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A Cellular Automata Model of Bone Formation

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Abstract

Bone remodeling is an elegantly orchestrated process by which osteocytes, osteoblasts and osteoclasts function as a syncytium to maintain or modify bone. On the microscopic level, bone consists of cells that create, destroy and monitor the bone matrix. These cells interact in a coordinated manner to maintain a tightly regulated homeostasis. It is this regulation that is responsible for the observed increase in bone gain in the dominant arm of a tennis player and the observed increase in bone loss associated with spaceflight and osteoporosis. The manner in which these cells interact to bring about a change in bone quality and quantity has yet to be fully elucidated. But efforts to understand the multicellular complexity can ultimately lead to eradication of metabolic bone diseases such as osteoporosis and improved implant longevity. Experimentally validated mathematical models that simulate functional activity and offer eventual predictive capabilities offer tremendous potential in understanding multicellular bone remodeling. Here we undertake the initial challenge to develop a mathematical model of bone formation validated with *in vitro* data obtained from osteoblastic bone cells induced to mineralize and quantified at 26 days of culture. A cellular automata model was constructed to simulate the *in vitro* characterization. Permutation tests were performed to compare the distribution of the mineralization in the cultures and the distribution of the mineralization in the mathematical models. The results of the permutation test show the distribution of mineralization from the characterization and mathematical model come from the same probability distribution, therefore validating the cellular automata model.

Keywords

Bone; Bone Formation; Osteoblast; Cellular Automata; Agent-Based Model; Mathematical Models of Bone Formation; Permutation Tests

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1. Introduction

At the macroscopic scale bone is a static, hard tissue. Specifically a composite, bone is comprised of collagen fibers giving rise to bone's relative elasticity and a hydroxyapatite mineral phase giving rise to its compressive strength [1]. While much less obvious at the macroscopic scale, bone is a highly dynamic tissue that maintains a tightly regulated homeostasis by responding to cellular interactions. At the microscopic level, bone consists of cells that create, destroy and monitor the bone matrix. Osteocytes, which live within the bone in a fluid-filled network are uniquely positioned to sense mechanical load [15].

By coordinating the activity of the bone-resorbing osteoclasts and the bone-forming osteoblasts, an intricate and highly efficient feedback system for the modification or maintenance of bone quality and quantity is achieved. This regulation is altered by a variety of factors including genetic and metabolic diseases, metastases, medications and altered loading environments (e.g., extended bed rest and implant introduction) [3, 10, 12, 18, 19].

Understanding these interactions is critical to exploiting bone's innate remodeling ability to prevent osteoporosis, halt metastatic bone progression, improve implant longevity and develop functional tissue-engineered substitutes. In *vitro* cellular systems have been used for years to study bone cell interactions [1]. In these systems bone cells are utilized under a specific set of experimental conditions to test a hypothesis or challenge a given paradigm. However, the results of the experiments cannot be extended beyond the specific model, environment and conditions established in the experiment. As such there is tremendous variation in experimental results across laboratories and extrapolating findings back to the *in vivo* system is challenging at best.

In this paper we present the first part of a much larger endeavor. The goal is to construct biomimetic multi-scale mathematical models to understand the underlying mechanisms, pathways and multicellular interactions that regulate bone remodeling. Our approach begins with the development of a mathematical model of osteoblast functional behavior based upon *in vitro* characterization studies. The mathematical models were developed to simulate the behavior of the osteoblasts in these exact culture conditions.

Moving research forward in the bone field will require the development of new model systems and approaches. For example, in *vitro* systems that incorporate the multicellular interactions of the osteocytes, osteoclasts and osteoblasts and enable the systematic investigation of soluble, cell contact and cellular communication interactions will far outpace research using isolated model systems. However, the findings of these evaluations will still be highly dependent upon the experimental conditions and an exhaustive experimental strategy is simply not feasible. In moving research forward the mathematical field provides exciting opportunities to develop model systems validated from *in vitro* studies that can offer eventual extrapolative potential. To maximize the effectiveness of these model systems the approach employed should not rely on a governing state equation but should provide an approach that can reflect biologic complexity and parallel biologic discovery. The development of cellular automata (CA) models and CA-complied agent-based models (ABMs) of bone dynamics offer that potential.

To that end we have begun the development of CA models of bone cell activity with the eventual goal of developing a multicellular ABM composed of individual CA models representing each of the three bone cell types (osteocytes, osteoblasts and osteoclasts) and accurately modeling their functional behavior (sense stimuli, create bone and degrade bone). In the ABM the single cell type CA models will interact and be modified based upon those interactions incorporating feedback that adequately reflects the *in vivo* environment. Ultimately the goal is to develop an ABM capable of predicting bone remodeling as a function of environmental conditions. Applications of this work could include modeling the effects of implant wear (particulate debris generation) and subsequent effects on bone cells to improve implant design and longevity or utilizing modeling data from mechanical stimulation studies to design more effective loading strategies for developing tissue engineered bone constructs in bioreactors.

1.1. Osteoblast Morphology and Function

Bone remodeling is an ongoing process throughout the lifetime of an individual. Specifically, remodeling is a coupled process that first requires the removal of old bone which is followed by the production of new bone to fill the removed space. In an optimal environment remodeling maintains a net balance between bone loss and gain. As we age, an imbalance results and net loss exceeds gain and osteoporosis ensues. Osteoblasts are the cells responsible for matrix production and mineralization (bone formation) during the bone remodeling process [10]. Osteoblasts secrete matrix proteins, including collagen type I that make up the osteoid, an organic precursor to mature mineral [2]. Mineralization occurs more slowly by the deposition and growth of hydroxyapatite crystals, $Ca_5(PO_4)_3(OH)$ [4].

1.2. Characterization

In vitro osteoblasts derive from two main sources: primary culture and immortalized lines. In primary systems cells are harvested from bone tissues in a series of collagenase digestions that degrade the bone matrix and release the cells (e.g., long bone). These systems can display tremendous variability and have a limited passage life, or finite lifespan, in culture requiring a continual tissue source. Immortalized lines are transformed with cancer genes or inducible factors that enable the cells to continue to proliferate in culture. These cells have a much longer passage life but are considered “osteoblastic” or “osteoblast-like” in that their origins at best allow the lines to resemble osteoblast activity. Moreover not all immortalized osteoblastic lines model every aspect of osteoblast behavior. For example, not all lines form bone in culture [13]. In addition cell lines can experience phenotypic drift and in extreme cases this gives rise to subclones of the line with markedly different behavior [6].

The MC3T3-E1 cell line was chosen for this study. The cell line is a preosteoblast line derived from mouse skull (calvarial) bone. These cells continue to proliferate in culture until chemically induced to differentiate. Once induced to differentiate they are capable of forming bone in culture. Typical osteoblastic MC3T3-E1 (subclone 4) images are shown in Figure 1. The image (A) on the left depicts uninduced preosteoblastic MC3T3-E1 cells, while the image on the right shows osteoblastic MC3T3-E1 cells which have been induced to mineralize. The images in (B) are of uninduced (left) and induced (right) MC3T3-E1 cells stained with fluorescent dyes for cell cytoskeleton visualization (rhodamine phalloidin/red)

and nucleus (DAPI/blue). As shown the cytoskeleton represents a highly organized internal framework comprised of actin filaments and can be disassembled and reassembled quickly [3]. In addition to providing cell shape and integrity the rapid assembly is important in cell movement, or motility [3]. An SEM image (C) of osteoblasts at the time of induction demonstrates a confluent monolayer of cells. Bone nodules formed in culture (D) are quantified by surface area following staining of the hydroxyapatite mineral; calcium stains red using alizarin red (left), and phosphates stain black using von Kossa stains (right).

1.3. Cellular Automata Model

CA models are flexible enough that they have been used in a variety of disciplines, including biology, chemistry and physics [22]. They are ideal for working with small areas or volumes and in systems where stochasticity occurs naturally. According to the current literature cells, including bone cells act stochastically as opposed to deterministically [20]. In other words two cells in identical conditions might not act in exactly the same way. Because of this mathematical models that take into account this stochasticity are crucial if we are to simulate the bone remodeling process. Moreover, with our particular goal of mathematically modeling osteoblast functional activity, CA models are ideal as the area to be modeled is small.

In the past CA models have been used to simulate a wide variety of biological processes [9], especially those which admit a high level of heterogeneity [16]. In particular CA models have been developed to model bone processes such as bone tissue formation inside a bone transplant [21] or tissue mechanics of bone functional adaptation [20, 21]. In this paper a CA model was constructed to simulate the ability of the osteoblastic cells to form bone in culture for quantification of individual cell type activity. This interdisciplinary approach allows for constant improvement and expansion of the mathematical models as new data from experiments become available, enabling CA fidelity to increase with biologic discovery. At the same time these mathematical models help suggest new *in vitro* experimentation and aid in deeper understanding of the bone formation process. By integrating CA models of osteoblast, osteoclast and osteocyte individual activity, the net effect of combined activity can be modeled.

Formally, a CA model consists of an n -dimensional matrix or lattice in which each element in the matrix is defined by a triplet $\langle I, S, F \rangle$, where I is the finite set of inputs, S is the finite or infinite set of all possible states that a cell can take and F is the function that determines the state the cell will take on the next step. This depends on input state pairs [5, 14]. The set of inputs is defined as a vector containing the states of the “neighboring cells” and the current state of the cell.

The goal is to develop an *in silico* model that mimics the behavior of the cells in culture. Unlike deterministic models, the CA model should represent the high variability that arises among characterization runs. This *in silico* model also allows for the completion of 10,000 simulations in a short amount of time.

To validate the mathematical model obtained statistical methods to compare culture images and simulated images were applied. One way to check the consistency between culture and

simulated images is by performing a permutation test. NA permutation test, a type of non-parametric randomization procedure, is one of the most commonly used statistical tools in modern research areas [7, 8, 11]. The permutation test is an efficient and highly attractive method because, unlike model-based statistical tests it does not require any particular assumptions other than the inference that observations are independent and exchangeable. Further, it works well for a small sample size.

The rationale for the permutation test is to compare the observed test statistic with those from re-arranged data by locating the observed test statistic with respect to the distribution of the test statistic. The null hypothesis states that the distribution of bone formation in the characterization described in Section 1.2 and the distribution of bone formation obtained from the CA model come from the same probabilistic distribution, regardless of the actual analytical forms of the distributions. This implies that the CA model is an accurate representation of the *in vitro* characterization. If the observed test statistic falls into two overlapping tails of the distribution, then it is interpreted as an unlikely outcome given the null hypothesis is true. In this case, the null hypothesis is rejected.

2. Materials and Methods

2.1. In Vitro Characterization

Preosteoblasts from the MC3T3-E1 cell line are seeded at an initial density of 2.5×10^3 cells/cm² onto polystyrene tissue culture wells and allowed to grow to 100% confluence; in other words, cells completely cover the surface of the wells. Cells are grown in minimum essential alpha medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Invitrogen) in an incubator at 5% CO₂ and 37°C. This supplemented medium is cell type specific and provides the necessary proteins and nutrients for optimal cell growth; the penicillin/streptomycin controls bacterial contamination. Upon confluence cells were induced to differentiate with the addition of 50 µg/mL ascorbic acid (Vitamin C) and 10 mM β -glycerophosphate to the culture medium. Cells were fed with this supplemented medium every three days. For quantification of bone formation alizarin red and von Kossa assays were performed. After formalin fixation alizarin red staining was performed followed by a 30-minute incubation period. For von Kossa staining cells were fixed in 4% paraformaldehyde in phosphate-buffered saline and then exposed to UV light for up to 60 minutes in a 5% silver nitrate solution before imaging. The percent area of culture well covered by each stain was quantified using measurement features in the ImageJ software at the following time points post-switch: one day, two days, four days, eight days, 16 days and 26 days. At each time point three control wells (not induced to mineralize) and nine switch wells (induced to mineralize) were analyzed per assay.

2.2. CA Model

Our CA model utilized a three-dimensional matrix B with dimensions M , N and $T+1$. The parameters M and N represent the dimensions of the monolayer of cells within *in vitro* cultures described in Section 1.2. In the simulation $M=75$ and $N=75$ were set to 1.5 cm each to represent the well diameter utilized in cultures. Since osteocyte bodies are approximately 5 – 20 µm in diameter and osteoblast bodies are 20 – 30 µm in diameter, a

single square in the CA model represents a set of 100 – 1600 osteocytes and 36 – 100 osteoblasts.

The parameter T represents time; one time step in the simulation coincided with one day in the *in vitro* culture. Each two dimensional array $B(:, :, t)$ simulated the state of the tissue culture well at $T = t$. The model was run for $T = 26$ to reproduce the 26-day culture conditions described in Section 1.2. The set of states in the CA model was the set of nonnegative real numbers representing the amount of bone formed by each group of osteoblasts; the larger the number, the more mineral produced. In the CA model the neighborhood of a cell was defined as the seven by seven grid surrounding the cell (See Figure 2).

At the beginning of each simulation the matrix B was identically zero. A random number i was selected from a normally distributed random variable I . The observations collected from the experiments described in 1.2 were used to determine the mean and standard deviation associated with I , which correlates to the number of initial clusters forming within culture wells at early time points (See Table 1). In each simulation i determined the number of elements of the matrix B which became non-zero elements representing initial sites of mineralization. These sites were chosen randomly with each having the same probability of being chosen. Once i number of sites in the matrix B were selected they were changed in the matrix B from zero to a random number selected from a uniform distribution on $(0,1)$, representing the volume of matrix material deposited.

Each time step in the simulation represents one day in the *in vitro* characterization. At each step values of the entries in the matrix may increase. To determine whether a specific element of the matrix increases in value (i.e. whether bone is formed at a specific site) the average of the values of each site in the matrix was compared to the average of the values of neighboring (Figure 2) sites. As a result sites surrounded by mineralization were more likely to increase bone formation. Similarly, bone localized and formed nodules in culture. As stated in Section 1.3 a permutation test was used to assess whether the mathematical model accurately represented the *in vitro* characterization.

3. Results

3.1. Characterization Results

In vitro results are shown in Figure 3. While few switch wells showed bone formation beginning at 16 days, all switch wells showed formation by 26 days of culture. Image A depicts representative images of calcium nodules stained with alizarin red ($n = 9$) by 26 days in comparison to control wells ($n = 3$) where cells were not chemically induced to differentiate. Image B depicts representative phosphate nodules stained with von Kossa at 26 days. A control well is pictured on the left, and a switch well is pictured on the right. The results of this study indicate the average percent area covered by alizarin red within switch wells ($n = 9$) at 26 days was 20.6%, and the average percent area covered by von Kossa ($n = 9$) was 20.5%; here, there is no statistically significant difference.

3.2. CA Model Results

Given the high variability of our CA model, we ran 10,000 Monte Carlo simulations to obtain an average area of culture well covered by bone formation. This analysis resulted in an average area of 27.83% and a standard deviation of 11.78%. Figure 4 shows nine such simulations.

Figures 3 and 4 represent the characterization performed *in vitro* and the simulations run *in silico*, respectively. The nine switch wells in Figure 3A were compared to the nine runs of the CA presented in Figure 4 by use of a permutation test. Three control, or uninduced, wells are also included in Figure 3, but were not included in the runs of the CA presented in Figure 4 as they were not of significant interest to compare. The percentage area of each of the wells covered by bone is displayed with their respective well in both Figure 3A and Figure 4.

It is worth noting that in Figure 4 dark blue color illustrates that no bone or mineralization formation was observed in that portion of the well. A lighter blue color represents mineralization which precedes bone formation. This initial mineralization is not discernible in Figure 3 as it does not bind to alizarin red. The inclusion of this mineralization in the *in silico* simulations is important for further experimentation that is not relevant to the results presented in this manuscript. Warm colors in the *in silico* experiments shown in Figure 4 represent bone. Warmer colors depict greater bone density.

The goal of the CA was to have both the percentages from the *in vitro* characterizations and *in silico* simulations come from the same distribution and for this stochasticity to arise from the basic principles guiding the bone formation process. Instead of exactly replicating each of the *in vitro* characterizations with our CA model, we want it to demonstrate the same stochasticity and variation shown *in vitro*.

In addition the CA model was used to track bone nodule formation throughout the duration of the characterization. Figure 5 shows amounts of mineralization simulated by two different runs of the CA model.

In this study, the percent areas covered by mineral were obtained for both culture images and simulated images. The permutation test was performed by creating a two-sample data set, $x_1, x_2, \dots, x_9$ (3.75%, 26.9%, 18.51%, 4.41%, 25.93%, 13.11%, 28.10%, 40.08%, 24.48%) and $y_1, y_2, \dots, y_9$ (25.39%, 36.50%, 5.42%, 23.47%, 9.21%, 6.12%, 18.47%, 6.54%, 30.65%), where x_i 's and y_i 's represent the percent areas covered from culture and simulated images, respectively. It was assumed that all x_i 's came from an unknown distribution F_x , and all y_i 's came from an unknown distribution F_y . The null hypothesis of the test indicated that the two distributions were the same regardless of the actual analytical forms of the distributions.

The difference between the two sample means was chosen to be the test statistic for the permutation test. The following steps were completed to carry out the test:

1. Calculated the observed value for the test statistic, which is the difference in means between the two samples.

2. Pooled the observations from two samples, and randomly drew two subsets from the pooled data without replacement. Then calculated the corresponding test statistic from the resulting two subsets.
3. Repeated Step (2) until all possible permutations were exhausted. The collection of all calculated test statistics is the distribution of the test statistic under the null hypothesis.
4. Calculated the p -value, which is the proportion of permutations in which the absolute value of the permuted test statistic is greater than or equal to the absolute value of the observed test statistic.

We reject the null hypothesis if the p -value is less than or equal to the significance level, α , which is commonly set at 0.05 in practice.

The computer language **R** [17], version 3.2.2, was used to carry out the permutation test. The observed value of the test statistic (the difference between two means) was 2.61%, and the p -value was found to be 0.6389. Therefore, there was sufficient evidence to conclude that the two samples, which represent data from culture images and simulated images, were not statistically different from one another. In other words, the proposed mathematical model was statistically proven to be a valid model for producing results that are consistent with the results obtained from *in vitro* quantification of osteoblast activity.

4. Conclusion

The work presented here is the first of a series of CA models which are being developed in order to better understand the mechanisms of bone formation and bone metabolism. This manuscript describes a mathematical model created to simulate *in vitro* culture in which osteocytes are induced to mineralization and after 26 days, the area covered by mineral is recorded. The CA model presented here is an *in silico* representation of the characterization performed in the lab.

The permutation test was run to compare the results from *in vitro* culture conditions and mathematical models. Given the high p -value of the test it was concluded that both the *in vitro* cell cultures and *in silico* simulations came from the same distribution. As additional characterizations are performed and more data become available, the CA model will be further calibrated. A well-calibrated CA model provides the freedom to run many *in silico* experiments for differing conditions which will help elucidate the effects of various factors, such as mechanical load, on bone formation.

The osteoblast CA described here models with considerable accuracy the area covered by osteoblastic bone formation. Future work includes several additions to the current model as well as the incorporation of other standalone CA models.

The current model will be expanded to incorporate elemental composition of mineral formed, concentration of alizarin red dye present within stained cultures, osteoblastic alkaline phosphatase activity (expressed during the formation of hard mineral) and the roles of biological markers/cytokines in bone formation.

Currently, we are characterizing osteoclast bone resorption in culture. Precursor cells have been cultured on bovine bone wafers and induced to fuse and undergo osteoclastogenesis. Osteoclasts will be stained for tartrate-resistant acid phosphatase (TRAP), an acidic enzyme that selectively breaks down mineral matrix, and TRAP activity will be quantified. Further, bone wafers will be stained with toluidine blue, and percentage of wafer surface resorbed will be determined. A CA model will be developed to simulate osteoclast activity *in vitro*. Together with the bone formation model describe here, the resorption model will contribute to an ABM of bone remodeling.

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Highlights

- In this paper we present the first towards the larger goal to construct biomimetic multiscale mathematical models to understand the underlying mechanisms, pathways and multicellular interactions that regulate bone remodeling.
 - The team will work to isolate each cell involved in the bone remodeling process in *in vitro* characterizations and experiments to analyze the mechanisms underlying this complex process by using mathematical modeling and statistical tools.
 - As a first step, this manuscript presents the isolation of osteoblasts in an *in vitro* characterization and the accompanying cellular automata model that mimics the behaviour of osteoblastic cells.
- This paper is truly multidisciplinary approach to understanding the bone formation process. The team includes two biomedical engineers, two statisticians and two mathematical modelers. By taking a multidisciplinary approach, we are able to utilize the strengths of three different areas to better understand the dynamics of bone formation.
 - One feature that makes this paper unique is the use of statistical methods to validate the cellular automata model.
 - The use of statistical tools to assess how effectively the mathematical model represents the biological phenomenon provides an innovative way to improve mathematical modeling of highly stochastic biological processes.
 - We are hoping that making mathematical models more realistic becomes a trend in mathematics.

Figure 1.

(A) Phase contrast imaging of uninduced (left) and induced (right) MC3T3-E1 cells. Uninduced cells are pictured at four days post-seeding, and induced cells are pictured at 16 days post-switch. Scale bars represent 100 μm . (B) Rhodamine phalloidin (red) and DAPI (blue) fluorescence images identifying actin and nuclei of osteoblastic cells in culture. Uninduced (left) MC3T3-E1 cells are pictured at four days post-seeding, and induced (right) cells are pictured at four days post-switch. Scale bars represent 50 μm . (C) SEM image showing the confluent MC3T3-E1 cells at induction. Some cell connections were broken in the fixative process. (D) Bone nodules (formation) were quantified with alizarin red staining, which shows calcium, and von Kossa staining, which shows phosphate. The area of each well is 1.9 cm^2 .

Figure 2.
Progression of Cellular Automata

Figure 3.

A. Whole well images of alizarin red stains at 26 days post-switch. The characterization was run in triplicate and repeated twice for a total of three characterizations. Uninduced/control and induced/switch wells and corresponding percentages covered by stain are given. The area of each well is 1.9 cm^2 . B. Representative whole well images of von Kossa stains at 26 days post-switch. An uninduced/control well is pictured on the left, and an induced/switch well is pictured on the right. The area of each well is 1.9 cm^2 . C. The average percent area covered by alizarin red within induced/switch wells was 20.6%, and the average percent area covered by von Kossa within induced/switch wells was 20.5%; the difference is not significantly different. Here, error bars represent standard errors of the means.

Figure 4.
Nine different cellular automata images for different runs after 26 simulated days

Figure 5. The figure on the left shows the mineralization area at each day on one CA run. For the figure on the right, alizarin red dye was extracted from each well and its concentration determined at each time point. Concentrations are given, and error bars represent standard errors of the means.

Table 1

Parameters used in CA Model

Step	Function	Variables	Explanation
t = 0	$I = \lfloor ini \rfloor$ I number of initial mineralization sites	$ini = \lfloor (.001 * M * N) \rfloor$ $M \times N$ r $b = \lfloor (.001 * M * N) \rfloor$	Initial number of mineralization sites Matrix dimensions Randomly generated number $r \sim N(b, 1)$ 0.1% of initial sites
t = 1 to t = 26	If $xa > A$ $B(i, j, t) = B(i, j, t) + \frac{7y^2}{8}a$ else $B(i, j, t) = B(i, j, t)$	a A $x \sim \mathcal{N}(8, 8)$ $y \sim \mathcal{U}(0, 1)$	Average mineralization of neighborhood Average mineralization of entire matrix Site mineralizing depends on neighborhood, the values μ and σ obtained from observation Mineralization amount is non negative