MULTISCALE MODELING AND SIMULATION OF LIMB CHONDROGENESIS

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by

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CHONDROGENESIS

Abstract
by
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Mathematical and computational models are playing an increasingly prominent role in developmental biology. Many biological phenomena have interactions and behaviors that operate across multiple spatial and temporal scales, so accurate simulation of the phenomena requires those scales to be explicitly modeled. Agent-based modeling is a paradigm, typically discrete and stochastic, whereby entities or agents interact locally with other entities and their environment, and global patterns can emerge from the local interactions of many agents.

In this thesis we describe a discrete, multiscale agent-based stochastic model for the behavior of limb bud precartilage mesenchymal cells in high-density cell culture. The model employs a biologically motivated reaction-diffusion process, and cell-matrix adhesion (haptotaxis), as the bases of chondrogenic pattern formation, whereby multicellular condensations are generated in a self-organizing fashion. The cells are extended, multipixel objects that can change shape in the plane and “round up” by moving pixels into a virtual third dimension. Chemical reactions, molecular diffusion, and diffusion of cells operate on different physical and temporal scales.

We calibrated the model using experimental data and study sensitivity to
changes in key parameters. We have found that not only does this model reproduce the experimental data, but that additional morphogenetic features of the micromass culture system are simulated as well. Simulations show that spot and stripe patterns (which also correspond to the nodules and bars of the developing limb skeleton in vivo), are close in parameter space and can be generated in multiple ways with single parameter variations. Our simulations have disclosed two distinct dynamical regimes for pattern self-organization involving transient or stationary inductive patterns of morphogens. An important implication is that some developmental processes do not require a strict progression from one stable dynamical regime to another, but can occur by a succession of transient dynamical regimes tuned (e.g., by natural selection) to achieve a particular morphological outcome. We discuss these modes of pattern formation in relation to available experimental evidence for the in vitro system, as well as their implications for understanding limb skeletal patterning during embryonic development.
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CHAPTER 1

INTRODUCTION

1.1 Overview

Mathematical and computational models are playing an increasingly prominent role in developmental biology [18]. These models serve to provide tests of the plausibility and robustness of proposed mechanisms of morphogenesis and pattern formation. Equally importantly, they can suggest previously unknown but experimentally testable consequences or hypotheses from known biological interactions, and they afford a convenient way of moving between simulation and experiment.

Models of biological phenomena can be characterized as discrete, continuous, or hybrid. Discrete models represent biological entities as individual distinct computational elements, and these elements are given state, actions, behaviors and interactions that typically have a direct relationship to experimental observations of the corresponding biological entities. Discrete models allow events to only occur at a discrete set of points in time, and they also commonly represent space in discrete units. Continuous models allow events to occur continuously over time, and space is typically also continuous; they use differential equations to describe the evolution of system state variables. Continuous models are well-suited for analyzing large numbers of homogeneous entities with homogeneous interactions.
Hybrid models combine discrete and continuous representations of different biological entities which are then coupled together. Models are also categorized as stochastic or deterministic. Stochastic models use random variables to introduce variability in behaviors, interactions and initial conditions; while deterministic models lack randomness and always produce the same result from the same initial conditions.

The choice of discrete, continuous or hybrid combinations often depends upon the entities being represented and the level of detail desired for the interactions between the entities. In biological applications, continuous models have been used to describe oceanic microbial cycles [8], microbial growth dynamics [49], the spread of species through an ecosystem [53], and biofilm formation [61]. Discrete methods that have been applied to biological systems include cellular automata [17], lattice-gas-based cellular automata [5] [55], the cellular Potts model [23], and agent-based modeling [60].

Agent-based modeling is a paradigm, typically discrete and stochastic, whereby entities or agents interact locally with other entities and their environment, and global patterns can emerge from the local interactions of many agents. Agent-based modeling has gained popularity due to the strong conceptual connection between the modeled entities, their behaviors and their interactions with their implementation as computational elements, which gives them intuitive and explanatory capabilities not provided by other modeling paradigms. Heterogeneity in the agent state, agent behaviors and interactions between agents and the environment can be easily incorporated into agent-based models. The complexity of agent-based models means there are few analytical techniques for studying their global behavior except to run many simulations. However, there is a growing
body of literature that studies continuous limits of discrete microscopic systems \[2, 7, 45, 48, 56\]; though the discrete systems are typically simplified in that agents are point particles without extended shape undergoing random walk. Current research \[8\] looks at the continuous limit of 2D extended cells with cell-cell interaction and cell-cell adhesion.

Of increasing importance in recent modeling efforts is the introduction of multiple spatial and temporal scales. Many biological phenomena have interactions and behaviors that operate across different scales, e.g. cells versus small molecules or proteins, so accurate simulation of the phenomena requires those scales to be explicitly modeled. Multiscale models have been developed for a variety of areas including cancer and tumor growth \[1, 59\], the immune system \[31\], blood clots \[65\], microbial systems \[64\], ecological predator-prey systems \[25\], and morphogenesis \[10, 51\].

In this thesis we describe a discrete, multiscale agent-based stochastic model for the behavior of limb bud precartilage mesenchymal cells in high-density cell culture. The model employs a biologically motivated reaction-diffusion process, and cell-matrix adhesion (haptotaxis), as the bases of chondrogenic pattern formation, whereby the biochemically distinct condensing cells, as well as the size, number and arrangement of the multicellular condensations, are generated in a self-organizing fashion. Improving on an earlier lattice-gas representation of the same process, it is multiscale (i.e., cell and molecular dynamics occur on distinct scales) and the cells are represented as spatially extended objects that can change their shape. We present this model not only as an example of post-hoc analysis of experimental findings, but also as a tool for experimental design and hypothesis testing. In particular, the cellular and molecular features we model (cell movement
and shape change, cell-cell signaling, cell adhesion, among others) are found in a variety of in vitro systems derived from developmental and cancer cell biology. The plausibility and limitations of postulated interactions and mechanisms, for example, reaction-diffusion as the basis for mesenchymal pattern formation, can be judged in simulations with this model, the strength of which is the facility with which it can be revised based on new information and experimental outcome.

1.2 Biology Background

The developing limbs, or limb buds, of embryos of vertebrate organisms such as chickens or mice, contain an interior population of loosely packed cells (“mesenchyme”), enclosed by a thin layer of embryonic skin, or ectoderm. The precartilage mesenchymal cells undergo a process of chondrogenic (i.e., cartilage) pattern formation whereby they first become more tightly packed at discrete sites, forming “condensations.” These precartilage condensations then differentiate into nodules or bars of cartilage which form the primordia of the developing limb skeleton (reviewed in [42]). The limb skeleton of the chicken and most vertebrate groups develops in a proximodistal fashion; that is the skeletal elements closest to the body form temporally earlier and successively more physically distant ones form temporally later.

The limbs of Hamburger and Hamilton stage 24-25 [24] leg buds of 5-day chicken embryos take the form of mounds of tissue (“limb buds”). Cell cultures can be prepared using precartilage mesenchymal tissue isolated from the distal 0.3 mm [9] of these limb buds. When grown in high density (“micromass”) culture, the same limb bud-derived cells undergo pattern formation that is similar in spatial scale and cellular and molecular mechanisms to that in the embryonic
limb. These cell cultures form a limb development subsystem that can be easily manipulated experimentally to further explore the underlying cellular and molecular mechanisms. In this thesis, we strive to model with greater biological fidelity this high density culture system in pursuit of a computational simulation system that can be calibrated with the experimental cell culture system and produce additional biological hypotheses that can be empirically tested.

Skeletal pattern formation in the developing vertebrate limb depends on interactions of precartilage mesenchymal cells with factors that control the spatiotemporal differentiation of cartilage. The most basic skeletogenic processes involve the spatial separation of precartilage mesenchyme into chondrogenic and non-chondrogenic domains. In vitro [34, 39] as well as in vivo [11], TGF-βs and other members of this superfamily of morphogens induce precartilage condensation by a process that involves the upregulation of the secreted extracellular matrix protein, fibronectin [15, 39]. Mesenchymal cells accumulate at sites of fibronectin deposition due to increased cell-matrix adhesive interactions [19, 20, 22] and then transiently adhere directly to one another by virtue of cell-cell adhesion molecules [46, 62], the enhanced expression of which are also induced by TGF-β. Cartilage differentiation, or chondrogenesis, follows at the sites of condensation in vitro and in vivo (reviewed in [44]).

In certain developmental processes, such as angiogenesis (sprouting of capillaries), and invasion by cancer cells of surrounding tissues, pre-existing multicellular structures become more elaborate. Precartilage condensation, in contrast, is an example of a developmental process in which cells that start out as independent entities interact to form multicellular structures. Others in this second category include vasculogenesis (the initial formation of blood vessels), the formation of
feather germs, and the aggregation of social amoebae into streams and fruiting bodies.

Earlier work has suggested that interactions between diffusible activators and inhibitors of chondrogenesis can explain the approximately periodic patterns of chondrogenesis in the developing limb \[26, 40, 43\] and in micromass cultures \[32, 37, 39\]. In particular, the morphogen TGF-\(\beta\) acts as an activator of pre-cartilage condensation by positively regulating its own production \[39\], as well as that of fibronectin \[15, 34\]. The nature of the lateral inhibitor of condensation is more elusive. Recent work suggests that it depends both on signaling via fibroblast growth factor receptor 2 (FGFR2) \[41\] and the juxtacrine (cell-cell contact-activated) Notch receptor \[21\], both of which appear on cells at sites of incipient condensation. Here we assume that the experimentally based lateral inhibitory effect is due to a diffusible morphogen, though other modes of propagation of an inhibitory signal are possible.

1.3 Prior Work

In a previous study by Kiskowski et al. \[32\], a discrete biological lattice gas model for high-density cultures of precartilage mesenchymal cells was derived for the embryonic vertebrate limb. This model, which was based on the physical notion of a lattice gas, in which individual particles are free to move from point to point on a lattice at discrete time steps, accurately simulated the formation of patterns of mesenchymal condensations observed in micromass cultures of such cells. In these simulations the distribution and relative size of the condensations corresponded to in vitro values when appropriate quantities for cell behavioral parameters were chosen, and the simulated patterns were robust against small
variations of these values. Moreover, the simulated patterns were altered similarly
to the cultures when cell density and exposure to, or expression of, molecular
factors represented in the model, were altered in a fashion analogous to their
counterparts in the living system.

In this earlier model each of the limb precartilage mesenchymal cells, and each
molecule from a core subset of the molecules they secrete (the diffusible activator
morphogen TGF-$\beta$, a diffusible inhibitor of TGF-$\beta$s effects, the extracellular ma-
trix (ECM) protein fibronectin), was represented as a single particle (pixel) on a
common grid. Default motion of the cell particles was random, but cell movement
was also biased by the presence of fibronectin particles produced and deposited by
the cells according to a set of rules involving TGF-$\beta$ and inhibitor particles. The
latter in turn were produced in a cell-dependent fashion according to a reaction-
diffusion scheme whose network structure was suggested by in vitro experiments
[34, 39, 41].

While, the model of Kiskowski et al. [32] places cells, morphogens and ECM on
a common spatial grid and interactions operate on the same temporal scale. Ef-
forts by Chaturvedi and coworkers to model the three-dimensional development of
the limb incorporates multiple scales [10, 28]. Their implementation combines the
cellular Potts model, a modified discrete model from statistical mechanics [23], for
representation of the individual cells and their behavior with a set of partial differ-
etial equations for the reaction-diffusion process mapped across the entire spatial
field. To obtain the appropriate patterns for regions of the three-dimensional space
corresponding to different segments of the limb, the reaction-diffusion equations
had to be tuned with different parameters which is an appropriate assumption,
but it leaves open the question of how the limb development process decides to
change from one segment type to another in a self-organizing manner.

The most detailed continuous model for vertebrate limb development is that of Hentschel et al. [26] with a system of eight partial differential equations (PDEs) constructed from cellular-molecular interactions experimentally determined for the avian and mouse limb bud. While this large continuous system is difficult to analyze and numerically solve, it has been determined to have smooth solutions that exist globally in time [6]. In the morphostatic limit, where cell identity is established independently of cell arrangement, the eight PDE system can simplified to a two-equation system for morphogen evolution [4]. Mathematical analysis [6] has shown that spots and stripes are stable patterns in these generalized Turing systems for greater than two dimensions.

1.4 Contribution of This Thesis

The ability of the model of Kiskowski et al. [32] to simulate both qualitative and quantitative aspects of precartilage condensation formation and distribution suggested that the core genetic network-cell behavioral mechanism that underlies this biological lattice gas might be sufficient to account for pattern formation in the limb cell micromass system and corresponding features of in vivo limb development. However, the model deviated from biological reality in several important ways:

- Mesenchymal cells in vitro are initially surrounded by a small layer of ECM which separates them by less than a cell diameter. Those which undergo condensation round up, reducing their surface area, but do not move away from adjacent noncondensing cells. Therefore, unlike the situation in the model of Kiskowski et al. [32], mesenchymal condensation in micromass culture
does not involve accumulation of cells at particular sites with concomitant depletion of cells in surrounding zones.

- The representation of cells, morphogens and ECM on a common grid is physically unrealistic. This is not simply a matter of pixel scale: molecular substances can indeed form deposits and gradients on the same linear scale as cells (∼10 µm), and a molecular pixel could be considered to correspond to thousands of molecules. Nonetheless, the dynamics of morphogen transport is continuous and is represented in an inauthentically saltatory fashion by pixel displacement on a grid of the same mesh size as that supporting cell translocation.

- Whereas the model made the assumption that cells halt their motion when they encounter suprathreshold levels of extracellular fibronectin [32], this does not agree with measurements [13, 16] indicating that cells actually slightly increase their speed of motion as they enter condensation centers and have a finite probability of escaping from these foci.

Despite the successes of the model of Kiskowski et al. [32], it was unknown whether removing its artifactual aspects and replacing them by more realistic assumptions would lead to similarly authentic results. We have therefore designed a more sophisticated model that overcomes each of the listed deficiencies of the earlier one. The cells in the new model are extended, multipixel objects that can change shape in the plane and “round up” by moving pixels into a virtual third dimension. The model cells are separated by less than a cell diameter, condense without denuding the regions surrounding condensation centers, and are not irreversibly trapped upon entering a center. Finally, two grids of different mesh size are used for cell and molecular dynamics.
Our model implements a discrete stochastic reaction-diffusion system of two morphogens similar in form to the simplified morphostatic limit system of Hentchel et al. [4]. One of the key reasons to investigate a discrete, stochastic reaction-diffusion model is to determine how important is the stochastic interplay between reaction-diffusion and cell dynamics for pattern formation of mesenchymal condensation. We feel we have made important contributions in this direction. Besides matching average statistical measures, the simulation pictures look like the micromass pictures. They pass the face validity test. There is variation in the condensations sizes, the condensation shapes, the distances between condensations, and boundaries of the condensations. These are all characteristics which were not available in the previous biological lattice gas cellular automata model, and are a direct consequence of using a discrete, stochastic reaction-diffusion model coupled with a multipixel cell representation. Using a continuous reaction-diffusion would have constructed an “overly perfect” chemical field pattern that avoided the complexities of stochastic behavior at the cellular level.

We calibrated the model using experimental data and study sensitivity to changes in key parameters. We have found that not only does this improved model reproduce the experimental data accounted for by the model of Kiskowski et al., but that additional morphogenetic features of the micromass culture system are simulated as well. Simulations also show that spot and stripe patterns (which also correspond to the nodules and bars of the developing limb skeleton in vivo), are close in parameter space and can be generated in multiple ways with single parameter variations.

Moreover, potential dynamical properties of the developmental process not seen in the earlier simulations, and not capable of being distinguished on the basis
of existing experimental data, were disclosed in simulations using the new model, which has therefore provided motivation for further empirical tests. Our simulations have disclosed two distinct dynamical regimes for pattern self-organization involving transient or stationary inductive patterns of morphogens. An important implication is that some developmental processes do not require a strict progression from one stable dynamical regime to another, but can occur by a succession of transient dynamical regimes tuned (e.g., by natural selection) to achieve a particular morphological outcome. We discuss these modes of pattern formation in relation to available experimental evidence for the in vitro system, as well as their implications for understanding limb skeletal patterning during embryonic development.
CHAPTER 2

COMPUTATIONAL MODEL

In a previous study [32], Kiskowski et al. presented a biological lattice gas model that remains the most successful computational model to date for pattern formation in the limb cell micromass system. The present model more closely reflects biological reality in several important respects. First, the cells in our model are extended, multipixel objects that can change shape. Adjacent cells are separated by less than a cell diameter, condense without denuding the regions surrounding condensation centers, and are not irreversibly trapped once entering a condensation. Chemical reactions, molecular diffusion, and diffusion of cells operate on different physical and temporal scales. Finally, key simulation parameters are calibrated to corresponding physical measurements, where available, including the physical area of a cell, which is equal to the average surface area of a limb cell in the micromass.

In the following sections, we describe the details of our discrete, multiscale agent-based stochastic computational model whereby the cells and molecules are represented as discrete agents that occupy spatial grids of differing resolution and interact according to a pre-defined set of rules.
2.1 Spatial Model

The spatial environment that cells and molecules occupy is modeled as a two-dimensional discrete grid. The implementation provides support for multiple overlaid grids of various spatial scales. In our current model, we only employ two scales; one for the cellular level and another finer resolution scale for the molecular level. The coarsest resolution spatial scale (the cellular level for our model) is considered to be the base spatial scale, and all other grids are an integer ratio size of that base grid. A ratio size of two corresponds to four times the area resolution for a two-dimensional grid, so four pixels of the finer resolution grid fit into one pixel of the base spatial grid. The base spatial grid can be defined as a square or rectangular grid of any height and width, and all of the grids overlay one another and cover the same physical area.

Our model supports both periodic and no-flux boundary conditions. With periodic boundary conditions, cells or molecules crossing a grid boundary will immediately appear on the opposite side of the grid, while with no-flux boundary conditions, the grid boundary acts as a barrier or obstacle that prevents cells and molecules from moving in that direction. No-flux boundary conditions can create situations whereby cells or molecules get stuck in corners or have less possibility of moving away from a boundary. We have not attempted to adjust this behavior at the boundary because such physical obstacles can accurately represent features of the physical domain, i.e., the edge of a cell-culture dish or the epithelial covering of a limb bud.
2.2 Agent-based Cell Representation

Each cell is a discrete agent represented as a set of seven contiguous pixels operating on the base spatial grid as shown in Figure 2.1(A). We chose the simplest multipixel representation of limb mesenchymal cells subject to the following biological constraints: (i) cells have essentially isotropic geometry, that is they do not elongate in the direction of migration but rather probe their environment by extending short randomly oriented projections; (ii) the cell nucleus is also isotropic but is relatively unchanging in shape and comprises more than half the cell volume; (iii) cells in fibronectin-rich, condensing areas of the micromass round up such that their cross-section in the plane of the culture is significantly reduced [13]. We maintain four pixels in a two-by-two square (kernel) configuration that represents the portion of the cell that contains the nucleus and allow the remaining pixels to occupy the border region around the nucleus. Cells that round up shrink their spatial area to five pixels as shown in Figure 2.1(C), the lost pixels moving into a virtual third dimension.

Cell diffusion is implemented as a random walk (see Algorithm 1). If the cell moves, then all of its seven (or five) pixels move one pixel in a given direction. Cells can also fluctuate in shape, yet such fluctuations maintain a structural representation of the nucleus by preserving intact a two-by-two square block of pixels (see Algorithm 2). Therefore, shape fluctuations are restricted to the motion of the three border pixels around the nucleus which either move to new positions on the border or displace a nuclear pixel; Figure 2.1(B) gives an example of both types of fluctuations for a cell changing shape.

The rates and probability by which cells move and change shape are parameterized separately from movement of molecules so that they can be calibrated to
Figure 2.1. (A) Three cells on the spatial grid each occupying seven pixels. (B) Cell changes shape. The region of the cell that contains the nucleus, indicated by the four gray pixels, is structurally maintained; two border pixels move to new locations (one moving down and the other moving left), and one border pixel (upper right) displaces a nucleus pixel, by moving down while the nucleus pixel gets shifted to the right. (C) Cell rounding up on fibronectin. The surface area with fibronectin is reduced with two border pixels moving into a quasi third dimension above the cell.
**Algorithm 1** moveWithProbability(p)

```plaintext
if random number < p then
    Randomly pick direction (up, down, left, right).
    if movement does not overlap another cell then
        move cell.
    end if
end if
```

the scale of actual biological cells. Unlike the representation of cells in the cellular Potts model \[10, 23, 36\], the properties of the cell are not described with energy expressions that are minimized as the simulation executes. Instead each cell is a discrete agent that behaves according to a pre-defined set of rules, and model parameters are used to adjust these rules.

**Algorithm 2** changeShape()

```plaintext
if cell size is 7 pixels then
    Randomly pick 3 border pixels.
else
    Randomly pick 1 border pixel.
end if
if selected border pixels do not overlap another cell then
    Set new border pixels for cell.
end if
```

Analysis of cell movement and shape within precartilage condensations \[13\] indicates that cells have a smaller contact area with substratum close to the condensation center and that the rate of movement of those cells slightly but significantly increases. We model this behavior by shrinking the area of cells associated with levels of fibronectin above a threshold value from seven pixels to five pixels (see Figure 2.1(C)), and increasing the rate at which those cells can move and
change shape. Likewise once a cell ventures onto a fibronectin-rich region, it has the tendency to remain there with a low probability of leaving the condensation.

2.3 Molecular Model and Reaction-Diffusion Mechanism

The reaction-diffusion mechanism, introduced by Turing [58] as a means for producing self-organizing spatial patterns, is based upon the interaction of slow diffusing, positive feedback activator molecules and fast diffusing, negative feedback inhibitor molecules. In our model, a discrete number of activator and inhibitor molecules occupy each pixel on the grid, and each molecule is considered to have a spatial extent of just one pixel. Each type of molecule has its own spatial grid independent of the other molecule types, so any number of molecule types can be defined, each with their own scale relative to the base spatial scale.

We model the reaction dynamics of the activator and inhibitor molecules at each pixel as follows: let $U_t$ and $V_t$ be the concentration of the activator and inhibitor, respectively, at time $t$ and let $\phi_t$ be an indicator function for the existence of a cell at a pixel at time $t$.

$$\Delta U_t = \min \{ \max U_t, (k_1 U_t + B_U) \phi_t + k_2 V_t \}$$ (2.1)

$$\Delta V_t = \min \{ \max V_t, k_3 U_t \phi_t + k_4 V_t \}$$ (2.2)

Equation (2.1) shows the change over time for each pixel on the grid of the activator morphogen concentration based upon a proportion (as defined by chemical reaction rates) of the current activator and inhibitor concentrations. Equation (2.2) shows the corresponding change over time for each pixel on the grid.
for the inhibitor morphogen. The activator morphogen is considered to be a positively self-regulating molecule and a positive regulator of the inhibitor; thus, the chemical rate parameters $k_1$ and $k_3$ both have positive values. The inhibitor morphogen is considered a negative regulator of activator that decays over time; thus, the values of the chemical rate parameters $k_2$ and $k_4$ are both negative.

The change of activator and inhibitor morphogens is calculated for each pixel on the grid for each time step of the simulation, and the concentrations of the two morphogens are updated at each pixel.

\[ U_{t+1} = \max\{0, U_t + \text{round}(\Delta U_t)\} \]  

(2.3)

\[ V_{t+1} = \max\{0, V_t + \text{round}(\Delta V_t)\} \]  

(2.4)

In keeping with the biology, we consider cells to respond to low concentrations of morphogens and therefore represent morphogen molecules as discrete entities. Consequently, the morphogen concentrations $(U_t, V_t)$ are whole numbers, and changes in the concentrations at a time step are rounded to the nearest integer and prevented from becoming negative. Nonetheless, we treat the chemical rate parameters $(k_1, k_2, k_3, k_4)$ for the two morphogens as averages of the reaction rates and allow them to assume real number values.

In our model, production of the activator and inhibitor molecules, as represented by the $k_1$ and $k_3$ parameters, can only occur in the presence of a cell. In contrast, the decay of activator and inhibitor, as represented by the $k_2$ and $k_4$ parameters, are considered to occur independently of cell presence. Cells are initially randomly distributed on the grid, and secrete a small basal amount $(B_U)$ of activator morphogen, which provides the initial concentration of activator; inhibitor
concentration starts at zero.

In any physicochemical reaction, there are limitations on how much reagent a single cell can realistically produce during any period of time. For this reason, our model provides separate parameters ($MAX_U$, $MAX_V$) for the maximum amount of activator and inhibitor that can be produced during a single reaction step. The maxima are imposed on individual pixels of the molecular grid rather than across the entire cell to reflect the polarization of limb mesenchymal cells [27]. This allows for small morphogen gradients to be present across the spatial extent of an individual cell through spatially polarized secretion of morphogens. The peaks of activator concentration produced by the reaction-diffusion dynamics define a large prepattern equal in spatial area to the fibronectin-rich patches, containing from thirty to fifty cells within a single patch, so polarization plays a role for the cells on the border region of the patch; while cells in the patch interior perceive a relatively constant morphogen concentration across their entire spatial extent.

Molecular diffusion from any pixel can occur randomly toward any of the four neighboring pixels (up, down, left, right). The diffusion rate ($D_U$, $D_V$) is scaled into a probability factor $0 < p < 1$ and a time step $n$ such that $D = pn$. The probability determines the chance that a molecule will diffuse, and the time step indicates how many opportunities a molecule has to diffuse for a single simulation iteration. If the molecule diffuses, then one of the four neighboring pixels is picked with equal probability. The chemical reaction operates at a much slower rate than molecular diffusion, so the time scales are separated, with diffusion calculated at a small time step and the reaction calculated at a longer time step. Algorithm 3 shows how the diffusion is performed at a finer time scale.
Algorithm 3 calculateReactionDiffusion()

Calculate chemical reaction for each pixel on grid.

\textbf{for} \ i = 1 \ \textbf{to} \ n \ \textbf{do}

Calculate activator and inhibitor diffusion for each pixel on grid.

\textbf{end for}

2.4 Fibronectin Production

Fibronectin is a non-diffusing, extracellular matrix molecule whose spatial distribution forms the template for precartilage condensations. As the concentration levels of the activator morphogen increase in the presence of a cell, that cell produces fibronectin mRNA which can then be translated into fibronectin protein molecules and secreted. We implement a simple threshold-sensing mechanism such that once the activator concentration exceeds the threshold value in the spatial presence of a cell, the cell differentiates into a fibronectin-producing cell. We separate the trigger for cell differentiation from the actual production of fibronectin by the cell to emulate delays seen in experiments.

When a cell produces fibronectin, a single multimolecular unit is secreted with random probability for each of the pixels on the molecular grid occupied by the cell, and each molecule is allowed to perform an initial small diffusion of at most one pixel \[32\]. Production of fibronectin molecules continues until a maximum concentration level is reached at a pixel, although cells may still continue to produce fibronectin on pixels that have not yet reach the maximum. The production rate of fibronectin, the duration of such production, and the maximum amount of fibronectin allowed per pixel, can be adjusted with model parameters.
2.5 Main Simulation

All of the various pieces including the cellular dynamics, the molecular dynamics, and fibronectin production can be brought together into the complete simulation code as shown in Algorithm 4. The scheduling of cell actions like movement and shape change is performed in an asynchronous manner and in random agent order. Molecular diffusion and chemical reaction is performed in a synchronous manner for the complete molecular grid. This is done in the normal way by utilizing a temporary matrix for holding the intermediate calculations, then that temporary matrix is copied to the molecular grid thus updating the whole grid in one step. Fibronectin production can be performed either asynchronously or synchronously because the calculation is not dependent upon neighboring cell states, so we perform the calculation synchronously, which is slightly more efficient.

Algorithm 4 Main Simulation

for each simulation iteration do
    Generate randomized list, $R$, of agents.
    for each agent in $R$ do
        moveWithProbability(p)
        changeShape()
    end for
    calculateReactionDiffusion()
    Determine if any cells have reached threshold for differentiation.
    Calculate fibronectin production for each differentiated cell.
end for
CHAPTER 3

RESULTS

3.1 Model Calibration

In attempting to calibrate our model parameters with known empirical parameters our objective is to correlate the in silico spatial and temporal patterns with in vitro experiments. For spatial patterns, we consider the size, shape, and distribution of the fibronectin-rich spatial domains. For temporal patterns, we consider the reaction rates of activator and inhibitor production, the diffusion rates of both cells and molecules, the onset of fibronectin production, the production rate of fibronectin, and the fluctuations of shape and movement of cells on fibronectin. The actual value for the set of key parameters used in the simulation and their corresponding physical measurements, if known, are shown in Table 3.1. Parameters for the stationary regime and stripe patterns, described in more detail below, are provided in Table 3.2.

3.1.1 Diffusion Rates

Diffusion rates for the activator and inhibitor play a vital role in defining the wavelength of the Turing patterns produced by the reaction-diffusion dynamics that ultimately determine the size and distribution of the fibronectin-rich
### TABLE 3.1

**CALIBRATED SIMULATION PARAMETERS TO KNOWN PHYSICAL VALUES FOR OSCILLATORY REGIME**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Physical Value</th>
<th>Simulation Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter/area</td>
<td>$15 , \mu m , / , 177 , \mu m^2$</td>
<td>7 pixels</td>
</tr>
<tr>
<td>Cell spatial grid</td>
<td>$1.4 , \times , 1.0 , \text{mm}$</td>
<td>$280 , \times , 200 , \text{pixels}$</td>
</tr>
<tr>
<td>Molecular spatial grid</td>
<td></td>
<td>$560 , \times , 400 , \text{pixels}$</td>
</tr>
<tr>
<td>Spatial ratio cells : molecules</td>
<td>$10000 : 1$</td>
<td>$28 , \text{pixels} : 1 , \text{pixel}$</td>
</tr>
<tr>
<td>Simulation temporal scale</td>
<td>$17.07 , \text{sec}$</td>
<td>1 iteration</td>
</tr>
<tr>
<td>Reaction temporal scale</td>
<td>$17.07 , \text{sec}$</td>
<td>1 iteration</td>
</tr>
<tr>
<td>Diffusion temporal scale ($n = 200$)</td>
<td>$85.3 , \text{msec}$</td>
<td>1 diffusion step</td>
</tr>
<tr>
<td>Basal activator production ($B_U$)</td>
<td>Unknown</td>
<td>28</td>
</tr>
<tr>
<td>Activator self-regulation ($k_1$)</td>
<td>Unknown</td>
<td>0.3356</td>
</tr>
<tr>
<td>Activator regulation of inhibitor ($k_3$)</td>
<td>Unknown</td>
<td>0.16</td>
</tr>
<tr>
<td>Inhibitor regulation of activator ($k_2$)</td>
<td>Unknown</td>
<td>-1.1</td>
</tr>
<tr>
<td>Inhibitor decay ($k_4$)</td>
<td>Unknown</td>
<td>-0.4615</td>
</tr>
<tr>
<td>Maximum activator produced ($MAX_U$)</td>
<td>Unknown</td>
<td>8000</td>
</tr>
<tr>
<td>Maximum inhibitor produced ($MAX_V$)</td>
<td>Unknown</td>
<td>8000</td>
</tr>
<tr>
<td>Cell differentiation threshold ($CDT$)</td>
<td>Unknown</td>
<td>7000</td>
</tr>
<tr>
<td>Activator diffusion rate ($D_U$)</td>
<td>$10 , \mu m^2/\text{sec}$</td>
<td>27 pixels/iteration</td>
</tr>
<tr>
<td>Inhibitor diffusion rate ($D_V$)</td>
<td>Unknown</td>
<td>108 pixels/iteration</td>
</tr>
<tr>
<td>Cell diffusion rate</td>
<td>$0.42 , \mu m^2/\text{min}$</td>
<td>1 pixel/60 iterations</td>
</tr>
<tr>
<td>Cell diffusion rate on fibronectin</td>
<td>$0.62 , \mu m^2/\text{min}$</td>
<td>1 pixel/40 iterations</td>
</tr>
<tr>
<td>Parameter</td>
<td>Stationary</td>
<td>Stripes</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>---------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Cell diameter/area</td>
<td>7 pixels</td>
<td>7 pixels</td>
</tr>
<tr>
<td>Cell spatial grid</td>
<td>$280 \times 200$ pixels</td>
<td>$280 \times 200$ pixels</td>
</tr>
<tr>
<td>Molecular spatial grid</td>
<td>$560 \times 400$ pixels</td>
<td>$560 \times 400$ pixels</td>
</tr>
<tr>
<td>Spatial ratio cells : molecules</td>
<td>28 pixels : 1 pixel</td>
<td>28 pixels : 1 pixel</td>
</tr>
<tr>
<td>Basal activator production ($B_U$)</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Activator self-regulation ($k_1$)</td>
<td>0.3146</td>
<td>0.3356</td>
</tr>
<tr>
<td>Activator regulation of inhibitor ($k_3$)</td>
<td>0.1584</td>
<td>0.16</td>
</tr>
<tr>
<td>Inhibitor regulation of activator ($k_2$)</td>
<td>-1.1</td>
<td>-1.1</td>
</tr>
<tr>
<td>Inhibitor decay ($k_4$)</td>
<td>-0.4615</td>
<td>-0.4615</td>
</tr>
<tr>
<td>Maximum activator produced ($MAX_U$)</td>
<td>8000</td>
<td>35</td>
</tr>
<tr>
<td>Maximum inhibitor produced ($MAX_V$)</td>
<td>8000</td>
<td>35</td>
</tr>
<tr>
<td>Cell differentiation threshold ($CDT$)</td>
<td>2400</td>
<td>2200</td>
</tr>
<tr>
<td>Activator diffusion rate ($D_U$)</td>
<td>30 pixels/iteration</td>
<td>27 pixels/iteration</td>
</tr>
<tr>
<td>Inhibitor diffusion rate ($D_V$)</td>
<td>130 pixels/iteration</td>
<td>108 pixels/iteration</td>
</tr>
<tr>
<td>Cell diffusion rate</td>
<td>1 pixel/60 iterations</td>
<td>1 pixel/60 iterations</td>
</tr>
<tr>
<td>Cell diffusion rate on fibronectin</td>
<td>1 pixel/40 iterations</td>
<td>1 pixel/40 iterations</td>
</tr>
</tbody>
</table>
patches. Lander and coworkers calculate that the effective diffusion coefficient for a molecule the size and shape of the morphogen Decapentaplegic (Dpp) to be $10 \mu m^2/sec$ [33]. Since TGF-β, which we assume to be our activator morphogen based upon cell-culture experiments [34, 39], is a morphogen of the same molecular class as Dpp, we take the diffusion coefficient of Dpp to be our activator diffusion rate, although the actual value may differ due to varying capacities of members of the superfamily to bind to variable microenvironments [47]. In the present model the diffusion rate for the activator morphogen in the reaction-diffusion system was found to be a key parameter for determining the size of the resultant patterns (see below). If the diffusion rate is too slow, the activator does not spread out across a sufficiently large area to produce broad condensations; in contrast, if the diffusion rate is too fast, the activator spreads out too much, thus preventing patterns from even forming.

The inhibitor morphogen, elicited when cells in incipient condensations are exposed to one or more ectodermally-produced fibroblast growth factors [41], must spread at a faster rate than the activator morphogen for stable patterns to be generated according to the reaction-diffusion dynamics. We performed a number of simulations that varied the ratio between the activator diffusion rate, which was kept constant, and the inhibitor diffusion rate. Consistent pattern formation was obtained when the inhibitor diffuses at a rate four to eight times faster than the activator. At a slower than four-fold ratio, the patterns degraded in consistency until the point where no patterns were produced at all, which occurred when both diffusion rates were almost equal (Table [3.3]). Beyond the eight-fold ratio, consistent patterns were still produced (results not shown). The relatively small ratio between the two diffusion rates makes the hypothesis of a diffusible inhibitor
of condensation formation \cite{38, 41} biologically plausible.

The diffusion rate for cells is considerably slower than for the activator and inhibitor molecules. Cui \cite{13} used phase-contrast microscopy and video-based cell tracking to measure the movements of cells during developing chicken limb precartilage mesenchyme over the time period of condensation formation. He determined an average cell diffusion coefficient of $0.506 \, \mu m^2/min$, and his data are consistent with cells moving slightly faster in condensations (see also \cite{16}). We implement this in a qualitative fashion by making cells associated with fibronectin diffuse faster than cells not associated with fibronectin (see Table 3.1).

Using the experimental values for activator and cell diffusion coefficients greatly facilitated choosing other parameters such that appropriately sized and spaced condensations formed in silico. This contrasted with parameter searches performed with non-biological choices of activator and cell diffusion coefficients. In those cases no realistic patterns formed in scores of simulations.

3.1.2 Reaction Rates

Parameters for the reaction rates must fall within an appropriate “morphogenetic” region of parameter space in order for Turing-type patterns to be produced. Within this morphogenetic region, two types of behavior are observed for the morphogen concentrations: steady-state equilibrium with stable patterns and oscillatory behavior with transient patterns. The oscillatory behavior was induced by imposing the maxima ($MAX_U$, $MAX_V$) on production. Otherwise a steady-state would be attained, but at an unrealistically high concentration level of activator. As these reaction rates are unknown, we take the approach of choosing parameters that correspond to our understanding of the qualitative behavior
TABLE 3.3
VARIATION OF AVERAGE PEAK INTERVAL AND AVERAGE ISLAND SIZE OVER A RANGE OF DIFFUSION RATIOS

<table>
<thead>
<tr>
<th>Activator-to-Inhibitor Diffusion Ratio</th>
<th>Average Peak Interval (mm)</th>
<th>Average Island Size (10^{-3} mm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>0.21 (0.009)</td>
<td>11.26 (1.26)</td>
</tr>
<tr>
<td>Simulations</td>
<td>1 : 3 (27 : 81)</td>
<td>No Patterns</td>
</tr>
<tr>
<td></td>
<td>0.218 (0.011)</td>
<td>11.867 (0.248)</td>
</tr>
<tr>
<td></td>
<td>0.2 (0.008)</td>
<td>13.167 (0.754)</td>
</tr>
<tr>
<td></td>
<td>0.213 (0.014)</td>
<td>13.464 (0.752)</td>
</tr>
<tr>
<td></td>
<td>0.204 (0.01)</td>
<td>14.179 (0.596)</td>
</tr>
</tbody>
</table>

of cells in culture.

3.1.3 Onset and Rate of Fibronectin Production

It was previously shown that brief, transient exposure of precartilage mesenchymal cells to exogenous TGF-β early in the culture period is sufficient to induce the production of precocious condensations a day later [34]. The cells responded to TGF-β by immediately producing elevated levels of fibronectin mRNA even though secretion of the fibronectin protein molecule in the discrete condensation pattern did not occur until the next day. In another study, the expression of FGFR2 protein (the mediator of FGF induction of the inhibitor at sites of incipient condensation) was found to be transient: it was no longer detectable once
actual condensation was underway [11]. These results suggest that the signaling system that induces the spatiotemporal pattern of condensation (i.e., the reaction-diffusion mechanism under our hypothesis) does not have to maintain stable peaks of activation throughout the entire period of precartilage condensation. A set of transiently formed peaks could provide a prepattern for condensations occurring at a later time. Based upon these empirical observations, we selected reaction parameters in our model giving oscillatory behavior in order to test the idea that transient signaling could lead to subsequent condensation-like patterns.

The physical area of a biological cell determines the area of a pixel on the grid. This value, along with the assumed activator morphogen diffusion rate, permits the temporal scale of the morphogen diffusion, the chemical reaction, and the overall simulation to be calculated (cell area $\times$ spatial ratio of cells to molecules $\times$ activator diffusion rate = $177\mu m^2$ $\div$ cell pixels $\times$ $1$ cell pixel $\div$ $4$ molecular pixels $\times$ $27$ molecular pixels $\div$ $10\mu m^2$ iteration $= 17.07$ sec / iteration). The chemical reaction occurs on the same time scale as the overall simulation, but due to the separation of time scales diffusion occurs faster at $17.07$ sec $\div$ $200 = 85.3$ msec for each diffusion step. Since in vitro experiments have not provided definitive values for the time course of morphogen activities, and the various morphogens may have other roles beyond induction of fibronectin production, we did not attempt to calibrate the duration of morphogen activity in our simulation to any known quantities. Instead, given the knowledge that cells respond quickly to TGF-β, we triggered cell differentiation early in our simulation, when the sum of units of activator across an entire cell reached a threshold value of 7,000. However, the transient signal of the morphogen concentrations occurs over a period of 500 simulation iterations which corresponds to a little over two hours. While this appears to be somewhat faster than the corresponding change
in vitro, it is well within an acceptable range that could be fine tuned once more is known about timing of events in the micromass culture system.

3.2 Simulation of Condensation Patterns

Consistent with the experimental constraints described above, we searched for a parameter set in the model that reproduces the formation of precartilage condensation patterns. We calculated the average interval of the centroids (“peak interval”) \([37]\) and the average island size of the fibronectin patches \([32]\) for 5 simulation images and compared the values to those obtained from 12 in vitro condensation images such as that in Fig. 3.1A. The results (Fig. 3.2) indicate that our enhanced model reproduces the pattern of precartilage condensations equally well as the model of Kiskowski et al. \([32]\).

Different views of one simulation with parameters chosen within the standard range are shown in Figs. 3.1B-D. The distribution of condensations (Fig. 3.1B) conforms very well to the photograph of the 72 h culture (Fig. 3.1A) although the cells in the individual in silico condensations are not tightly packed as they are in the in vitro ones. This is not unexpected, since the model at present lacks representation of a cell-cell adhesion molecule, several of which are upregulated at condensation sites in limb mesenchyme \([46, 62]\). The shape change of the model cells once they encounter fibronectin does nonetheless lead to a realistically higher cell density in condensed vs. noncondensed regions of the simulated cultures.

The simulated distribution of fibronectin (Fig. 3.1C) conforms to the distribution of condensations, as expected from immunolocalization studies \([15]\). The distribution of activator peaks at the time-point shown in Fig. 3.1D maps out the set of eventual condensations. Previous experimental studies show TGF-\(\beta\) local-
Figure 3.1. *In vitro* and oscillatory regime simulation images for spot-like precartilage condensations. (A) Discrete spot-like precartilage condensations that have formed after 72 hours in a micromass culture of 5-day leg bud apical zone limb mesenchymal cells, visualized by Hoffman Contrast Modulation optics. Actual size of the microscopic field is $1 \times 1.4$ mm, and each condensation contains approximately 30-50 tightly packed cells. (B) Spatial grid of equal physical size to (A) containing over 6000 cells produced by simulation using the parameter values in Table 3.1 showing clusters of fibronectin-producing differentiated cells (white), non-differentiated cells (blue gray), and empty space between cells (black). Each cluster contains on average $\sim 30$ cells. (C) Spatial grid of same simulation as (B) showing fibronectin-rich patches (black) produced by the differentiated cells. (D) Spatial grid of same simulation as (B) showing activator concentrations at time slightly after the initial onset of cell differentiation. The color bar indicates the range, with magenta for high concentration and light blue for low concentration.
Figure 3.2. Average peak interval versus average island size for 12 experimental (circle) and 5 simulation (square) points using parameter values in Table 3.1 with different random initial conditions. All simulations were run for 3000 iterations with periodic boundary conditions.
ization to anticipate the formation of condensations by up to a day \[34\], and to trigger the subsequent production of fibronectin after a brief, transient exposure \[34\]. The model, with different parameter choices, leads to realistic condensation patterns with either transient (as in the simulation shown in Figs. 3.1B-D) or stable activator patterns (see below).

We explored the robustness of the parameter set by varying key parameters independently (± 5%); results can be seen in Figure 3.3. Minor variation of the inhibitor strength on activator \((k_2)\) by either +5% or 5% produced little change in the resulting condensation patterns. Instead, the temporal dynamics were modified, causing an increase and decrease in the period of the morphogen oscillations, respectively, with the +5% and 5% changes. For a decrease of 5% in the activator self-regulation \((k_1)\) or an increase of 5% in the activator regulation of inhibitor \((k_3)\), smaller condensation patterns were produced with the condensations spaced further apart from one another. For a 5% increase in the activator self-regulation \((k_1)\) or a decrease of 5% in the activator regulation of inhibitor \((k_3)\), condensation patterns greatly expanded in size such that the condensations touched one another, producing a pattern of interconnected stripes instead of spots. Similar results were also obtained if the inhibitor decay \((k_4)\) was increased by 5%. For a 5% decrease in the inhibitor decay \((k_4)\), the chemical reaction was effectively damped and no patterns were produced.

Consistent with observations of limb precartilage development in vitro and in vivo, our simulation results indicate that cells can form condensations patterns by undergoing small displacements of less than a cell diameter, packing more closely by changing their shapes, while maintaining a relatively uniform cell density across the entire spatial domain.
Figure 3.3. Variation in some of the key parameters induces morphological changes in the resultant spatial patterns from distinct spots to connected spots to stripe-like patterns. Average peak interval versus average island size for variations in the some of the key parameters are shown: +5% (diamond) and -5% (filled diamond) for activator self-regulation ($k_1$), +5% (triangle) and -5% (filled triangle) for activator regulation of inhibitor ($k_3$), +5% (down triangle) and -5% (filled down triangle) for inhibitor regulation of activator ($k_2$), +5% (plus) for inhibitor decay ($k_4$). The colored points are a gradient of variations: 1% (red), 2% (orange), 3% (green), 4% (blue), 5% (violet).

Also shown are the five simulations (square) using the standard parameter values in Table 3.1 and the mean for the twelve experiments (circle). All simulations were run for 3000 iterations with periodic boundary conditions.
Given the possibility that choices of spatial domain and boundary conditions could lead to simulation artifacts, we sampled various alternatives in combination and investigated changes in the resulting condensation patterns.

With respect to the spatial domain, we ran simulations with rectangular grids of various widths and heights (data not shown); this produced no noticeable effects on the size, shape, or distribution of the condensations. We conclude that the total area of the spatial domain determines only the number of condensations.

We also ran simulations with periodic and no-flux conditions. In periodic conditions grid boundaries are connected together simulating a continuous space, whereas the no-flux boundary acts as a barrier. Both types of boundary condition produced similar results for the size, shape, and distribution of the condensation patterns other than the expected pattern truncations under no-flux conditions (data not shown).

3.3 Two Dynamical Regimes in Condensation Pattern Formation

Our simulations disclosed two regimes of behavior in the reaction-diffusion system of morphogens. In one regime, the maximum concentration levels for the two morphogens is characterized by a stationary value; this regime appears when the chemical reaction is slow, i.e., the production rate of the activator morphogen is balanced with the production rate of the inhibitor morphogen (Fig. 3.4B). In the other regime, the concentrations levels for the two morphogens had an oscillatory behavior; concentrations increase up to a peak value, decrease back down to almost zero, and then continually repeat that cycle (Fig. 3.4A). The oscillatory regime occurs when the chemical reaction is fast but a cap exists for the maximum amount of morphogen produced for a single reaction step.
Figure 3.4. Dynamics of oscillatory and stationary regimes. (A) Oscillatory regime produces transient patterns that repeat over time but are spatially stochastic. (B) Stationary regime produces stable patterns with minor stochastic fluctuations around an equilibrium concentration. Graphs show the maximum concentration value for a single pixel across the entire molecular grid (that pixel lies within an activator peak as in Fig. 3.1D and 3.5D but may shift from peak to peak as concentrations vary) for activator (black +) and inhibitor (blue) morphogens.
Both regimes for the reaction-diffusion system can produce condensations patterns in the range of experimental values for size and distribution. (See Figs. 3.1 and 3.2 for the oscillatory regime and Figs. 3.5 and 3.6 for the stationary regime).

The limits on morphogen production ($MAX_U, MAX_V$ in Table 3.1) induce the oscillatory regime by restricting production of activator while still allowing production of inhibitor, whose concentration has not yet reached the limit. The result is that inhibitor concentrations build up in the system; the inertia of inhibitor concentration dampens activator production throughout the whole system, which quickly accelerates and reduces the activator concentration down to basal levels. Cells continue to produce a basal amount of activator, so over time conditions are reproduced for the onset of morphogen pattern formation. The dynamics repeat, with transient patterns being formed, though the spatial arrangement of the peaks varies unpredictably from one oscillation to the next.

Variations in the limits on morphogen production in the oscillatory regime produced minimal changes in the average peak interval and average island size of the fibronectin patch distribution (Table 3.4). The oscillatory regime is more robust for higher limits and breaks down when the concentrations are low. In contrast, the stationary regime operates in the lower concentration levels of the morphogens. The oscillatory regime is robust to a noisy threshold level for cell differentiation. Simulations where each cells threshold is randomly assigned from a normal distribution, $N(9000, 1000)$, instead of a constant value, produce only slight variation in the average peak interval and average island size despite the large deviation in the threshold levels. However, the stationary regime is sensitive to the threshold level for cell differentiation as a modest variation, $N(2400, 170)$, completely disrupts the spatial distribution of the fibronectin patches (data not
Figure 3.5. *In vitro* and stationary regime simulation images for spot-like precartilage condensations. (A) Discrete spot-like precartilage condensations that have formed after 72 hours in a micromass culture of 5-day leg bud apical zone limb mesenchymal cells, visualized by Hoffman Contrast Modulation optics. Actual size of the microscopic field is 1×1.4 mm, and each condensation contains approximately 30-50 tightly packed cells. (B) Spatial grid of equal physical size to (A) containing over 6000 cells produced by simulation using the stationary parameter values in Table 3.2 showing clusters of fibronectin-producing differentiated cells (white), non-differentiated cells (blue gray), and empty space between cells (black). (C) Spatial grid of same simulation as (B) showing fibronectin-rich patches (black) produced by the differentiated cells. (D) Spatial grid of same simulation as (B) showing activator concentrations at simulation iteration 400 after most cells have differentiated. The color bar indicates the range with magenta for high concentration and light blue for low concentration.
Figure 3.6. Average peak interval versus average island size for 12 experimental (circle) and 5 simulation (square) points using the stationary parameter values in Table 3.2 with different random initial conditions. All simulations were run for 3000 iterations with periodic boundary conditions.
The formation of patterns in the stationary regime is sensitive to the period that cells are exposed to activator morphogen and to the threshold level for cell differentiation. If the exposure time is too short, small, irregularly spaced condensations are produced. If the exposure is too long, irregularly shaped condensations are produced. While the stationary regime produces stable activator peaks, those peaks tend to wander spatially over time due to the underlying cell diffusion. The oscillatory regime is less sensitive to the threshold level for cell differentiation, and a single transient pulse provides a well-defined exposure period.

3.4 Formation of Stripe Patterns

While the focus of our model has been on producing the spot patterns typically seen in leg cell cultures [14, 15], with a slight adjustment to parameters the exact same model can produce stripe patterns (Fig. 3.7B-D). This is significant because uncontrolled variations in the preparation of cultures grown under the same conditions as the spot-producing ones occasionally give rise to stripe patterns (Fig. 3.7A). When the reaction-diffusion system progresses to spot patterns, it goes through a brief period of partial stripe formation until dominant activator peaks stabilize the system into spot patterns. Reducing the limits on morphogen production ($MAX_U$, $MAX_V$ in Table 3.2) prevents peaks of activator morphogen from dominating and stable stripe patterns are maintained. This corresponds to theoretical analysis by Shoji and coworkers of reaction-diffusion systems with linear kinetics and constant constraints [54]; they show stripe patterns are generated instead of spot patterns if the upper and lower constraints are equal distance from the equilibrium. Similar to the formation of spot patterns in the stationary
regime, formation of stripe patterns is sensitive to the duration of the period in which cells are exposed to activator morphogen.
Figure 3.7. *In vitro* and simulation images for stripe-like precartilage condensations. (A) Stripe-like precartilage condensations that have formed in a micromass culture of 5-day leg bud apical zone limb mesenchymal cells, visualized by Hoffman Contrast Modulation optics. (B) Spatial grid containing over 6000 cells produced by simulation using stripe parameter values in Table 3.2 showing stripes of fibronectin-producing differentiated cells (white), non-differentiated cells (blue gray), and empty space between cells (black). (C) Spatial grid of same simulation as (B) showing fibronectin-rich stripes (black) produced by the differentiated cells. (D) Spatial grid of same simulation as (B) showing activator concentrations at time slightly after the initial onset of cell differentiation. The color bar indicates the range, with magenta for high concentration and light blue for low concentration.
4.1 Advantages of Agent-based Modeling

A benefit of the agent-based approach to cell culture simulation described here is the ease with which the contribution to biological phenomena of specific experimentally determined details can be evaluated as to their sufficiency and necessity. Furthermore, because very simple agent-based models can often capture biological features, it is also useful to determine if basic models retain such behaviors when they are made more realistic.

Agent-based representations of developmental and other cellular systems afford a convenient way of moving between simulation and experiment. The model described here can provide a general framework for an interdisciplinary approach to studying cells in culture. The strategy can take the form of the following investigative cycle: (i) establishing a core biological model involving various simplifications and assumptions; (ii) computational implementation and calibration; (iii) comparison of in silico experiments with in vitro biological experiments; (iv) suggestions for new biological experiments based on disparities between in silico and in vitro results; (v) new biological experiments and biological model improvement and/or revision of the computational model.
4.2 Enhancements over Previous Model

In the case studied here, in vitro pattern formation in limb bud precartilage mesenchyme, an earlier model was a simple biological lattice-gas model consisting of single-pixel agents representing biological cells, morphogens and secreted macromolecular matrices on one uniform grid \cite{32}, which captured many quantitative aspects of the pattern formation process. Our enhanced model incorporates multipixel cells with variable geometry and separation of spatial scales in the form of modeling cells and morphogen and extracellular matrix molecules on grids of differing mesh size. Realistic values of morphogen and cell diffusion have also been introduced.

These experimentally motivated modifications and constraints yielded a model that continues to generate, in a robust fashion, realistic patterns of cell condensation. In particular, our more sophisticated model produces fibronectin-rich patches corresponding to condensations in micromass cultures in size, shape, cell number, and spatial distribution. In contrast to the earlier model, but in keeping with biological findings, coverage of the virtual culture surface remained continuous as cells moved small distances, changed their shape in response to the deposited fibronectin matrix, and maintained their movement within, and capacity to leave, condensations once these had formed.

We have demonstrated that parameter choices can be found for our model that reproduce the experimental distribution and size range of precartilage condensations in experimental micromass cultures. The performance of the model was equal to that of Kiskowski et al. \cite{32}, despite the imposition of realistic scaling and experimentally determined constraints.
4.3 Multiple Scales

We initially separated the spatial scales in our desire for greater biological fidelity. However, we discovered that the multiple spatial scales required the introduction of multiple temporal scales, specifically between the morphogen diffusion and morphogen chemical reaction but also with cell diffusion, in order for the parameters to be within the proper range for production of condensations of the appropriate size and distribution. Without the separation of time scales, the chemical reaction parameters would have to be scaled down to the morphogen diffusion time scale thus resulting in calculations which would be too imprecise due to rounding errors for low concentrations. Interestingly, if we performed the separation of time scales first, it would have likely led us to separating the spatial scales anyways because the cellular grid is too coarse for accurate simulation of our calibrated value for molecular diffusion. Our model provides a case study for how calibration to known experimental values may require introduction of multiple scales in order to provide simulation accuracy.

4.4 Calibration

The match between the experimentally determined diffusion coefficient of an activator morphogen of the same molecular family as the one in our model and the model value required to produce condensations of the appropriate size and distribution suggests that our model is physically reasonable. Since the computational model provides fine control over the specification of how cells respond to morphogen concentrations and thus alter their functional and differentiated states, how they produce and secrete morphogens and extracellular matrix molecules (i.e., fibronectin), how they behave on extracellular matrices by, for example, changing
their shape and motile, there is ample opportunity to introduce new experimental data. Experimental values of measured parameters provide constraints on unknown values which can be evaluated computationally and experimentally.

4.5 Model Predictions

The model has allowed us to study the interplay between reaction-diffusion processes, fibronectin production and cell-fibronectin interaction in greater detail than previously possible. In particular, our simulations disclosed two regimes in the interplay of the reaction-diffusion system of morphogens with fibronectin production and cell behavior. In one regime, stationary morphogen patterns were produced, followed by cell rearrangement into patterns of condensation. In the second regime, morphogen patterns were transient and oscillatory in time, and the induced fibronectin production (and consequent cell rearrangement) occurred with a delay. In addition, the dynamical characteristics of the second regime provide a natural explanation for apparent oscillatory effects of limb precartilage cell responses to TGF-β seen in previous experimental studies \cite{34}. The transient regime also exhibits less sensitivity than the stationary regime to several key system parameters, giving it plausibility as the more robust pattern-forming mechanism. However, in order to suppress second-generation condensation patterns due to the recurrence of activator peaks in this regime, we assumed that cell differentiation to a morphogen-nonresponsive state occurs rapidly relative to the period of oscillation. This assumption is obviously not needed for simulations in the stationary case; indeed, stable pattern formation in this regime would be consistent with extended (i.e., over a period of a day or more) susceptibility to perturbation by exogenous TGF-β. We are currently performing in vitro experi-
ments analogous to earlier studies on the first day of development [34] to test this predicted difference, as well as some others.

The capacity of our model to generate both spots and stripes of precartilage condensation under slightly different parameter choices corresponds well to experimental results, where either morphotype may be generated under similar initial conditions. Because the developing limb itself generates its skeleton in the form of spots and stripes of precartilage condensation, this result of our simulations supports the applicability of the core molecular-genetic mechanism we have used to the understanding of both in vitro and in vivo chondrogenic pattern formation. Moreover, the flexibility and generality of the framework presented here makes it suitable for representing and testing other experimentally motivated models for periodic patterning in which cell movement and shape change is involved, such as the formation of feathers and hairs [29, 63] and teeth [52, 57].

4.6 Cell-Cell Adhesion

Our model generates realistic patterns of precartilage condensation in high-density culture without the need to postulate direct cell-cell adhesive interactions. This feature appears to reflect biological reality. First, although the separation of condensing from noncondensing cells superficially resembles sorting out by differential adhesion (see [30] for a recent model of the latter process based on a free energy minimization principle), haptotactic binding to fibronectin is sufficient to recruit limb precartilage mesenchymal cells, or even inert particles, into condensations [19]. Second, while as mentioned above, several cell-cell adhesive proteins, including N-CAM [62] and N-cadherin [46] are expressed at sites of condensation, their loss does not impair condensation-dependent skeletogenesis [12, 35].
4.7 Limitations and Future Work

We note that in both the oscillatory and stationary cases the region of parameter space that leads to realistic fibronectin-patch and condensation patterns corresponds to activator morphogen peaks that are on the spatial scale of the condensations themselves. For the oscillatory regime, a small number of those peaks have relatively high, possibly nonphysiological, activator and inhibitor concentrations (assuming morphogen units represent one or more protein molecules). If morphogen dynamics in these cultures is indeed oscillatory [34], this may represent an inauthentic aspect of our model, resulting from the use of the classic diffusion-dependent Turing-type morphogen scheme. We are therefore exploring alternative embodiments of the model using juxtacrine signaling the role of which is suggested by recent demonstration of involvement of the Notch signaling pathway in the inhibitory branch of the condensation patterning network [21]. Recent analyses have suggested that introducing juxtacrine signaling into the dynamics can bring reaction-diffusion pattern forming systems which are otherwise biochemically implausible into more realistic parameter domains [50]. We note that our multipixel representation will enable the incorporation of cell asymmetry and polarity (a known feature of limb mesenchymal cells [27]) in future models employing cell relay mechanisms.
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