for limiting pattern formation to certain cell types and conditions.

The patterns in this experimental system are also reproduced by other reaction-diffusion models (19). The family of reaction-diffusion equations have in common rapid diffusion of the inhibitor and nonlinear inhibition and activator autocatalysis. We selected this particular reaction-diffusion model because the structure of the equations corresponded best to known physiological variables and relationships in our experimental preparation. Our finding that these cells self organize according to reaction-diffusion principles may ultimately be modeled by other members of the family of reaction-diffusion models as more is learned about the physiology of these cells. What is remarkable here is that our experimental preparations have produced patterns that are strikingly like those produced by an entire family of reaction-diffusion models.

The population we are modeling here is of a single cell type and two of its morphogens. Other investigators have looked at systems that involve more than one cell type (see, for example, ref. 20). Such heterogeneous systems of pattern forming cells are often found in embryologic phenomena. Our homogeneous model may be more typical of the situation in adult tissue.

It is important to note that the patterns we see in the culture dishes are patterns of cells, but the patterns in our mathematical model are patterns of concentration of a chemical, the "activator" morphogen. We focused on a two-variable model (activator U and inhibitor V) on the grounds that this model predicted the spatial patterns correctly, without requiring any assumptions about cell behavior. Arguably, the minimal model that explains the patterns is the best model; following Occam's razor, we choose the simpler explanation.

In addition, we are following the excellent study by Kondo and Asai (21), which uses a model that, like ours, is a two-variable reaction-diffusion equation, with a chemical activator and inhibitor. And, as in our study, they compare an observed pattern of cells with a simulated pattern of activator chemical, which they set in correspondence to each other ("The striking similarity between the actual and simulated pattern rearrangement strongly suggests that a reaction-diffusion wave is a viable mechanism for the stripe pattern of *Pomacanthus*").

Although we do not think a model including cells is necessary to explain the patterns, we also studied a three-variable model, including cells, following Keller and Segel (22), and more recently, the model of Painter, Maini, and Othmer (23). In addition to the activator u and inhibitor v, we add a variable n representing cell density. A differential equation for n was derived following Painter, Maini, and Othmer, which combines a tendency to diffuse with a tendency to follow the activator chemical U. The full model would be:

$$\frac{\partial u}{\partial t} = \nabla \bullet (D\nabla u) + f(u,v)$$
$$\frac{\partial v}{\partial t} = \nabla \bullet (\nabla v) + g(u,v)$$
$$\frac{\partial n}{\partial t} = \nabla \bullet \left(\nabla n - \frac{\chi_0}{(1+u^2)} n \nabla u \right)$$

$$f(u,v) = \gamma \left(\frac{u^2}{(1+ku^2)v} - cu \right)$$
$$g(u,v) = \gamma \, (u^2 - cv + s)$$

We carried out simulations using this three-variable model and found that the predictions of this model are very similar to the predictions of the simplified two-variable model. Fig. 5 illustrates this similarity.

Rationale for model reduction. In retrospect, the correspondence between the patterns of *U* and the patterns of *n* can be understood directly from the equations. Consider the equations simplified to one spatial dimension *x*; at equilibrium, the steady-state solution for *n* is

let the flux $$0 = \frac{\partial}{\partial x} \left(\frac{\partial n}{\partial x} - \frac{\chi_0}{(1+u^2)} n \frac{\partial u}{\partial x} \right)$$

$$J = \frac{\partial n}{\partial x} - \frac{\chi_0}{(1+u^2)} n \frac{\partial u}{\partial x}$$

Because we assume that no cells leave the domain, $J = 0$. Therefore, by integrating directly, we get

$$n = Ce^{\arctan(u)}, \quad where \; C = e^{\chi_0}$$

and so *n* is a monotonically increasing function of *U*. Hence the distribution of *n* at equilibrium should parallel the distribution of *U***.** In other words, the distribution of cells

follows the distribution of activator, hence the third equation adds little explanatory value.

Additional Model Predictions. According to our model, the autocatalysis of U would be predicted to saturate. Experimental data from ref. 16 support this prediction. As shown in Fig. 6, Ghosh-Choudhury (16) report that BMP-2 autocatalysis saturates. In this experiment, BMP-2 production is measured as the transcriptional activity of the BMP-2 promoter ("relative luciferase activity"), which levels off with increasing levels of BMP-2.

In contrast, an important alternative model, also due to Gierer and Meinhardt (24), predicts that U would increase autocatalytically without bound:

$$f(U,V) = -bU + \frac{U^2}{V} .$$

A second prediction of our kinetics is based on the observation by Koch and Meinhardt (25) that the Gierer-Meinhardt kinetic scheme with saturation produces patterns only if the degradation rate of the inhibitor exceeds that of the activator. Because patterns are formed by our model, then it predicts that the inhibitor degrades more rapidly than the activator. The experimental findings indicate the inhibitor is indeed eliminated more rapidly, in our experiments about twice as fast. Koch and Meinhardt (25) give a numerical criterion (in appendix A, ref. 25), which is met by our system. For our model to produce patterns, the ratio of inhibitor degradation rate to activator degradation rate would have to be $>(2/a_0 - 1)$. In the terminology of Koch and Meinhardt (25), a_0 is the equilibrium value of the activator (our U_0), which is ≈ 1.1. The criterion then reduces to requiring that $2 > (2/1.1 - 1)$, or $2 > 0.8$, indicating that our model satisfies the criterion.

A third consideration that lends support to our kinetics is the observation of Murray (19) that the model with saturation is more likely to support stable pattern formation than the model without saturation in the region of our parameter estimates. Because the model

produces stable patterns, it predicts that the parameters fall into a certain region in "Turing space." The biologically determined parameters fall clearly into the pattern formation region for the saturation model but not for the model without saturation (figure 7 in ref. 19). (In our case, $a = 0$, $D = D_V/D_U \approx 200$, and $b = \mu/\nu \approx 0.5$, with μ and ν being the degradation rates of the activator and inhibitor.)

Refinement for case of homologous inhibitors. At very low levels of MGP, such as in the $MGP^{(-/-)}$ mouse, some activity of its homologous genes, such as noggin and chordin, may be induced. This could be accommodated by a minor revision of the model in which V is replaced with $V + C$, where C is a constant representing compensatory inhibitory activity of the homologous genes. Such a simulation was run by using $C = 0.05$; the resulting pattern (shown below) is not significantly affected by the modification. A level of 5% was chosen as an example, because the compensatory activity in the knockout mouse should be significantly lower than normal, otherwise it would not have a phenotype.

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