ON THE PERMANENT LIFE OF TISSUE OUTSIDE THE ORGANISM

A Thesis in
Materials Science and Engineering

by

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ABSTRACT

Growth and maintenance of cells/tissue outside the body is central to the practice of biomedical sciences and technology. *In vitro* culture techniques, developed almost a century ago, are fundamental enabling tools for the study of life processes and are routinely employed in many applications that impact human health care. Emergence of the field of tissue engineering, focused on developing tissue surrogates for transplantation, has renewed the emphasis on critically examining the conditions under which we culture cells/tissue outside the body. It has been well recognized that isolation of cells from the complexity of their native physiological environment results in adaptive responses that often limit cell viability and function *in vitro*. Bridging the gap between physiological complexity and *in vitro* culture environments is critical to the successful implementation of the tissue engineering strategy. We show herein that a compartmentalized bioreactor based on the principle of continuous-growth–and-dialysis provides stable culture conditions that better simulate the physiological environment. The bioreactor was used to grow mineralizing, collagenous bone tissue up to 150 μm thick from an inoculum of isolated murine (mouse calvaria MC3T3-E1, ATCC CRL-2593) or human (hFOB 1.19 ATCC CRL-11372) osteoblasts over uninterrupted culture periods up to a year. Proliferation and phenotypic progression of an osteogenic-cell monolayer into a tissue comprised of ≥6 cell layers of mature osteoblasts in the bioreactor was compared to cell performance in conventional tissue-culture polystyrene (TCPS) controls. Cells in the bioreactor basically matched results obtained in TCPS over a 15 d culture interval, but loss of insoluble ECM (iECM) and ~2X increase in apoptosis rates in TCPS after 30 d indicated progressive instability of cultures maintained in TCPS with periodic refeeding but without subculture. By contrast, stable cultures were maintained in the bioreactor for up to a year, suggesting that extended-term
tissue maintenance is feasible with little-or-no special technique. Month’s long culture
interval in the bioreactor lead to progression of pre-osteoblasts to osteocyte-like cells
embedded in mineralized matrix observed in normal bone and production of visually-
apparent (macroscopic) bone. Challenging bioreactor-derived bone tissue at different stages
of development with metastatic breast cancer cells (MDA-MB-231) known to invade the
skeleton created a system-in-crisis that captured early stages breast cancer colonization of
bone. In situ confocal microscopy revealed sequential steps of breast cancer cell adhesion,
penetration, and degradation of the 3D bone-like osteoblast tissue over co-culture intervals
ranging from 3 to 10 days. Bioreactor enabled direct observation of interactions of breast
cancer cells with engineered 3D bone like tissue and simulated an important step in the
progression of the disease that may be a target for therapeutic intervention. Using bone as a
model tissue, this study demonstrates that complex physiological and pathological processes
of great importance to human health can be simulated using engineered cellular
environments for potential applications in drug discovery and toxicology.
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Chapter 1

Introduction

1.1 In Vivo and In Vitro

Fundamental biological processes can be studied within the living organism (in vivo) or in a controlled artificial environment outside the body (in vitro). Investigations of biological phenomena in vivo using animal models has the distinct advantage that the responses to particular dose are integrated across the length scales of cells, tissues and organs, and are, therefore, representative of physiological complexity. However, from an experimental design standpoint, it is not very easy to separate cause and effect because the response to a dose cannot be limited to a particular physiological compartment. In vivo studies offer very limited control over the experimental conditions and it is difficult to tease out the relationships between the numerous variables involved. Therefore, the very complexity that confers physiological relevance to in vivo studies often makes them difficult to interpret (Table 1).

Isolation of cells, tissues or even organs into a controlled artificial environment has several advantages. In vitro techniques provide greater control over the experimental conditions and the ability to limit the number of variables. In other words they allow us to isolate a control volume defined by the biological
structure under investigation and its environment. Techniques that make it possible to isolate, grow and sustain cells and tissues in controlled environments outside the body were developed in the early part of the 20th century by pioneers such as Harrison and Carrell.1-3 The incredible power of this approach to provide fundamental insights into biological phenomenon was illustrated by the very first experiment utilizing the technique. In 1907, Harrison2 isolated fragments of the nerve tube of larval frog into a medium of clotted frog lymph in a hollowed out glass slide and observed the outgrowth of nerve fibers from the explants. Harrison’s seminal experiment2,4 provided, for the first time, the means to observe living processes outside the body under controlled conditions. The experimental setup allowed Harrison to separate the growth of the embryonic nerve tissue from the overall complexity of embryonic development. Harrison’s method was perfectly suited for testing his hypothesis that the nerve fibers were outgrowths from the neurons and it helped him furnish experimental evidence to resolve definitively one the major scientific controversies of his time on the development of the nervous system.2,4

Early in vitro culture techniques including those used by Harrison and Carrell were primarily culture of explants or fragments of tissue. The shift from culture of tissues to the culture of isolated cells became possible only with the development of enzymatic methods of cell separation in the 1950s.5 Rapid
adoption of these methods has resulted in culture of isolated cells being the norm today. These developments have been consolidated into the standard practice of monolayer cell culture where cells are cultured on a 2D substrate surrounded by a medium that sustains the metabolic requirements of the cell. In almost a century since their development, in vitro culture techniques have become integral to the practice of biomedical sciences and technology. These enabling techniques remain to this day, fundamental tools for the study of life processes and are routinely employed in many applications that impact the delivery of human health care.

1.2 The Gap between In Vivo and In Vitro

In spite of the widespread success in the use of in vitro techniques, it is important to recognize that there are profound differences between in vivo and in vitro cellular environments. In vivo, cells held together by cell-cell contacts and the extracellular matrix in a 3D structure, interact with neighboring cells of the same type and/or different type with their responses coordinated by a variety of biochemical and mechanical factors. In other words, cells function within the context of a highly specialized microenvironment that is specific to the cell type and the anatomical location. Growth, differentiation and ultimate fate of cells is determined by complex interactions between the cell and its microenvironment.
Culture of cells outside the body using standard 2D monolayer techniques deprives cells of their physiological context and brings into sharp focus the impact of culture conditions on cellular function. Physical, biological and biochemical factors affect cellular function within this controlled environment (Fig. 1-1). To illustrate the nature of these environmental effects, consider the affect of changes in osmolarity of the medium on cell volume. Decreasing the osmolarity of the medium surrounding a cell leads to a compensatory response of cell swelling; increase in osmolarity leads to cell shrinkage. In addition to these physical factors, biological factors like cell-cell and cell-matrix interactions condition cellular responses. Absence of proper environmental cues often results in a less differentiated phenotype in vitro. Thus, there is strong experimental evidence that cells undergo several adaptive responses when transferred from their physiological environment to a radically different exogenous environment. In aggregate, these adaptive responses have been characterized as “culture shock” and they are often thought to limit cell viability and function in vitro.

1.3 Bridging the culture gap

The basic principles underlying in vitro culture techniques have not changed significantly over the many years of their development. It is only with
the emergence of fields like tissue engineering\textsuperscript{14} that the environment in which we routinely grow cells is being critically appraised. The gap between conventional cell culture and the physiological environment is driving efforts to develop engineered 3D cellular environments that can better simulate physiological complexity.\textsuperscript{6,15} These approaches have the potential to integrate physiological responses across the length scales of cells and tissues under controlled conditions enabling us to model complex biological processes \textit{in vitro}.

Working on the overall theme of the influence of the microenvironment on cellular responses, this study showed that improved simulation of physiological complexity can be achieved through suitable engineering of the cellular environment. Particular focus was on the stability of culture environment - an issue that has not received much attention in the literature. Stable culture environments were engineered by separating cell growth and cell feeding functions and through retention of cell-secreted growth factors and cytokines in the cellular microenvironment.\textsuperscript{16} Using bone forming cells as a model system, it was shown that stable culture environments promote assembly of isolated cells into complex biological structures that can develop/mature over extended periods.\textsuperscript{16} These \textit{in vitro} grown structures permit a high level of control over important experimental variables yet provide sufficient biological complexity (\textit{e.g.} three-dimensional, tissue-like cell density) that outcomes are a
reasonable predictor of the whole-organism physiological response to the experimental variables. These tissue surrogates will be used as model systems to study biological phenomenon of importance to human health care such as bone formation and breast cancer colonization of bone.
References


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Table 1: Comparison of *in vivo* and *in vitro* techniques

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<td>1. Easier to separate cause and effect under controlled conditions and isolate the effect of individual variables.</td>
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<td>2. Results obtained at the different length scales have to be integrated to create informed hypotheses about function at the organism level.</td>
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<td>reality.</td>
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<td>are largely preserved.</td>
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<td>problems by breaking them down to their individual components.</td>
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<td>5. Difficult to study molecular level interactions including signaling</td>
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<td>and regulatory mechanisms.</td>
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<td>6. Expensive, time-consuming and requires the maintenance of animals</td>
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<td>under the required test conditions for extended periods.</td>
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<td>7. Studies using animals have to consider the inherent heterogeneity</td>
<td>7. Permits greater control over test conditions and therefore repeatability.</td>
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<td>of subjects and require careful statistical analysis.</td>
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Figure 1-1: Cells and/or tissues are cultured in an artificial environment within a defined control volume. Physical, biological and biochemical factors that condition the cellular response within the control volume are listed.
Cellular Environment and Cellular Function

Physical Factors
- Temperature (typically 37°C)
- Gas composition (5% CO₂)
- Medium composition, pH, and Osmolarity
- Oxygen tension
- Mechanical stress

Biological Factors
- Presence of cytokines, growth factors, serum-derived proteins, or other macromolecules in the medium either by design or through secretion by the cells as part of their normal function.

Cell-Cell, Cell-ECM and Cell-Substrate Interactions.

Figure 1
Chapter 2

Literature Survey

This chapter starts with a short tutorial on bone growth and development and introduces critical aspects of the physiology of bone-forming cells. Historical review of in vitro models used to study these cells revealed specific design considerations for meeting the need for improved bone cell culture models.

2.1 Osteogenesis In Vivo

2.1.1 Bone Development

Bone is a highly specialized organ that performs multiple functions within the body. In addition to providing structural support, bone acts as a storehouse of essential minerals and is the site for hematopoiesis. Bone consists of a network of interconnected cells, mineralized extracellular matrix and spaces that include bone marrow cavity, vascular supply, canaliculi and lacunae. Growth, development and maintenance of the structural integrity of bone require the coordinated actions of a complex cellular network.

The primary modes of bone formation are endochondral and intramembranous. The process of endochondral bone formation that gives rise to the long bones and the vertebrae requires the presence of a cartilaginous template that is replaced by or remodeled into bone. Structurally, endochondral bone is further organized as either a dense outer
shell called the cortical bone or as a relatively thin inner network of connecting rods and plates called trabecular bone. Intramembranous bones like the flat bones of the skull, scapula and the ilieum form directly through differentiation of the mesenchymal cells into bone forming osteoblasts without the need for an intermediate cartilage model.

The principal functional activities involved in bone development and maintenance are the formation of bone by osteoblasts and resorption of bone by osteoclasts. Removal of bone from one site and resorption of bone at a different site occurs through a process called "modeling" and is responsible for the shape and structure of bone during development. In adults, resorption and new bone formation occur at the same site resulting in regeneration of bone. This process called "remodeling" continues throughout life and becomes the dominant process by the time bone reaches its peak mass in the 20s. Remodeling results in complete regeneration of the adult skeleton every 10 years.

Bone remodeling occurs through a tightly regulated sequence of osteoclastic bone resorption followed by osteoblastic bone formation. The specific sequence of events involved are osteoclast resorption and apoptosis followed by migration of osteoblast precursors to the resorption site, proliferation and differentiation of osteoblasts, formation of the extracellular matrix and its mineralization and finally cessation of osteoblast activity. Remodeling occurs through focal and discrete packets through the agency of a temporary cellular unit called the "basic multicellular unit" (BMU) comprising of osteoblasts and osteoclasts, central vascular capillary, nerve supply and related connective tissue. It is estimated that in healthy human adults, 1 million BMUs operating at any given time and over a course of a year 3-4 million BMUs are initiated to support the highly dynamic function of bone tissue. The resorption stage of the bone remodeling sequence takes place
over the time frame of 10-14 days whereas formation of new bone at the site of resorption takes up to 6 months. Delicate balance between resorption and bone formation that is the hallmark of the remodeling process becomes disrupted in several pathological conditions including osteoporosis and breast cancer metastasis to bone resulting in loss of the structural integrity of bone.

2.1.2 Cellular Origins of Bone Cells.

Osteoblasts and osteoclasts are derived from precursor cells within the bone marrow. The bone marrow consists of cellular elements belonging to either the stromal lineage or the hematopoietic lineage. The hematopoietic group is responsible for giving rise to blood cells such as lymphocytes, erythrocytes, granulocytes, megakaryocytes and monocytes. Osteoclasts are derived from hematopoietic cells of the monocyte/macrophage lineage. Osteoblasts belong to the stromal group and are derived from multipotent mesenchymal stem cells, which also give rise to bone marrow stromal cells, chondrocytes, muscle cells and adipocytes. The complex sequence of steps involved in the transformation of these early progenitor cells into functional osteoblasts and the various factors that regulate the lineage development are only beginning to be understood. The focus of this study was the functional expression of the osteoblast phenotype through the sequence of proliferation, extracellular matrix formation, mineralization and finally osteocytic transformation.

2.1.3 Bone Formation

Bone consists of a mineral phase (70-90%) dispersed within an organic matrix (osteoid) consisting primarily of Type I collagen. The rest of the organic matrix is composed
of non-collagenous proteins such as osteocalcin, osteonectin, bone sialoprotein, fibronectin, vitronectin, thrombospondin as well as several proteoglycans, glycosaminoglycans and lipids (Table 1). The organic matrix of bone also sequesters several cytokines and growth factors derived from bone cells and other cells that play important roles in modulating growth and differentiation of bone cells. These include growth factors such as Insulin-like growth factor (IGF-I), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF-β), Bone Morphogenetic Proteins (BMP) and cytokines such as IL-1 (Table 2).

Synthesis, deposition and mineralization of the organic matrix of bone is carried out by osteoblasts. Bone formation involves proliferation of preosteoblasts and their differentiation into functional osteoblasts capable of deposition, organization and progressive mineralization of the organic matrix of bone. Stages of pre-osteoblast proliferation, differentiation, extracellular matrix formation, mineralization and osteocytic transformation advance through a tightly regulated spatial and temporal sequence.

In the final stage of development osteoblasts can either undergo programmed cell death (apoptosis) or transform into bone lining cells or become trapped within the bone matrix as osteocytes. The proportion of osteoblasts that follow a particular fate is highly variable and depends on several factors including age, species and anatomical location. In the human cancellous bone it has been estimated that about 65% of the osteoblasts undergo apoptosis with 30% transforming into osteocytes. In addition to pre-osteoblasts, active osteoblasts, bone lining cells and osteocytes, a number of transitional stages have been identified in the developmental program of the osteoblasts. These transitional stages include preosteoblastic osteoblast, osteoblastic osteocyte (Type I pre-osteocyte), osteoid-
osteocyte (Type II pre-osteocyte), Type III pre-osteocyte, young osteocyte and old osteocyte. Characteristic feature of this developmental program is that osteoblasts at different stages of differentiation are part of a functional network of cells that communicate with each other through gap junctions.\textsuperscript{26,27,28} As a result, bone tissue has been described as a functional syncytium with cells ranging from osteoprogenitors to mature osteocytes connected in a vast network,\textsuperscript{28} and coordinating responses to various mechanical and biochemical stimuli through dynamic cell-cell contacts.

Various stages in the osteoblast differentiation are identified on the basis of morphology, function, responsiveness to hormones such as parathyroid hormone and the expression of a suite of molecular markers.\textsuperscript{29} Pre-osteoblasts are actively dividing cells that express bone specific markers such as alkaline phosphatase and osteonectin.\textsuperscript{4} Osteoblasts are characterized as non-dividing, cuboidal shaped cells, with large eccentric nucleus and one to three nucleoli. Osteoblasts have extensive rough endoplasmic reticulum and golgi areas consistent with their primarily secretory function.\textsuperscript{4} Osteoblasts can be distinguished from pre-osteoblasts by the upregulation of number of bone markers including osteocalcin, bone sialoprotein and type I collagen, alkaline phosphatase, vitamin D3 receptor and others.\textsuperscript{29} As osteoblasts lay down the matrix, they become trapped within matrix and undergo transformation to osteocytes.\textsuperscript{30,31} Nascent osteocytes radiate several cell processes\textsuperscript{32,33} towards the mineralizing matrix and have organelles very similar to those of osteoblasts. As the osteocytes become embedded deeper in the matrix, changes in morphology and cell organelle distribution occur.\textsuperscript{34} Mature osteocytes have a characteristic stellate shape, are embedded within spaces called lacunae and extend long thin cellular processes that contact other osteocytes and osteoblasts.\textsuperscript{32} Osteocytic transformation results in up to 70\% decrease in cell volume compared with original osteoblasts, and simplification of organelles with
decrease in golgi apparatus, decrease in the size of mitochondria and decrease in endoplasmic reticulum.\textsuperscript{25,34} Osteocytes with an estimated half life of 25 years in humans have a very long life span compared to the 3-4 month life span of the osteoblasts.\textsuperscript{33} Osteocytes die as a result of senescence, degeneration, and/or entrapment by osteoclasts.\textsuperscript{30} Several aspects of osteocyte function and lifecycle are yet to be defined because it is very difficult to study osteocytes outside the body.\textsuperscript{30} Osteocytes are non-dividing, and they do not maintain their differentiated state and function when isolated from the matrix that surrounds them in their native state. As a result of this, several fundamental questions regarding osteocytic transformation and the function of osteocytes are not yet fully understood.\textsuperscript{3,29,30} In summary, the complex developmental program of osteoblasts associated functionally with the process of bone formation ranges from pre-osteoblasts to osteocytes with a number of transitional stages between these two stages and is modulated by a number of cellular and molecular level interactions.\textsuperscript{33,35} In spite of significant progress in our understanding of the developmental program of osteoblast and the various transitional stages there are still considerable gaps in our knowledge. Filling these gaps requires experimental models that can define the various stages not only in terms of morphology but also by the levels of expression of specific molecular markers like alkaline phosphatase, osteocalcin, osteonectin, parathyroid hormone related protein, as well as markers considered to be specific to osteocytes such as E11 and DMP1.

\textbf{2.1.4 The Osteoblast Mileu}

Growth, development, and function of osteoblasts is modulated by number of local factors through both autocrine and paracrine mechanisms.\textsuperscript{20} The vast majority of these factors are secreted by the osteoblasts themselves (Table 2).\textsuperscript{5} Mediators of osteoblast response are also derived from other cells in the marrow environment and from the bone
matrix which has a high affinity for systemic and local factors. The complexity of the osteoblast milieu is defined by cell-cell, cell-matrix interactions as well as the presence of a number of regulators in the local environment of the osteoblasts that include growth factors such as insulin-like growth factor (IGF-I), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF-β), bone morphogenetic proteins (BMP) and cytokines such as IL-1. For extensive discussion of these regulatory factors see References 17 and 20.

2.2 Osteogenesis In Vitro

Tissue culture techniques have contributed tremendously to our understanding of the mechanisms of bone formation. Bone has been studied in vitro as an organ, tissue or as isolated cells. This section traces the historical development of these techniques outlining the advantages and disadvantages associated with each of these approaches.

2.2.1 Organ Cultures

Strangeways and Fell pioneered the use of organ cultures to study the embryonic development of skeletal tissue. Early organ cultures involved isolation of explants from the chick embryo at different stages of development into a semi-solid medium of clotted plasma and embryo extract. Cultures were incubated in test tubes, watch glasses or in hollowed out glass slides. Watch glass cultures were further enclosed in a petridish with a layer of moist cotton at the bottom to provide a humidified culture environment. In the first successful adoption of the technique, Strangeways and Fell demonstrated the growth and development of cartilage from the leg buds of a 3-day chick embryo over a 14 day culture interval. These early experiments revealed the potential of the technique to study the developmental processes of bone under controlled conditions. Organ culture technique was
further refined and developed by Fell to study the development of avian embryonic long bones. In 1929, Fell published the first paper\textsuperscript{37} dealing with calcification of bone \textit{in vitro} using organ cultures of isolated femora and tibiae from 5 $\frac{1}{2}$ to 6 day chick embryo. In the 1930s Fell used this technique to establish the importance of alkaline phosphatase in mineralization,\textsuperscript{37,39,40} to demonstrate the osteogenic capacity of the periosteum and endosteum \textit{in vitro}\textsuperscript{38} and to investigate the \textit{in vitro} development of the avian knee joint.\textsuperscript{41} These early studies and Fell’s contributions in particular are highlighted here because they represent a significant progress from static histological studies to the dynamic study of the development of bone. Fell’s pioneering studies form the basis for our understanding of different stages of cartilage and bone cell differentiation during the process of embryonic bone development. Organ cultures have been used since then to describe the embryonic and fetal development of the chick long bone\textsuperscript{42} and to study bone development in mammalian models like fetal and rat long bones.\textsuperscript{43} Organ cultures have also been found to be particularly suitable to evaluate the action of vitamins and hormones on bone\textsuperscript{44} and to carry out resorption studies.\textsuperscript{43,45}

\textbf{2.2.2 Culture of Bone Slices and Explants}

Culture of slices of bone derived from the long bone, calvaria or metatarsal tissue provide another \textit{in vitro} alternative to study bone.\textsuperscript{46-48} The most successful study employing slices of bone tissue\textsuperscript{49} \textit{in vitro} was carried out by Rose in 1960 using tissue culture chambers made of cellophane.\textsuperscript{50-52} Rose was among the first to recognize the importance of retaining cell-secreted factors in the cellular environment for maintaining the differentiated state of the cells. Culture chambers employed by Rose separated cell growth and cell feeding functions through use of a dialysis membrane and represented a significant innovation in technique.
(see chapter 3 for extended discussion). Rose reported outgrowth of osteoblasts and fibroblasts from embryonic chick bone slices in culture.\textsuperscript{49} Although calcification was not demonstrated, explants were maintained for extended periods up to weeks.\textsuperscript{49,52} Utility of these early studies in understanding the cellular basis of bone formation was limited by the heterogeneity of the cell populations involved. In the absence of modern genomic and proteomic tools it was difficult to identify and separate the responses of bone cells from other cell types involved. Therefore, the primary contribution of these early studies using slices of bone tissue \textit{in vitro} is limited to short-term studies on the metabolism of bone and in determining the effect of hormones on bone tissue.\textsuperscript{46-48}

Explant cultures of the periosteum and endosteum have also been used to study bone formation.\textsuperscript{38} Culture of periosteum was initiated by Fell who showed that explants of periosteum from 6-10 day chick embryos could form osteoblast-like cells that produced an osteoid. Culture of folded periostea, developed by Nijweide, is a particularly useful form of the explant technique.\textsuperscript{53} Nijweide and Vanderplas showed that in cultures of folded periostea from 16-18 day old embryonic chick calvaria, osteoprogenitor cells at the center of the fold differentiate into matrix-secreting osteoblasts.\textsuperscript{53} Folded periostea preserve critical cell-cell contacts and the overall 3D structural organization of the tissue and serve as a functional model for the study of osteogenesis \textit{in vitro}. Culture of folded periostea from embryonic chick calvariae has been used to study hormonal regulation,\textsuperscript{54,55} metabolic effects\textsuperscript{55}, the regulation of calcium transport\textsuperscript{53,55}, to demonstrate osteoid formation\textsuperscript{53,56} and mineralization in the presence of β-glycerophosphate\textsuperscript{57}, and to study the effect of dexamethasone on bone formation \textit{in vitro}.\textsuperscript{58}
2.2.3 Culture of Isolated Bone Cells

The real breakthrough in our understanding of the physiology of bone forming cells came through the development of enzymatic methods of isolation of bone cells by Peck and his colleagues\(^59,61\) in the 1960s. Peck\(^61\) employed buffered collagenase to isolate rat calvarial osteoblasts and cultured them using monolayer cell culture techniques. Isolated cells were shown to be viable in culture for days although matrix deposition or bone formation was not noted.\(^61\) In subsequent studies, it was shown that osteoblastic cells synthesize collagen, respond to ascorbic acid with enhanced collagen synthesis\(^59\) and react to hormones such as parathyroid hormone.\(^62\) Isolation of osteoblasts in culture under these controlled conditions made it possible for the first time to undertake a wide range of biochemical and metabolic studies. It was possible, for example, to study calcium transport,\(^63,64\) oxygen consumption and lactic acid production\(^65\) over short-term incubation periods.

Methods of enzymatic isolation developed by Peck and his co-workers and the refinements of these methods\(^66\) provided the basis for much of subsequent work on understanding the function of bone forming cells. Sequential extraction of bone cells using proteases such as trypsin and collagenase\(^67-71\) resulted in isolation of subpopulations of osteogenic mouse calvarial cells. A further advance in developing homogenous cell populations was the dissociation and cloning of fetal rat and mouse calvarial cells\(^72-79\) in the early 1980’s. Cloning allowed isolation of cell populations based on specific characteristics such as growth patterns, responsiveness to parathyroid hormone or alkaline phosphatase activity. Formation of discrete mineralized bone nodules \textit{in vitro} was reported in cultures of fetal rat calvarial cells in the presence of ascorbic acid and \(\beta\)-glycerophosphate.\(^80\) Ability of cultured osteoblasts to form mineral deposits was found to be dependent on several factors...
including initial cell density, length of culture interval and the presence of ascorbic acid and organic phosphates like \(\beta\)-glycerophosphate.\(^{72-74,80-89}\) Isolated osteoblasts in culture were shown to synthesize several proteins involved in bone formation including osteocalcin, type I collagen, osteonectin and osteopontin.\(^{22,90}\) Identification of these proteins and enzymes and their temporal expression during the bone development process was actively investigated.\(^{22,91}\) Studies using cultures fetal rat and chick calvarial osteoblasts identified the developmental sequence of osteoblasts consisting of proliferation, extracellular matrix formation and mineralization and co-related this sequence with the gene expression of specific proteins involved in bone formation.\(^{22}\)

### 2.3 In Vitro Models of Bone Formation: An Assessment

In summary, bone cells/tissue in culture isolated from different sites (long bones, calvaria, mandibles or the iliac crest) and from different species (rat, mouse, avian, human) using a variety of isolation techniques (enzymatic dissociation, outgrowths from explants) have contributed to our understanding of bone formation (Table 3). These isolation and culture techniques continue to be in use today.\(^{92}\) Careful analysis of these disparate studies reveal important issues that are critical to understanding past efforts at culturing bone cells and devising better culture models for the study of bone formation. Some of these issues are discussed in the next few sections.

#### 2.3.1 Homogeneity versus heterogeneity

A convincing argument can be made that the overwhelming trend in the historical development of bone culture models is a reductionist shift from the heterogeneity and complexity of tissue/organ/explant cultures to the simplicity and homogeneity of cultures of isolated bone cells. Shift to the study of homogenous cell populations was driven
by a need to isolate the cellular level responses of specific population of bone cells from various other influences (other cell types, biochemical factors) in the bone microenvironment. However, a purely reductionist approach brings into question the physiological relevance of culturing isolated bone cells deprived of critical cell-cell and cell-ECM interactions. Choice between organ cultures and cultures of isolated bone cells is ultimately an experimental design issue and has to be resolved by the needs of the problem being investigated. Traditionally organ cultures have been used for studying the systemic effects of various hormones, drugs or other agents on bone and while cultures of isolated bone cells have been used to investigate cellular and molecular level interactions involved in the formation of bone. Culture of tissue explants have been the bridge between the length scales of cell and organ.

Another way of resolving the issue of length scale is to define a functional unit of structure with the necessary complexity to simulate the physiological process of bone formation. Osteoblasts have been known to lose their differentiated state when cultured as a monolayer and reassume their differentiated state when cultured as multiple layers. There is also extensive evidence in the literature that multiple-layered osteoblast tissue is required to demonstrate mineralization in vitro. Tissue surrogates grown from isolated cells can integrate the requirements of 3D structure, increased cell-cell contact and cell-ECM interactions in the developmental processes of the bone cell and in the formation of bone in vitro. Developing 3D tissue from isolated cells in a controlled manner has the potential to realize functional biological structures that can bridge the gap between culture of isolated bone cells and organ cultures. Further, tissue engineering methods differ fundamentally from cultures of explant tissues because they allow us to build complexity in a controlled manner using relatively homogenous cells as the starting materials. Aspects of cell
proliferation and stages of differentiation can be controlled by providing suitable environmental cues through use of scaffolds, specially designed culture vessels called bioreactors and by controlling the presence of various biochemical factors in the cellular environment. Numerous studies in recent years have adopted this approach to develop 3D bone tissue from isolated bone cells seeded on to scaffolds and cultured in vitro in dishes, spinner flasks, perfused cartridges or rotating vessels.

2.3.2 Length of Culture Interval

The length of culture interval is a critical variable in studies of bone formation in vitro. Demonstration of osteogenesis in vitro requires continuous culture intervals of weeks to months because the underlying physiological processes of bone cell proliferation, extracellular matrix formation and mineralization takes place over extended periods up to months. Quick survey of the historical development of in vitro bone cultures shows that the most successful studies of osteogenesis involve extended culture intervals. The classic example is the study by Binderman and his colleagues where they demonstrated for the first time mineralization in cultures of isolated bone cells after 8 weeks of continuous culture. In vitro cultures of bone cells must not only be viable over these extended culture intervals but they must simulate the different stages in the development of bone forming cells in a physiologically relevant manner. Recent studies have also shown that the formation of bone in vitro is dependent on the length of culture interval.

2.3.3 Effect of the Microenvironment.

Biochemical Factors: Growth and function of bone cells is regulated by the presence of a number of local factors in their microenvironment. Osteoblasts secrete a number of regulatory factors that in turn control the growth and differentiation of these cells through
autocrine and paracrine mechanisms. Importance of retaining these biochemical factors in the cellular microenvironment was first articulated by Rose in the 1960s and has been elegantly demonstrated by Tenenbaum and Heersche using cultures of folded chick periosteaum. Success of periosteal osteogenesis model has to do largely with the fact that folding the tissue not only increases cell-cell contact but also provides a microenvironment in which the cell-secreted products are sequestered. To demonstrate this effect, periostea were cultured without folding under two different configurations. In the first configuration cell-secreted products were allowed to accumulate in the immediate environment of the tissue. In the alternate configuration cell-secreted factors could diffuse away from the tissue leading to reduced local concentrations. Under these experimental conditions it was found that osteo-differentiation took place only in those situations where the cell-secreted products were trapped within the local cellular environment. Refinements of the experimental setup employing Diaflo membranes with different molecular weight cut-offs showed that allowing large molecules (100-300kD) to accumulate in the local cellular environment was critical for inducing differentiation of the osteoprogenitor cells. Recognition of these ideas has to the led to the discovery of several factors in the bone microenvironment that play a critical role in the development of the bone cell. Soluble factors that influence bone formation include bone morphogenetic proteins (BMP), fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and Interleukin-1 (IL-1). Complete description of the complex role these factors play in the cellular physiology and development of bone is beyond the scope of this work. However, it is critical to incorporate the effect of local regulators of bone formation in the development of in vitro models of osteogenesis. Recent studies using 3D cell culture models have confirmed the importance of the mass transfer of biochemical factors in the development of bone tissue.
**Influence of Medium/Substrate:** Early *in vitro* studies of bone tissue were carried out in the semi-solid medium of plasma clot/embryo extracts and with air as the gas phase. Refinement of explant culture techniques have resulted in culture of explants on a floating raft/grid/gel at the interface between liquid medium and air to ensure sufficient gas exchange. Development of techniques for the culture of isolated bone cells resulted in widespread adoption of fluid medium consisting primarily of buffered salt solution with addition of various nutrients and vitamins. The conventional practice is to culture monolayer of cells in polystyrene dishes/flasks completely surrounded by a fluid medium containing nutrients and to incubate these dishes in 5% CO$_2$ in air.

Higher CO$_2$ tensions used in conventional culture have been shown to stimulate bone cell proliferation and bone formation and calcification in organ cultures. In studies examining the role of oxygen tension, lower O$_2$ tension has been shown to stimulate bone matrix formation and calcification in some culture systems. Studies of oxygen consumption are diverse in terms of cells/tissues used and therefore it is difficult to extrapolate the results of these studies to arrive at a specific oxygen requirement for bone cells. Ensuring optimal delivery of this critical nutrient is critical to the development of *in vitro* bone cell culture models. Oxygen tensions have to be tailored to the thickness of the cell/tissue layer and the specific metabolic requirements of the cells being cultured. Conventional polystyrene flasks are relatively impermeable to CO$_2$ and O$_2$ and therefore oxygen supply is mostly through the dissolved oxygen supplied through the medium. Oxygen diffusivity in aqueous solutions is rather low. Depending on the rate of oxygen consumption, cells in conventional culture may be placed in anoxic conditions or hyperoxic conditions. Therefore, the level of medium in the culture dishes (between 2-5mm) has to be
carefully controlled to provide sufficient oxygen tensions for the metabolic requirements of the cells. Local oxygen concentrations regulate cell behavior in many different ways and therefore it is important to consider not just the bulk oxygen concentration but also the effects of oxygen gradients on cellular function. In summary, supply of oxygen in optimal pericellular concentrations is a critical design consideration for developing better in vitro models of bone formation.

**Mechanical Stress:** Growth and development of bone is conditioned by mechanical loads imposed on the skeleton. Number of model systems have been developed to study the effect of stretching and pressure forces on bone and organ cultures. More recently fluid shear stresses have been shown to have an important role in regulating the development of bone tissue in 3D cell culture models. Although, mechanical conditioning of in vitro bone cells/tissue is an important area of research, it is not being addressed directly through this study.

Review of the literature on bone cell physiology and historical development of the in vitro methods used to study the function and development of bone cells, revealed fundamental requirements for the design of culture models that can bridge the gap between multiple length scales and demonstrate osteogenesis in vitro. These requirements include 3D structural organization, optimal mass transport of nutrients and oxygen tension, stability of the culture environment achieved through retention of cell-secreted biochemical factors and the ability to grow/maintain tissue over extended time periods. Design and implementation of a compartmentalized bioreactor that meets these requirements is described in the next chapter.
2.4 3D models of Bone Formation

Identification of improved methods of hard tissue repair, augmentation or replacements represents a major clinical and socioeconomic need. The field of bone tissue engineering has emerged in recent years to meet this need through the development of engineered bone tissue from a combination of cells, scaffolds and growth factors. Rapid growth in the field has led to the development of several 3D models of bone formation employing the basic strategy of culturing cell-scaffold constructs under controlled conditions in devices called bioreactors. Bone tissue engineering approach has received extensive attention in recent literature. Examples of primary studies, reviews, and even books devoted to the subject indicate the extent of interest in the field.

Central to the development of engineered bone tissue are the design and implementation of bioreactors that provide a controlled physical environment and suitable microenvironmental cues to direct the assembly of isolated bone cells into 3D tissue. Numerous studies in recent years have investigated the use of bioreactors to engineer bone tissue to repair or replace bone lost due to disease or injury. Many different bioreactor designs such as spinner flasks, rotating wall vessels, perfusion reactors and rotating vessels have evolved for this purpose in recent times (Refer to Table 4 for a few examples drawn from many refs.), each with unique attributes and drawbacks. Common design considerations underlying these different approaches to bioreactor development are discussed below -

2.4.1 Cell-scaffold Interactions: The surface of the scaffold is designed to promote cell-attachment through careful choice of scaffold material and biomaterial surface engineering. The internal structure of the 3D scaffold is designed to promote efficient cell seeding, to
provide a suitable microenvironment for the cells, to allow for transfer of fluids and to provide a template for the development of 3D tissue. Scaffolds made of biodegradable polymers are designed to degrade and to be ultimately replaced by the developing tissue.  

2.4.2 Mass Transport of Nutrients and Oxygen: Critical limiting factors in the growth and maintenance of 3D tissue are the delivery of soluble nutrients and oxygen to the cells while simultaneously removing metabolic waste products. Design of bioreactors has to take into account concentration gradients in nutrients and oxygen concentrations that may develop through the thickness of the tissue and the tissue/scaffold constructs. Without adequate nutrient supply, the scaffolds may either develop a necrotic core or diminished cell survival and function at the center of the scaffold compared to cell/tissue layer at the surface. Bioreactor designs take different approaches to address this fundamental issue. Spinner flasks provide increased mass transport through continuous mixing of the medium. However, the turbulence generated in spinner flasks can be detrimental to tissue development. Mass transport through the tissue construct could be increased by continuous rotation of the culture vessel itself or by continuous direct perfusion of medium through the cell/scaffold constructs. Rotating wall vessels employ dynamic laminar flow under conditions of low shear stress for efficient mass transport.

2.4.3 Mechanical conditioning: Bioreactors have also been designed with specific emphasis on mechanical conditioning to the 3D bone tissue constructs. Mechanical conditioning can be provided through direct application of stress or through application of fluid shear stress.
2.4.4 Mass transport of biochemical factors: A design feature that has received far less attention in the development of bioreactors is the mass transport of biochemical factors. Source of biochemical factors could be either endogenous (cells) or exogenous (added to the bioreactor environment). It is well recognized that cell-secreted growth factors and cytokines play important roles in modulating cellular function through both autocrine and paracrine mechanisms. Retaining these factors within the cellular microenvironment could be critical for the growth/differentiation and maturation of the 3D bone tissue. (For complete discussion see section 2.3.3 in this chapter). Continuous or scheduled perfusion of medium typically employed in bioreactors can result in removal of cell-secreted regulatory factors, perturbation of the cellular environment and instability in cultures. There is experimental evidence to suggest that increased flow rates through 3D bone constructs lead to diminished cell survival and function that could be a result of the loss of cell-secreted regulatory molecules.155

As a consequence of the above factors, we have focused our attention on the design and implementation of a compartmentalized bioreactor based on the principle of continuous-growth and dialysis. The compartmentalized bioreactor was a significant departure from existing designs because of its emphasis on stability of the pericellular environment (Table 5). Extraordinarily stable culture environments were achieved in the bioreactor through the separation of cell-growth and cell-feeding functions by a dialysis membrane. Carefully selected molecular weight cut-off resulted in continuous supply of nutrients and removal of low molecular weight waste products while retaining cell-secreted biochemical factors. Whole device was enclosed by gas permeable films that provide sufficient oxygen tension to meet the metabolic requirements of the cells. Continuous dialysis resulted in controlled delivery of nutrients and removal of waste products. As a
result of the compartmentalized design, it was not necessary to disturb the medium in the cell chamber through the course of the month’s long experiment. At the same time, nutrients from the medium reservoir gradually dialyzed into the cell chamber while low-molecular weight nutrient were continuously removed from the cell chamber. Hence a decided advantage to the simultaneous-growth-and-dialysis critical to this study is that the microenvironment remained substantially constant. Design features that further distinguish the bioreactor include scaffold-free design that permitted direct observation of the growth of 3D bone- like tissue and its interaction with metastatic cancer cells through in situ confocal microscopy. Scaffold free design also permitted routine examination of tissue through phase-contrast microscopy and relatively easy isolation of tissue (avoiding the complexity of separating tissue from a 3D scaffold) at different stages of development for various analyses. The surface area of the films was designed to be equivalent to standard T-25 tissue culture dishes (25 cm²) to enable direct comparison culture conditions in the bioreactor and standard 2D cell culture. The bioreactor was designed, in principle, to accommodate application of mechanical stress on the cells. This design feature, however, was not evaluated in this particular study.

The implementation of the compartmentalized bioreactor for extended term culture of bone cells is discussed in the next chapter.
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List of Table Legends

Table 1: Principal extracellular matrix proteins secreted by the osteoblasts and their sequence of expression.

Table 2: Factors in the bone microenvironment secreted that condition the growth and differentiation of the osteoblasts.

Table 3: Historical time line of the development of *in vitro* models of bone formation.

Table 4: Representative bioreactors for bone tissue engineering applications.

Table 5: Unique design features of the compartmentalized bioreactor
Table I: Principal extracellular matrix proteins secreted by the osteoblasts and their sequence of expression. Adapted from Reference (22)

<table>
<thead>
<tr>
<th>Principal Extracellular Matrix Proteins</th>
<th>MW (kDa)</th>
<th>Characteristics</th>
<th>Sequence of Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>~320</td>
<td>Principal structural component of bone extracellular matrix.</td>
<td>Expressed early, during the proliferation period. Gene expression gradually downregulated with mRNA maintained at low osteocalcin basal level at subsequent stages of differentiation.</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>60</td>
<td>Acidic glycoprotein. Arg-Gly-Asp containing sequence mediates cell attachment. Binds to Hydroxyapatite.</td>
<td>Expressed early and late in the osteoblast development sequence.</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>33</td>
<td>Phosphorylated glycoprotein, binds calcium, cell attachment.</td>
<td>Associated with mineralization.</td>
</tr>
</tbody>
</table>
Table 2: Factors in the bone microenvironment secreted that condition the growth and differentiation of the osteoblasts.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW(kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>7.5</td>
</tr>
<tr>
<td>IGF-II</td>
<td>7.5</td>
</tr>
<tr>
<td>aFGF</td>
<td>~16</td>
</tr>
<tr>
<td>bFGF</td>
<td>~16</td>
</tr>
<tr>
<td>IL-1</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>25</td>
</tr>
<tr>
<td>BMP</td>
<td>18.5</td>
</tr>
<tr>
<td>OIF</td>
<td>22-28</td>
</tr>
<tr>
<td>PDGF</td>
<td>30</td>
</tr>
<tr>
<td>EGF</td>
<td>~6.4</td>
</tr>
<tr>
<td>BDGF</td>
<td>11</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Growth factors and cytokines secreted by osteoblasts

Factors derived from the Bone and other sources

IGF- Insulin Derived Growth Factor
aFGF- Acidic Fibroblast Derived Growth Factor
bFGF-Basic Fibroblast Derived Growth Factor
BMP- Bone Morphogenic Protein
OIF-Osteoinductive Factor
PDGF- Platelet Derived Growth Factor
IL-Interleukin
EGF-Epidermal Growth Factor
BDGF-Blood Derived Growth Factor

* Cytokines are polypeptides (proteins) that regulate many cell functions. Cytokines that act on the same cell that produced them are called autocrine factors; those that act on other cells are called paracrine factors; those that act systemically (through the vascular system) are referred to as endocrine factors. Molecules that regulate cell division are referred to as growth factors.
Table 3: Historical time line of the development of *in vitro* models of bone formation.

<table>
<thead>
<tr>
<th><strong>Organ Cultures</strong></th>
<th><strong>Explant Cultures</strong></th>
<th><strong>Culture of Isolated Osteoblasts</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fell and Robison (1929)</td>
<td>Cultivation of isolated femora and tibiae from 5½ to 6 day chick embryos using watch glass technique.</td>
<td>First successful demonstration of calcification of bone in <em>vitro</em>.</td>
</tr>
<tr>
<td>Fell (1932)</td>
<td>Culture of periosteum and endosteum from embryonic chick calvaria using hanging drop method.</td>
<td>Demonstration of the osteogenic capacity of the periosteum and endosteum in <em>vitro</em>.</td>
</tr>
<tr>
<td>Rose (1960)</td>
<td>Culture of bone explants from the long bone of embryonic chick in culture chambers made of cellophane</td>
<td>Weeks long culture of explants in specially designed culture chambers</td>
</tr>
<tr>
<td>Nijweide (1975)</td>
<td>Culture of folded periostea from embryonic chick calvaria</td>
<td>Study of osteoid formation, calcium transport and effects of hormones</td>
</tr>
<tr>
<td>Tenenbaum and Heersche (1982)</td>
<td>Culture of folded periostea from embryonic chick calvaria</td>
<td>Osteoid deposition and mineralization in the presence of β-glycerophosphate</td>
</tr>
<tr>
<td>Peck <em>et al.</em> (1964)</td>
<td>Isolation of osteoblasts in culture from rat calvariae using buffered collagenase.</td>
<td>Landmark study that pioneered isolation of osteoblasts using enzymatic techniques.</td>
</tr>
<tr>
<td>Smith <em>et al.</em> (1973)</td>
<td>Isolation of four different types of cells by mechanical separation from rat calvaria</td>
<td>Studies on the short term (hours) metabolism of isolated bone cells.</td>
</tr>
<tr>
<td>Binderman <em>et al.</em> (1974)</td>
<td>Isolation of osteoblasts in culture from rat calvariae using trypsin and EDTA</td>
<td>Weeks long culture of osteoblasts showing deposition and mineralization of extracellular matrix.</td>
</tr>
<tr>
<td>Wong and Cohn (1974)</td>
<td>Isolation of osteoblasts using sequential enzymatic treatment</td>
<td>Isolation of sub-populations of osteogenic cells</td>
</tr>
<tr>
<td>Rao <em>et al.</em> (1977)</td>
<td>Development of clonal populations from fetal rat and mouse calvarial cells</td>
<td>Increased homogeneity in cell populations</td>
</tr>
<tr>
<td>Leuben <em>et al.</em> (1977)</td>
<td></td>
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<tr>
<td>Kadis <em>et al.</em> (1980)</td>
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<tr>
<td>Aubin <em>et al.</em> (1982)</td>
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<tr>
<td>Ecarot-Charier <em>et al.</em> (1983)</td>
<td></td>
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<tr>
<td>Sudo <em>et al.</em> (1983)</td>
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<tr>
<td>Bellows <em>et al.</em> (1986)</td>
<td></td>
<td></td>
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<tr>
<td>Robey and Termine (1985)</td>
<td>Establishment of human bone cells in <em>vitro</em></td>
<td></td>
</tr>
<tr>
<td>Bellows <em>et al.</em> (1986)</td>
<td></td>
<td></td>
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<tr>
<td>Lian and Stein (1990)</td>
<td>Sequential progression of osteoblast differentiation using fetal rat and chick calvarial osteoblasts</td>
<td>Osteoblast development sequence</td>
</tr>
</tbody>
</table>
Table 4: Representative bioreactors for bone tissue engineering applications.

<table>
<thead>
<tr>
<th>Types of Bioreactors</th>
<th>Principle of Operation</th>
<th>Scaffolds</th>
<th>Cells</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotating-wall vessels (RWV)</td>
<td>RWV consists of two concentric cylinders- a stationary gas-permeable inner cylinder and a rotating outer cylinder. Scaffolds are placed in the annual space immersed in culture medium. Carefully selected rotational rates balance gravitational and centrifugal forces acting on the scaffolds resulting in microgravity-like conditions.</td>
<td>Microcarrier beads made of dextran based polymers.</td>
<td>Rat osteosarcoma cell line ROS 17/2.8 cells</td>
<td>[144]</td>
</tr>
<tr>
<td>Perfusion Culture Reactors</td>
<td>Continuous perfusion of medium through the scaffolds using a peristaltic pump.</td>
<td>Porous ceramic materials composed of β-tricalcium phosphate</td>
<td>Osteoblasts derived from the bone marrow of 7 week-old Fischer 344 male rats</td>
<td>[106]</td>
</tr>
<tr>
<td>Rotating Vessels</td>
<td>50 ml polypropylene centrifuge tube with a 30x40mm silicone window for gas exchange placed on roller culture apparatus rotating at 6 RPM</td>
<td>3D poly(DL-lactide-co-glycolide) PLGA foams</td>
<td>Osteoblastic cells from the marrow of 3-7 days old neonatal Lewis rats</td>
<td>[146]</td>
</tr>
<tr>
<td>Spinner Flasks</td>
<td>Scaffolds seeded with cells are attached to needles hanging from the cover of the flask. Magnetic stir bar at the bottom of the flask is used to effect mixing of the medium</td>
<td>3D poly(DL-lactide-co-glycolide)PLGA foams</td>
<td>Rat marrow stromal cells</td>
<td>[143]</td>
</tr>
</tbody>
</table>
Table 5: Unique Design Features of the Compartmentalized Bioreactor

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Design Implementations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass Transport of Nutrients</td>
<td>Continuous- growth and dialysis</td>
</tr>
<tr>
<td>Mass Transport of Biochemical Factors</td>
<td>Limited to the cell growth compartment by the dialysis membrane</td>
</tr>
<tr>
<td>Flow of Medium</td>
<td>Static</td>
</tr>
<tr>
<td>Delivery of Oxygen</td>
<td>Gas-permeable films made of a co-polymer of ethylene methacrylate designed to provide optimal oxygen tensions.</td>
</tr>
<tr>
<td>Cell-Scaffold Interactions</td>
<td>Scaffold free design. Cell-substrate function is performed by the dialysis membrane.</td>
</tr>
<tr>
<td>Microscopy</td>
<td>Optically clear gas-permeable films enable observation of tissue through phase contrast or confocal microscopy.</td>
</tr>
</tbody>
</table>
Chapter 3

Extended Term Culture of Bone Cells in a Compartmentalized Bioreactor

Abstract

A specialized bioreactor is used to grow mineralizing, collagenous tissue up to 150 μm thick from an inoculum of isolated murine (mouse calvaria MC3T3-E1, ATCC CRL-2593) or human (hFOB 1.19 ATCC CRL-11372) osteoblasts over uninterrupted culture periods longer than 120 d (4 months). Proliferation and phenotypic progression of an osteogenic-cell monolayer into a tissue comprised of ≥6 cell layers of mature osteoblasts in the bioreactor was compared to cell performance in conventional tissue-culture polystyrene (TCPS) controls. Cells in the bioreactor basically matched results obtained in TCPS over a 15 d culture interval, but loss of insoluble ECM (iECM) and ~2X increase in apoptosis rates in TCPS after 30 d indicated progressive instability of cultures maintained in TCPS with periodic refeeding but without subculture. By contrast, stable cultures were maintained in the bioreactor for more than 120 d, suggesting that extended-term tissue maintenance is feasible with little-or-no special technique. TEM ultramorphology of tissue derived from hFOB 1.19 cells recovered from the bioreactor after only 15 d of culture showed evidence of osteocytic-like processes
and gap junctions between cells like that observed in vivo, over-and-above elaboration of the usual osteoblastic markers such as alkaline phosphatase activity and mineralization (alizarin-red). Thus, the bioreactor design based on the principle of simultaneous-growth-and-dialysis was shown to create an extraordinarily-stable pericellular environment that better simulates the in vivo condition than conventional tissue-culture. The bioreactor shows promise as a tool for the in vitro study of osteogenesis and osteopathology.
1. Introduction

Cells extracted from the native, *in vivo* physiological state and placed into a culture system undergo adaptive responses often referred to as “culture shock” (1). The impact and duration of culture shock strongly depends on the stability of the pericellular (micro) environment, the extent to which this microenvironment simulates the *in vivo* condition, and the ability of cells to actively interact/transform the pericellular milieu by secreting a variety of macromolecules found in extracellular matrix (ECM) (2, 3). Indeed, culture shock and the accumulated damage cells sustain in culture has long been thought to limit cell viability *in vitro* (4, 5). Thus, one of the bioengineering objectives for any *in vitro* culture device is to create a stable pericellular environment simulating the *in vivo* condition in a manner that mitigates, rather than amplifies, the impact of culture shock. The conventional tissue-culture approach is to surround cells held in dishes or flasks with a buffered medium containing various nutrients (amino acids, glucose, serum proteins, vitamins, etc.). As cells grow, nutrients are depleted, waste products accumulate (especially lactic acid), and pericellular pH decreases to unacceptable levels. The typical solution to this problem is to exchange spent growth medium with fresh, either on a continuous basis (perfusion) or as a matter of scheduled maintenance. A decided disadvantage with either of these approaches is that the
aforementioned secreted macromolecules are removed along with waste products. In the case when periodic media exchanges are employed, the resulting oscillation in both pH and nutrient concentrations imparts an instability in cultures that can lead to excessive cell vacuolization, ruffled cell margins, and increasing rates of cell-surface detachment. Thus, conventional tissue-culture strategies, especially the flask-and-dish type serviced with periodic media exchanges, fail to maintain a stable pericellular environment critical to recovery from culture shock. As applied specifically to the culture of bone cells in vitro, continuous removal or periodic exchange of growth medium also means that cell/protein-mediated dissolution-precipitation reactions involved in mineralization-resorption are likewise perturbed. Thus it is difficult to simulate the natural developmental sequence of bone cells that includes proliferation, post-mitotic expression of a differentiated phenotype, extracellular matrix formation, and mineralization. As an example relevant to this work, transformation of mature osteoblasts into an osteocytic phenotype in vitro has not been reported to our knowledge.

More than a decade before widespread use of conventional tissue culture in biotechnology, G. G. Rose pioneered the “simultaneous-growth-and-dialysis” method (6) by culturing cells beneath a cellulosic membrane (commercial cellulose wrapping film in Rose’s implementation). The core idea was to
continuously feed cells with low-molecular-weight metabolites by dialysis through a cellulose membrane that also retained secreted high-molecular-weight macromolecules within the growth space created by the bounding membrane. Metabolic waste products such as lactic acid dialyzed out of the pericellular space and into the basal medium where waste could be removed without perturbing cells by wholesale growth-medium removal. Rose’s idea languished in the literature until its rediscovery more than twenty years later when simultaneous-growth-and-dialysis was bioengineered into a routinely usable, so-called compartmentalized culture device (7, 8), one version of which is shown Fig. 1. This bioreactor was shown to facilitate a number of advantages in the culture/maintenance of soft-tissue and hybridoma cells, perhaps most notably of which was the ability to sustain cells for more than 30 d without user intervention and the accumulation of biosynthesized macromolecules (such as IgG) within the growth space for periodic harvest (8). Unpublished work (Vogler) suggests advantages in the culture of recalcitrant primary cells and in vitro immunization as well.

Our interest in the development of improved orthopedic biomaterials and scaffolds for orthopedic tissue engineering (9-11) has led the need for in vitro test vehicles that permit extended-term (>30 d) contact of osteoblasts, osteoclasts, and co-cultures thereof with candidate biomaterials. Finding many limitations with
conventional culture, not the least of which was loss of culture integrity over long culture intervals, we were motivated to implement the compartmentalized bioreactor for bone-cell culture. Herein we report phenotypic development of two continuous osteoblast cell lines from an osteogenic-cell monolayer to a mineralizing, collagenous tissue; mouse calvaria MC3T3-E1 (ATCC CRL-2593) and human fetal osteoblasts (hFOB 1.19, ATCC CRL-11372). TEM ultramorphology of hFOB 1.19-derived tissue recovered from the bioreactor after 15 d shows evidence of osteocytic-like processes and regions of intercellular contacts between cells, suggesting that osteoblasts undergo complex phenotypic development within the bioreactor like that observed in vivo when osteoblasts are engulfed in a mineralizing tissue. Thus, the compartmentalized bioreactor shows promise as a tool for the in vitro study of osteogenesis and osteopathology.

2. Methods and Materials

Bioreactor Design and Implementation: The compartmentalized bioreactor (Fig.1, Panel a) separates a cell-growth space (A, 5 mL) from a larger-volume medium reservoir (B, 30 mL) with a dialysis membrane (C). Cells were cultured on a transparent, liquid-impermeable film (E) selected for cytocompatibility (12-14) and gas (O₂ and CO₂) permeability (see further below). During culture, cells were continuously bathed in pH-equilibrated and oxygenated medium dialyzing from the reservoir (B). At the same time,
metabolic waste products such as lactic acid dialyzed out of the growth compartment (A), maintaining low-pericellular concentrations. Serum constituents or macromolecules synthesized by cells with molecular weights in excess of the dialysis-membrane cutoff (6-8 kDa in our implementation) were retained and concentrated within the growth compartment. The entire vessel was ventilated through transparent gas-permeable films bounding cell growth and reservoir compartments (D, E). The assembled bioreactor had a 25 cm² cell-growth area. The medium reservoir volume was designed for medium-replenishment intervals ranging from 15-45 d, depending on the metabolic activity of the cells. Access to the cell-growth space and medium reservoir was through luer taper ports (J, K; Panel c Fig. 1) using standard pipettes. The bioreactor was designed to work with most standard inverted phase-contrast microscopes with minor stage modification and allowed adequate optical microscopy throughout the culture interval; although resolution at the cellular level naturally was compromised by the development of a thick, collagenous multi-cellular tissue. The bioreactor was machined from 316L stainless-steel stock (8). The body of the compartmentalized device consisted of four main rings (F, G H, I; Panel c). Two chambers (A, B) were created with three films (C, D, E) sandwiched between inner rings (F, G). The whole device was held together in a liquid tight fashion using 6 stainless steel screws, as can be seen in the laboratory implementation of Panel b. Access to, and venting of, growth and reservoir
chambers was through Luer-taper ports (J, K). The gas permeable films (G, H) forming the outer barriers for chambers (A, B) were ~3 mil thick and made by hot pressing Surlyn 1702 resin (DuPont, Wilmington, DE) by simultaneous application of heat (220 °C) and pressure (245 Pa) in a laboratory hot press (Model 2699, Fred S. Carver Inc.). The internal film barrier was cellulosic-dialysis membrane (Spectrapor-13266, Spectrum Medical Industries) and was hydrated in deionized water for at least 1 hr before assembly of the bioreactor. Fabricated bioreactors were filled with 0.1% sodium azide prepared in phosphate buffer saline, packaged in plastic bags, and sterilized using 10 Mrad γ-ray irradiation at the Breazeale Nuclear Reactor on the campus of the Pennsylvania State University. Sterile-packed bioreactors were opened within the confines of a laminar-flow biosafety cabinet, drained of azide storage solution, and 3X rinsed with basal medium using conventional aseptic technique just before cell inoculation as described below.

**Cells and Cell Culture:** Murine calvaria pre-osteoblasts (MC3T3-E1, ATCC CRL-2593) and human fetal osteoblastic cells (hFOB 1.19, ATCC CRL-11372) were obtained from American Type Culture Collection (ATCC). MC3T3-E1 were cultured in alpha minimum-essential medium (α-MEM) supplemented with 10% charcoal-stripped fetal bovine serum, and 1% penicillin-streptomycin. Post-confluent MC3T3-E1 were cultured in a differentiation medium additionally
containing 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate. hFOB 1.19 were cultured using Dulbecco’s modified Eagle medium-Ham’s F-12 (1:1) basal media supplemented with 10% charcoal-stripped fetal bovine serum, and 1% penicillin-streptomycin. Post-confluent hFOB 1.19 were cultured in a more complex differentiation medium additionally containing 50 µg/mL ascorbic acid, 10⁻⁸ M 1,25-dihydroxy vitamin D₃ and 10⁻⁸ M menadione. The hFOB 1.19 cell line is conditionally immortalized by transfection with a gene encoding for the temperature-sensitive mutant (tsA58) of the SV40 large T antigen. Transfection confers a 33.5 °C continuous proliferation “permissive” temperature and 39 °C quiescent temperature at which cells stop proliferating without undergoing apoptosis (15). We cultured hFOB 1.19 at 37 °C and obtained a doubling time similar to that observed at the permissive temperature (16).

Culture experiments were initiated in rinsed bioreactors (see above) by filling the growth compartment with approximately 5 mL of serum-containing medium containing a suspension of approximately 2X10⁴ cells/mL (sub-confluent cell density). The reservoir was filled with serum-free basal medium containing no proteins. Cells from the same inoculum were plated in 25 cm² standard tissue-culture-grade polystyrene (TCPS) dishes (Corning Life Science) which served as the controls. Cultures were maintained in a water-jacketed 5% CO₂ incubator (Model 3110, ThermoForma) held at 37 °C. After cells had
reached confluence (3-5 d), growth medium was replaced with differentiation medium and cells were permitted to grow in control plates and in the bioreactor without subculture. Medium was replaced in TCPS controls every 3-5 d as dictated by color of the methyl red indicator, with a hint of yellow (acid indicator) leading to full exchange. Basal media within the bioreactor reservoir (but not growth space) was refreshed every 15-30 d, again depending on the perceived color of the pH indicator, with a hint of yellow leading to full exchange of only the reservoir contents. For example, 120 d MC3T3-E1 cultures in the bioreactor were sustained with four replacements of basal media within the reservoir every 30 d.

**Cell Attachment and Proliferation Rates:** Short-term \((0 < t < 3 \text{ hr})\) cell attachment assays were performed as described previously \((16, 17)\). Briefly, cells were plated onto 15 identically-prepared plates at \(2 \times 10^4\) cells/cm\(^2\) in whole medium. TCPS culture dishes were the comparison controls. Dishes with the substratum used in the bioreactor were prepared by adhering Surlyn film into the bottom of TCPS culture dishes using double-sided adhesive tape. Cells were allowed to adhere from the sessile medium while incubated at 37 °C in a CO\(_2\) incubator. At various time intervals, a single plate was selected for destructive analysis (every 5 min from \(0 < t < 30\) min, every 10 min from \(30 < t < 60\) min, every 15 min for \(60 < t < 120\) min, and every 30 min for \(120 < t < 180\) min) by
discarding suspension medium and rinsing substrata 3X with PBS. Attached cells were released with trypsin and counted by hemacytometry. Surfaces were analyzed in triplicate. Proliferation assays were performed in the same basic way, except that after 3h attachment time, substrata were rinsed with PBS to remove non-adherent cells and refreshed with growth medium. Remaining cells were allowed to proliferate for 6, 12, 24, 48, and 72 h before destructive analysis. Long term (≥15 d) proliferation was monitored by SYBR green nuclear-staining. Duplicate TCPS and substrata removed from the bioreactor were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, stained with 0.02% SYBR Green in PBS (Molecular Probes), and visualized using an Olympus BX-60 epi-fluorescent microscope. Average nuclei number was determined by counting 15 representative spots on each substrate using image analysis (ImagePro Software, MediaCybernetics Inc).

**Alkaline phosphatase activity:** Alkaline phosphatase (ALP) activity was quantified at the end of each culture period using a chromogenic assay involving conversion of p-nitrophenyl phosphate to p-nitrophenol as described in (18). Briefly, cells washed in PBS were lysed by rinsing with PBS and 0.1% (w/v) Triton X-100 (Sigma) in PBS. Lysates were subjected to two freeze-thaw cycles, after which ALP reaction buffer (1:1 mixture of 0.75 M 2-amino-2-methyl-1-propanol and 2 mg/mL p-nitrophenol phosphate) was added and incubated at
37°C for 15 min. This reaction mixture was stopped with 0.05 N NaOH, and the absorption was measured at 410 nm with a Beckman Spectrophotometer (DU-70 series). A calibration curve was prepared by serial dilution of p-nitrophenol standard solution. A portion of the reaction mixture was used to measure total protein concentration using Bio-Rad protein assay kit. ALP activity was normalized to total cell protein. Experiments were conducted in triplicate for duplicate cultures. Results are reported as mean±standard deviation (1σ).

**Apoptosis:** Cell apoptosis was analyzed using the enzyme terminal deoxynucleotidyl transferase assay (TUNEL assay, Promega) to catalytically incorporate fluorescein-12-dUTP at 3'-OH DNA ends, so that apoptotic cells fluoresce green. All cells were stained red with SYTOX® Orange (Molecular Probes) so that apoptotic cells could differentiated from normal by fluorescent microscopy. At the end of each growth period, duplicate cultures were rinsed twice with PBS, fixed with 4% paraformaldehyde, and permeablized with 0.2% Triton X-100. Cells were then equilibrated in buffer for 5-10 min at room temperature, followed by incubation with TdT buffer (that included equilibration buffer, nucleotide mix, and TdT enzyme) in a humidified chamber for 60 min at 37°C. The reaction was terminated using 2X “SSC washes” for 15 min at room temperature according to the TUNEL assay protocol. A positive and negative control was prepared in parallel. After removing unincorporated fluorescein-12-
dUTP, cells were stained with SYTOX® Orange for 10 min at room temperature. After staining, substrata were mounted with ProLong® Gold and photographed with a Laser Scanning Confocal microscope (Olympus Fluoview300). Images were analyzed with ImagePro software (MediaCybernetics Inc.). The percentage of apoptotic cells in the whole cell population was calculated from 10-20 random spots on each substrate.

**Insoluble Extracellular Matrix (iECM) Protein assay:** Cells were incubated in a bioreactor or in TCPS for 15 and 30 d. After medium was removed, cell layers were washed twice with PBS and extracted in 4 M guanidine hydrochloride, 50mM Tris-HCl, 100mM 6-aminocaproic acid, 5 mM benzamidine hydrochloride, and 1mM phenylmethylsulphonyl fluoride, pH 7.4, at 4°C with constant rocking. Cells were separated from the extract by centrifugation (2000g X 5 min; the extract was routinely checked for cell debris by microscopy with trypan-blue staining to enhance visualization of cell debris). Protein concentration in the supernatant was quantified using Bio-Rad protein assay kit, and normalized with cell number for each growth condition.

**Hematoxylin and Eosin Staining:** Tissue excised from the bioreactor was fixed in 2.5% glutaraldehyde. Dehydration and paraffin embedding were carried using an automated tissue processor (Citadel 2000, Thermo Electron). 5µm
sections were cut using a microtome (Model 2040, Leica Micro System) and mounted on glass coverslips. Hematoxylin and Eosin staining was carried according to a standard protocol using automated staining equipment (Shandon Gemini, Thermo Electron) and the stained sections were observed under an optical light microscope (Olympus BX50, HiTech).

**Scanning and Transmission Electron Microscopy (SEM and TEM):**

Cultures were washed in buffer (0.1M, pH 7.2 sodium cacodylate) and fixed overnight with 2.5% glutaraldehyde in cacodylate buffer at 4°C followed by staining with 1% osmium tetroxide in cacodylate buffer for an hour at room temperature. Fixed cells were dehydrated using graded series of ethanol concentrations, dried in a critical point dryer (BALTEC SCD030, Techno Trade Inc.) using dry CO₂, mounted on to an aluminum stub, and sputter coated with 10 nm of gold/palladium in an automated sputter coater (BALTEC SCD030, Techno Trade Inc.). Cells were examined under an SEM (JSM 5400, JEOL, Peabody, MA) at an accelerating voltage of 20 kV. Energy dispersive X-Ray analysis was carried out using image analysis software (IMIX-PC v.10, Princeton Gamma Tech Inc.). For TEM, primary fixation with 2.5% glutaraldehyde and secondary fixation with 1% osmium tetroxide were followed by en bloc staining with 2% aqueous uranyl acetate for an hour. Dehydration was carried out using a graded series of ethanol concentrations followed by impregnation and
embedding in Spurr’s resin. Ultra-thin sections were cut with a diamond knife (Diatome Ultra 45) on a microtome (Ultracut UCT, Leica), placed on uncoated copper grids and stained with both 0.2% aqueous uranyl acetate and 0.2% lead citrate. The cross-sections were examined using TEM (JEM 1200 EXII, JEOL) and images were collected using an attached high-resolution camera (Tietz F224, Gauting).

3. Results and Discussion

Tables 1 and 2 compile semi-quantitative cell-growth data over 30 d for hFOB 1.19 and 120 d for MC3T3-E1 cells, respectively, in the bioreactor compared to that obtained in conventional tissue-culture grade polystyrene (TCPS). Neither cells grown on TCPS nor those grown in the bioreactor were subcultured over the entire culture interval (see Methods and Materials). For both hFOB 1.19 and MC3T3-E1, between 1-2 cell layers formed in TCPS compared to 4-6 layers in the bioreactor over 30 d culture interval (the exact number of layers varied with position in the bioreactor and how a cell layer was defined). Evidence of a mature osteoblastic phenotype was indicated by alkaline phosphatase activity which was positive for both cell lines in both culture devices. Mineralization was especially evident by von Kossa and formation of
nODULES in the MC3T3-E1 case, but hFOB 1.19 did not mineralize as well in either culture device. Based on the data summarized in Tables 1 and 2, it is evident that performance of hFOB 1.19 and MC3T3-E1 in the bioreactor was similar that of cells grown on TCPS in the first 15 d of culture. However, cell performance in the bioreactor clearly exceeded that for TCPS over a 15-30 d culture interval, with double the number of cell layers and increased alkaline phosphatase activity and alizarin-red staining. TCPS controls were not carried for more than 30 d because it was both visually and quantitatively evident that these cultures were failing (see further below).

Fig. 2 compares attachment and growth dynamics of hFOB 1.19 in the bioreactor and TCPS over $0 < t < 720$ hr culture period. In the short-term ($0 < t < 3$ hr), there was ~ 2X attachment preference for TCPS over the polymer-film substrata used in the bioreactor (see inset expanding time axis and expressing % inoculum on a linear axis; see also ref. (17) for more discussion of polymer-film cytocompatibility). However, proliferation into confluent monolayer (on TCPS) from this sub-confluent cell density ($3 < t < 72$ hr) and post-monolayer growth into multilayers ($72 < t < 720$ hr) was nearly the same in TCPS and the bioreactor. In fact, actual growth rates $k$ within $3 < t < 72$ hr were statistically identical at 95% confidence (TCPS = $0.23 \pm 0.01 \times 10^{-2}$ hr$^{-1}$; bioreactor = $0.29 \pm 0.03 \times 10^{-2}$ hr$^{-1}$). Data within the $72 < t < 720$ hr interval were too sparse for
statistical comparison, but it is interesting that the total-cell number (at constant surface area = 25 cm²) continued to significantly increase within both the bioreactor and TCPS at approximately the same rate, achieving ~5X increase in total cell number over this time span. The data show that the initial two-fold cell-attachment preference for TCPS persisted through the exponential-growth ($3 < t < 72$ hr) and post-confluence expansion ($72 < t < 720$ hr) phases. Further interpretation in terms of the data in Table 1, this persistent-attachment preference implies that more cells are packed into fewer cell layers on TCPS than within the bioreactor, consistent with the general observation that cells on TCPS appear thinner than in the bioreactor (compare, for example, Panel A, C Fig. 3); although a focused study would be required to be definitive in this regard.

Despite of the initial advantage on TCPS, hFOB 1.19 began to appear less robust than in the bioreactor within 720 hr (30 d), as evidenced by TEM images showing numerous apoptotic bodies, cytoplasmic vesiculation, and chromatin margination (Fig. 3) and a distinct loss of insoluble iECM (Fig. 4). TUNEL assay results (summarized in Fig. 5) confirmed that at 30 d, apoptosis was indeed ~2X greater in TCPS than in the bioreactor (37-47% in TCPS compared to 16-23% in the bioreactor), although 30 d apoptosis levels were roughly 3X 15 d levels in both devices. Examination of hFOB 1.19 tissue recovered from the bioreactor after 15 d culture by cross-sectional TEM (Fig. 6) revealed 3-4 layers of cells
(Panels A and B) with many cytoplasmic protrusions (arrowheads) and healthy-appearing nuclei showing no evidence of apoptosis. The general impression derived from an examination of many such TEM cross-sections is that cell layers closest to the substratum (presumably older cells) were flatter and more dish-shaped compared to cells within upper layers (presumably younger cells) that had a more cuboidal shape. No effort was made to quantify this visual interpretation. Arrow annotations on Fig. 6C point to what appear to be osteocytic-like processes between two cells and Fig. 6D shows a close contact between cells. Exocytosis of vesicles is evident in Fig. 6D.

Results obtained with MC3T3-E1 osteoblasts in TCPS and the bioreactor were similar to those seen for hFOB 1.19 with the notable exception that mineralization was much more striking with MC3T3-E1 cells. One of many large nodules (Panel A, SEM) that were found on and within MC3T3-E1 tissue after 70 d culture (see also Fig. 8 Panel A) was analyzed (Fig. 7). Panel B is a high-resolution cross-sectional view through a nodule similar to that shown in Panel A. Nodules such as these proved positive for calcium and phosphorous by energy-dispersive x-ray analysis (Panel C, SEM/EDAX of a nodule taken from a 30 d bioreactor). These nodules were enmeshed in a fibrous, apparently collagenous, network that was very evident in SEM preparations such as that shown in Fig. 8A. Histological preparations of MC3T3-E1 tissue recovered from
70 d bioreactors (Panel B) confirm substantial matrix development with osteoblasts oriented along sheets of matrix. TEM of von Kossa-stained 70 d tissue shown in Panel C is consistent with collagen fibers running in (IP annotation) and along (OP annotation) the plane with interspersed mineral nodules (arrows). We estimated that tissue taken from a 120 d bioreactor was $\geq 150 \mu m$ thick. After 120 d in culture, there was no indication that MC3T3-E1 tissue grown in the bioreactor was unstable and the absolute term-of-culture within the bioreactor is regarded as indefinite until experimentation finds an endpoint at which significant instability can be detected.
4. Conclusions

Evidence summarized in Fig. 2 and supporting micrographs (Figs. 3-8) show that long-term maintenance of hFOB 1.19 (ATCC CRL-11372) and MC3T3-E1 (ATCC CRL-2593) cells without subculturing leads to formation of cell multilayers within a thick, mineralized matrix that cannot be realized when cells are regularly passaged at confluence. Achievement and maintenance of such a 3D ‘tissue’ that promotes cell-cell contact has been shown to be critical to the development of osteogenic cells into a fully-differentiated osteoblast phenotype (19-22). We demonstrated that such tissue can be grown and maintained long-term (> 120 d) in a bioreactor based on the principle of simultaneous-growth-and-dialysis with improved efficiency over conventional tissue-culture labware (TCPS). In fact, tissue maintained 30 d in TCPS with medium replacement every 3 d became unstable, with 2X apoptosis rates and loss of insoluble extracellular matrix (iECM) compared to cells grown in the bioreactor, even though results obtained in TCPS and the bioreactor were similar in the first 15 d of culture. Furthermore, morphology of cells grown in the bioreactor suggested development of an osteoid structure with older cells taking on a flattened osteocytic-like phenotype with inter-cellular connections. The matrix surrounding up to 6 layers of cells was collagenous and mineralized. This comparison of the bioreactor to TCPS suggests that the eventual instability observed in TCPS was primarily due to the accumulated damage arising from
periodic medium replacement that removes not only waste products but also
‘luxury macromolecules’ secreted by the cells. Hence, the standard cell-culture
method tends to propagate, rather than mitigate, the effects of culture shock. By
contrast, the simultaneous-growth-and-dialysis culture method maintained an
extraordinarily-stable pericellular environment. With neither subculturing steps
to push cells closer-and-closer to the Hayflick limit (4) nor the environmental
stress of an ever-changing pericellular milieu, tissue grown with the bioreactor is
apparently stable for very long periods of time measured at least in months.
Thus the compartmentalized bioreactor shows promise as a tool for the *in vitro*
study of osteogenesis and osteopathology.
Acknowledgements

This work was supported, in part, by a grant from the Pennsylvania Department of Health. Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations or conclusions. This work was also supported by The Pennsylvania State Tobacco Settlement Formula Fund and NIH AG13087-10. This work was also supported, in part, by grants from the US Army Medical and Materiel Command Breast Cancer Program (W81XWH-06-1-0432), and the Susan G. Komen Breast Cancer Foundation (BCTR 0601044. Authors appreciate additional support from the Huck Institutes of Life Sciences, Materials Research Institute, and Departments of Bioengineering and Materials Science and Engineering of the Pennsylvania State University.


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Table 1. hFOB 1.19 Cells in Conventional Culture Compared to Compartmentalized Bioreactor

Table 2. MC3T3-E1 Cells in Conventional Culture Compared to Compartmentalized Bioreactor
Table 1. hFOB.1.19 Cells in Conventional Culture Compared to Compartmentalized Bioreactor

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<tr>
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<td>30</td>
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<tr>
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<td>2.97 ± 0.18</td>
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<td>-</td>
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<tr>
<td>Mineralized nodules</td>
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\(^{+}\) = observed; \(^{-}\) = not observed; \(^{a}\) measures of osteoblast maturity; \(^{b}\) (nmol/mg protein/min); \(^{c}\) extent of mineralization.
Table 2. MC3T3-E1 Cells in Conventional Culture Compared to Compartmentalized Bioreactor

<table>
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<th>Days in Culture</th>
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<td>Mineralized nodules</td>
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+ = observed; - = not observed; $^a$ measures of osteoblast maturity; $^b$(nmol/mg protein/min); $^c$ extent of mineralization; ND = not determined
List of Figure Legends:

**Figure 1:** Compartmentalized bioreactor design. Panel (a) is a cross-sectional diagram through the device showing separation of the cell-growth space (A) from the basal-medium reservoir (B) by a dialysis membrane (C). Cells are grown on gas-permeable but liquid-impermeable film (E). The device is ventilated through film (D), which is the same material as (E) as described herein but can be different. The whole device is brought together in a liquid-tight fashion using screws shown in the laboratory implementation Panel (b) and Panel (c) which is an exploded-view identifying separate components. Liquid-access is through leur-taper ports (J, K) which mate to standard pipettes. See Methods and Materials for theory of operation.

**Figure 2:** Growth dynamics of hFOB 1.19 on tissue-culture-grade polystyrene controls (TCPS, filled triangles) compared to the compartmentalized bioreactor (open squares; note logarithmic ordinate). Inset expands short-term attachment rates using a linear ordinate. Note that the initial two-fold cell-attachment preference for TCPS persists through the exponential-growth \((3 < t < 72 \text{ hr})\) and post-confluence expansion \((72 < t < 720 \text{ hr})\) phases.
**Figure 3:** Comparison of hFOB 1.19 on TCPS (Panels A, B) and in the compartmentalized bioreactor (Panels C, D) by cross-sectional TEM. Notice that cell layers are thicker in the bioreactor than on TCPS (compare Panels A, B to C, D), and formation of apoptotic bodies (arrows) after 30 d culture in TCPS.

**Figure 4:** Comparison of insoluble extracellular matrix (iECM) production by hFOB 1.19 on TCPS (dark bar) and in the bioreactor (light bar). Notice that iECM significantly decreased after 30 d on TCPS but remained constant in the bioreactor. Bar values are mean of duplicate samples measured in triplicate with standard deviation represented by error bars.

**Figure 5:** Representative confocal image of apoptotic bodies (green stain) in TCPS and in the bioreactor selected from 8-10 similar in-depth image planes probing hFOB 1.19-derived tissue. All cells were stained red (Sytox Orange, scale bar = 50 μm). Percent apoptotic cells quoted in each panel are the range of values observed within the image planes.

**Figure 6:** Ultramorphology of hFOB 1.19-derived tissue (15 d) recovered from the bioreactor by cross-sectional TEM. Arrows point to cell protrusions that
occasionally connect two cells, as shown in Panel C. Annotations: jNC = gap junction; MV = matrix vesicles; N = nucleus; rER = rough endoplasmic reticulum.

**Figure 7:** Analysis of mineral nodules taken from MC3T3-E1 cultured in a bioreactor. Panel A is an SEM of a large nodule taken from a 70 d bioreactor. Panel B is a high-magnification, cross-sectional TEM of a similar nodule. Panel C is an x-ray spectrum obtained by SEM/EDAX of a nodule taken from a 30 d bioreactor confirming Ca and P as major constituents.

**Figure 8:** Microscopic examination of matrix derived from MC3T3-E1 cultured in a bioreactor for 70 d. Panel A is an SEM of the outer layer showing a highly fibrous network with bone nodules. Panel B is an optical micrograph of a histologic workup showing layers of collagen with imbedded osteoblasts. Panel C is a cross-sectional TEM showing mineralized fibers running in (IP annotation) and out (OP annotation) of the plane.
Figure 1

Diagram a: Diagram showing the flow of gases and media through the system.

Diagram b: Photograph of the actual device.

Diagram c: Exploded view showing the different components and layers of the device.
Figure 2
Figure 3
Figure 4

The graph shows the iECM production (ng/cell) over different growth periods. The x-axis represents the growth period in days (15 days and 30 days), and the y-axis represents the iECM production. The bars indicate the production for TCPS (black) and BioR (gray). The asterisks (*) indicate statistical significance between the two groups.

Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A. Bone Nodule

B. Collagen

C. cfl, cfc

1 μm

50 μm

5 μm
Chapter 4

System in Crisis- Colonization of Bone Tissue by Metastatic Breast Cancer In Vitro

Abstract

Metastasis is the ultimate cause of mortality in over 90% of cancer patients. Metastasis is a multi-step process that starts with the shedding of neoplastic cells from the primary tumor, entry into circulation, exit into a distant organ and ends with colonization of the target organ. The final step in the metastatic cascade, colonization of distant tissue, is a critical step that determines the eventual disease outcome and is therefore a valuable target for therapeutics. Dynamic interaction between tumor cells and the host microenvironment determines the survival, growth and the eventual development of clinically relevant tumors in the target organ. Lack of model systems with sufficient biological complexity to serve as relevant surrogates for the host microenvironment is a significant impediment to understanding the cellular and molecular basis for colonization and discovery of therapeutic interventions. We show herein that a three-dimensional, mineralizing, bone-like tissue grown from isolated mouse calvarial pre-osteoblasts (MC3T3-E1) over months-long culture in a specialized bioreactor is an effective surrogate for studying bone-tissue colonization by metastatic breast cancer. Bioreactor derived bone-like tissue challenged with metastatic breast cancer cells (MDA-MB-231) known to invade the skeleton captured important hallmarks of metastatic colonization. In situ confocal microscopy revealed sequential steps of breast cancer cell adhesion, penetration, and apparent degradation of the 3D bone-like tissue over co-culture intervals ranging from 3 to 10 days. Bioreactor enabled direct observation of interactions of breast cancer cells with engineered bone-like tissue simulating
an important step in the progression of the disease that may be a target for therapeutic intervention.

**Introduction**

Tissue engineering has focused considerable interest in the growth of three-dimensional (3D), multiple-cell-layer tissues from isolated cells of purposely-selected type(s). It has been well recognized that these engineered tissues can be used to great advantage in drug discovery, development of therapeutic strategies, and toxicology by bridging the gap between conventional monolayer (2D) cell culture and whole animals. These *in vitro* tissue models confer considerable control over important experimental variables yet provide sufficient biological complexity that outcomes are a reasonable predictor of the whole-organism physiological response to experimental variables. A further advance in the use of engineered tissue for drug discovery is to create an *in vitro* “system in crisis” that simulates an *in vivo* disease state. Various candidate drugs and/or therapeutic strategies could then be screened to find those that most effectively resolve the purposely-imposed crisis. Tissue surrogates are useful in this pursuit because systemic aspects of disease can be broken down into physiological subsystems to be studied separately *in vitro*. Efficiency of drug discovery can be greatly improved with a clear understanding of the cellular and molecular basis of disease because opportune targets of therapeutic intervention can be clearly identified.

We have focused on metastatic breast cancer colonization of bone as a proof-of-principle problem with both stringent analytical requirements and great importance in human healthcare. Colonization is the final step in the multi-step process of metastasis that starts with the shedding of neoplastic cells from the primary tumor, entry of these cells into circulation, arrest in a distant organ and finally growth and tumor formation in the target organ. Cancers that colonize bone are particularly pernicious because, once bone
colonization occurs, the cure rate is almost zero.4-6 Cancers in bone progress with significant morbidity related to massive bone loss, bone pain, hypercalcemia, pathological fractures, and spinal cord compression.7 Of the major cancers that colonize bone (breast, prostate, and multiple myeloma of hematopoietic origin), breast cancer has perhaps attracted the most attention because of its devastating effects on women - approximately 25% of breast cancers metastasize and the target organ is bone in 46% of the primary cases. Cancer can reoccur after successful treatment of primary tumor and years of remission – in patients with first relapse the frequency of metastatic bone disease is much higher at 70%.8 Critical rate limiting steps in cancer colonization of bone include survival of tumor cells in the bone microenvironment, proliferation and organization into overt tumors and angiogenesis or recruitment of blood vessels necessary to sustain the tumor mass.9 Cancer cells can also remain dormant and persist as isolated cells for years or even decades ready to be activated into fully developed tumors under the right set of conditions.9 Cancer dormancy is a poorly understood stage in disease progression characterized by the presence of cancer cells in bone that have not progressed to form clinically detectable tumors. Difficulty in accessing the bone marrow cavity probably accounts for the fact that bone tumors are usually detected after cancer colonization has reached a very advanced stage, further complicating effective treatment strategies.

Bone degradation in metastatic breast cancer is thought to be primarily mediated by bone-resorbing cells (osteoclasts)10-13 that are activated by cancer cells. Excessive resorption results in the formation of osteolytic lesions that reduce the strength and integrity of bone. Consequently, drugs of the bisphosphonate family are currently used to inhibit osteoclasts. Bisphosphonate treatment slows lesion formation but does not result in the restoration of bone. Furthermore, it is not a cure in that it does not eliminate the cancer cells.14 Osteoblasts, responsible for bone formation, also play a critical role in cancer colonization.
Osteoblasts not only regulate osteoclast activity but also interact with cancer cells. As a consequence, we have focused our attention on understanding the cellular and molecular basis of cancer-cell colonization of bone and the related interactions with osteoblasts. These interactions include suppression of osteoblastic differentiation, increased osteoblast apoptosis, an immune-like osteoblastic stress response, and the up-regulated secretion of cytokines observed in conventional 2D cell culture. These findings imply a significant osteoblastic pathology associated with cancers in bone, in addition to (and perhaps coupled with) the known activation of osteoclasts. Understanding the effect of breast cancer on osteoblast function can lead to therapeutic strategies that not only inhibit development of osteolytic lesions but also restore or renew the normal functional activity of bone formation.

In spite of its clinical importance, the biology of cancer colonization is not completely understood. Lack of experimental models that simulate cellular interactions involved in breast cancer colonization of bone is a significant impediment to progress in the field. Whole animal models often obscure details of the colonization process, especially when the target is a difficult-to-access tissue such as bone. Models based on excised bone are not only technically problematic but also difficult to interface with modern microscopic methods of investigation. Further, most experimental models access only the end stage of the disease defined by presence of clinically relevant tumors – critical early stages that determine the eventual disease outcome remain largely “hidden” and inaccessible. In principle, a sub-set of the metastatic process can be studied in vitro if the model system under consideration retains sufficient biological complexity to be a reasonable surrogate for host tissue. Three-dimensional (3D) tissue models have become a focus of recent investigation for this reason. We have shown recently that a compartmentalized bioreactor based on the principle of simultaneous-growth-and dialysis can promote the development of isolated osteoblasts into mature 3D tissue over extended culture intervals up to a year. Resulting bone like
osteoblast tissue recapitulated progression of pre-osteoblasts to osteocyte-like cells observed in normal bone, including production of visually-apparent (macroscopic) bone. Challenging the bioreactor derived osteoblast tissue at various stages of development with metastatic breast cancer cells known to invade the skeleton revealed the sequential steps of cancer cell-tissue adhesion, penetration, and ultimate degradation of the host tissue, modeling a critical stage in the progression of the disease that may be a target for therapeutic intervention.

Materials and Methods

Cells and Cell Culture: Murine calvaria pre-osteoblasts (MC3T3-E1, ATCC CRL-2593) were a gift from Dr. Norman Karen at the University of Delaware. Human metastatic breast cancer cell line (MDA-MB-231, ATCC-HTB 26) labeled with green fluorescent protein (GFP) were a gift from Dr. Danny Welch from the University of Alabama at Birmingham. MC3T3-E1 were inoculated into the bioreactors at a sub-confluent density (10^4 cells/cm^2) and cultured in alpha minimum-essential medium (α-MEM) (10-022-CV, Mediatech Inc, Manassas VA) supplemented with 10% fetal bovine serum (FBS) and 100U/ml penicillin and 100μg/ml streptomycin. Once the cells reached confluence, the medium was replaced with differentiation medium containing the additional ingredients 50μg/mL ascorbic acid and 10mM β-glycerophosphate. MC3T3-E1 cultures were maintained in the bioreactor without sub-culture for extended intervals up to 10 months using culture conditions and protocols described in chapter 3. MDA-MB-231 cells were cultured in standard tissue culture dishes in Dulbecco’s modified Eagle medium (DMEM) containing 5% fetal bovine serum and 100U/ml penicillin and 100μg/ml streptomycin.
**Bioreactor Co-Cultures:** MC3T3-E1 osteoblast tissue in the bioreactor was stained with Cell Tracker Orange™ (Invitrogen Corp., Carlsbad, CA; prepared according to vendor instructions) by replacing original culture medium with fresh medium containing 2.5μM cell tracker orange for 45 minutes at 37°C, followed by 30 minutes incubation in dye-free culture medium. Co-cultures were initiated by inoculating a suspension of GFP-labeled MDA-MB-231 in osteoblast differentiation medium into the bioreactor on top on the MC3T3-E1 tissue at an estimated cancer cell/osteoblast ratio of 1/10. All operations were performed under sterile conditions in a level II biosafety cabinet. Bioreactor co-cultures were maintained for periods ranging from 3-10 days under standard culture conditions (5% CO₂ and 95% air) in a humidified incubator at 37°C. In situ confocal microscopy was used to follow the interaction of breast cancer cells with 3D osteoblast tissue. At the end of the culture interval, the bioreactor was dismantled and substratum film with adherent tissue was cut into pieces for various assays. Bioreactors cultured with MC3T3-E1 for equivalent periods of time under exactly the same conditions with similar medium changes but without the addition of cancer cells were used as controls.

**Confocal Microscopy:** *In situ* laser-scanning confocal microscopy of co-cultures in the bioreactor was performed using Olympus FV-300 laser scanning microscope (Olympus America Inc, Melville, NY). Sections were observed with a 40X Olympus UPlanF1 objective with a numerical aperture of 0.85. Cell Tracker Orange™ was excited using a 543 nm line from a helium-neon laser and collected through a 565 nm long-pass filter. GFP was excited using a 488 nm argon laser and collected through 510 nm long-pass and 530nm short-pass filters. A 570nm dichroic long-pass filter was used to split the emission. Serial optical sections were taken at 1μm intervals throughout the tissue. Confocal images were processed using Fluoview 300, Version 4.3b, Olympus America Inc. 3D optical
reconstructions of 2-D serial sections were obtained using AutoQuant AutoDeblur and AutoVisualize software (Version 9.3, Media Cybernetics, Inc., Silver Spring, MD).

**Transmission Electron Microscopy:** Tissue excised from the bioreactor was fixed overnight with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at 4°C. Secondary fixation with 1% osmium tetroxide in 0.1M sodium cacodylate was carried out for an hour was followed by enbloc staining with 2% aqueous uranyl acetate. Buffer washes in 0.1M sodium cacodylate (3 washes, each 5 minute duration) were carried out after each fixation step and after the enbloc staining step. Tissue was then dehydrated using a graded series of ethanol concentrations (50%, 70%, 85%, 95%, 100%) and finally immersed in 100% acetone. Infiltration with Spurrs resin was carried out gradually over 3 infiltration steps with each step carried out overnight at room temperature. Samples were embedded in Spurr's resin and cured overnight at 55°C. The cured block of embedded tissue was thin-sectioned with a diamond knife (Ultra 45, Diatome, Hatfield PA) on a microtome (Ultracut UCT, Leica, Bannockburn, IL). Thin sections of tissue were placed on uncoated copper grids and stained with both 0.2% aqueous uranyl acetate and 0.2% lead citrate. Cross-sections of tissue were examined using TEM (JEM 1200 EXII, JEOL, Peabody MA) and images were collected using an attached high-resolution camera (Tietz F224, Tietz Video and Image Processing Systems GmbH, Gauting Germany).

**Scanning Electron Microscopy:** Cultures were washed in buffer (0.1M, pH 7.2 sodium cacodylate) and fixed overnight with 2.5% glutaraldehyde in cacodylate buffer at 4°C followed by staining with 1% osmium tetroxide in cacodylate buffer for an hour at room temperature. Fixed samples were dehydrated using graded series of ethanol concentrations,
dried in a critical point dryer (BALTEC SCD030, Techno Trade Inc.) using dry CO₂, mounted on to an aluminum stub, and sputter coated with 10 nm of gold/palladium in an automated sputter coater (BALTEC SCD030, Techno Trade Inc.). Samples were examined under an SEM (JSM 5400, JEOL, Peabody, MA) at an accelerating voltage of 20 kV.

**Hematoxylin and Eosin Staining:** Tissue excised from the bioreactor was fixed in 2.5% glutaraldehyde. Dehydration and paraffin embedding were carried using an automated tissue processor (Citadel 2000, Thermo Electron, Waltham MA). 5μm sections were cut using a microtome (Model 2040, Leica Micro System, Peabody, MA) and mounted on glass coverslips. Hematoxylin and Eosin staining was carried according to a standard protocol using automated staining equipment (Shandon Gemini, Thermo Electron, Waltham MA) and the stained sections were observed under an optical light microscope (BX50, Olympus USA, Center Valley, PA).

**Results and Discussion:**

Figure 1 represents schematically results of interaction between GFP-labeled human breast cancer cells (MDA-MB-231) and the multiple layer, mineralizing osteoblast tissue in the bioreactor. We observed (Figure 2) that murine MC3T3E-1 osteoblasts maintained in the bioreactor for periods up to 10 months develop a tissue-like network (Panel A and B) of 6-8 layers of differentiated cells within a highly collagenous matrix that stain for alkaline phosphatase activity and mineralization by Von Kossa (Panel B), as well as form mineralized nodules (Panel C) that prove positive for calcium and phosphorous by scanning electron microscopy/energy-dispersive analysis of x-rays. 5 months of MC3T3-E1 culture resulted in formation of visually apparent macroscopic bone especially in the form of
deposits on the dialysis membrane (Fig. 2D). Injection of MDA-MB-231 human breast cancer cells (Figure 3, Panel A) directly onto the osteoblast tissue leads to cancer cell, adhesion and tissue penetration within 24 hours (Panel C) probably accomplished through the extension of long cellular processes (Panel B). In figures 4 and 5 are results of in situ confocal microscopy study of breast cancer cell penetration of, and replication within, an MC3T3E-1 osteoblast tissue developed in the bioreactor over 5 months of continuous culture. Figure 4, a collection of optical sections through the thickness of the tissue corresponding to 1-3 days of cancer cell/bone tissue co-culture indicates that solitary cancer cells (day 1), replicate (day 2) and co-localize with the osteoblasts and penetrate the entire thickness of the tissue (day 3). Figure 5 shows 3D reconstructions of optical sections like those shown in Figure 4 corresponding to days 1-3 of co-culture, respectively. We interpret these images to be multi-layers of MC3T3E-1 incorporated in a thick collagenous matrix (black, compare to Figure 2) into which columns of cancer cells penetrate in a few locations during the first day of co-culture (Panel B). Within 2 days of co-culture, breast cancer cells replicate (Panel B) and begin to organize into linear files especially evident in Panel C of Figure 4. Close inspection of both 2D optical sections and 3D reconstructions (Figure 5) suggests concomitant remodeling of the osteoid tissue. Before cancer-cell challenge, osteoblasts exhibit a rounded, cuboidal morphology. Over 3 days of cancer cell co-culture, osteoblasts take on a definitively elongated appearance and align with cancer cells. Similar morphological changes have been observed in osteoblasts exposed to breast-cancer-cell-conditioned medium in conventional culture. It was also observed that tissue from co-culture experiments was much more fragile than originating tissue not exposed to cancer cells, requiring very careful processing to prevent wholesale cell sloughing during the wash steps involved in preparation of specimens for histology and electron microscopy. It was plainly evident that the tissue matrix was eroded and easily separated from the base film of the bioreactors used in this work. We speculate that osteoblast tissue destruction occurs
through increased apoptosis and suppression of osteoblast differentiation that has been observed in conventional 2D tissue culture\textsuperscript{24}, as well as wholesale degradation of the thick extracellular matrix (ECM) in which osteoblasts are embedded (Figure 1) through the action of matrix metalloproteinases and cathepsin K synthesized by cancer cells.\textsuperscript{25-27} Further studies using genomic and proteomic tools can delineate the specific mechanisms responsible for degradation of osteoblast tissue by breast cancer cells observed in the bioreactor.

Cancer-related bone loss appears to occur through multiple pathways, including the osteoclast-mediated resorption.\textsuperscript{10,11,28} In particular, destruction of devitalized bone directly by cancer cells has been reported\textsuperscript{10,11,28} and it has been found that, late in metastasis when bone-degradation rate is highest, osteoclast cell numbers are actually in precipitous decline.\textsuperscript{29} These lines of evidence support the idea that osteoclasts are not solely responsible for excessive bone degradation and that cancer cells directly contribute to bone loss.

Degradation of the osteoblast-derived osteoid tissue by co-culture with breast cancer cells observed in the model system presented here (that purposely excludes osteoclasts) strongly suggests that yet another mechanism of bone loss is related to disruption of the bone-accretion process by destruction of osteoblastic tissue. There is clinical and experimental literature to support this concept. For example, quantitative histomorphometric analyses of bone biopsies from patients with hypercalcemia due to bone metastasis indicated a dramatic decrease in osteoblast activity.\textsuperscript{30} Histomorphometric analysis of rodents inoculated with lytic human breast cancer cells (MDA-MB-231) indicated that, even though administration of risedronate (a bisphosphonate) reduced the number of osteoclasts, slowed bone lysis, and significantly reduced tumor burden, there was no evidence of new bone deposition or repair. Similarly, administration of bisphosphonates to humans with osteolytic metastasis slowed lesion progression but did not bring about healing.\textsuperscript{31} All taken together, these observations strongly suggest that normal osteoblast function (\textit{i.e.} deposition of matrix) is not only
impaired in the presence of breast cancer cells but, in fact, osteoblastic tissue is destroyed by an orchestrated attack by cancer cells, possibly by enlisting a cooperative response by osteoblasts themselves.

Conclusions and Future Work:
We have shown that metastatic breast cancer challenge of 3D osteoblast tissue grown in a specialized bioreactor over 5 months of continuous culture permitted direct observation of interaction of breast cancer cells with the osteoblast tissue. Pathological outcomes such as tissue penetration and cancer cell filing which are not well modeled in conventional culture were clearly observed in the bioreactor. The bioreactor allowed us to control and select stages in osteoblast development for challenge with cancer cells. By varying the breast cancer cells/ osteoblast ratio the model allows us to probe the conditions under which cancer cells remain as solitary cells or associate to form tumors simulating early stages of cancer colonization of bone. A decided advantage of the bioreactor cultures is that medium in the cell culture chamber is not disturbed throughout the course of the experiment and various cell-secreted factors are allowed to accumulate within the cell chamber. Supernatant from the culture medium can be collected at periodic co-culture intervals to assay for specific factors that may be secreted by cancer cells over the course of their interaction with 3D tissue. Isolating the RNA from the osteoblast cultures at different co-culture intervals can elucidate the mechanisms that lead to changes in osteoblast physiology in the presence of breast cancer. The bioreactor model also permits study of the effect of specific drugs that may inhibit cancer cell penetration, tumor formation and osteoblast tissue destruction under controlled conditions over extended culture intervals. Further understanding of the cellular and molecular basis for breast-cancer interactions with osteoblasts and discovery of
therapeutic interventions will be greatly expedited by the use of three-dimensional bone
tissue models such as the one reported herein.
References


List of Figure Legends

**Figure 1:** Schematic showing the sequential steps of cancer cell attachment, penetration, replication and possible host tissue destruction observed in the bioreactor as a result of interactions between green fluorescent protein (GFP) labeled breast cancer cells and multiple layer 3D osteoblast tissue.

**Figure 2:** (A) Light micrographs of a Hemotoxylin and Eosin stained 70-day tissue (40X) reveal osteoblasts (pale red with dark-red nuclei) lining a collagenous matrix (pale pink). (B) Transmission electron micrographs (TEM) of 22-day tissue cross sections show up to 5 cell layers (1500X). (C) Scanning electron micrographs of the surface of a 70 day culture are studded with mineral nodules (1000X) that prove positive for calcium and phosphorous by energy-dispersive analysis of x-rays (not shown). (D) Bone chip recovered from a 5 month MC3T3-E1 bioreactor. X-ray diffraction (inset) indicates similarity with a bovine bone reference spectrum (lines).

**Figure 3:** Transmission electron micrograph (TEM) of a cross-section of an isolated MDA-MB-231 breast cancer cell crawling on the base film of the bioreactor used in this work (cultured for 3 days in osteoblast-conditioned medium) reveals an amoeboid morphology with protrusive structures at the leading edge, consistent with a migratory phenotype (Panel A, 1500X). TEM of breast cancer cells (BC in Panel B, 5000X) co-cultured for 10-days with a 60-day MC3T3-E1 tissue shows that cancer cells displaced osteoblasts originally adhering to the base film (see Figure 2) and exhibit protrusive structures that may be involved in penetration. (arrows; compare to Panel A). Fluorescence microscopy oft breast cancer cells
(green) in co-culture with osteoid tissue (red) organize in single-file order (Panel C, 40X), captured in cross-section by TEM (inset, 2000X).

**Figure 4**: Serial optical sections through an MC3T3-E1 derived osteoid tissue (5-month culture interval stained with Cell Tracker Orange™) co-cultured with GFP-labeled MDA-MB-231 breast cancer cells (green) for 3 days. Optical sections are from bottom [0μm] to top [16μm]) of the tissue. (40X, scale bar = 50 μm)

**Figure 5**: 3D reconstruction of serial optical sections taken through the thickness of the 5 month osteoblast tissue cultured in the bioreactor and subject to MDA-MB-231 breast cancer challenge over 3 day co-culture interval.
Figure 1
Figure 2
Figure 3
Figure 4
VITA

Dhurjati Ravi

Dhurjati Ravi was born in Kakinada in India on December 08, 1976. Dhurjati graduated from Kerala University with a degree in Mechanical Engineering. Dhurjati came to the United States to pursue higher education and received his Masters Degree in Materials from Penn State University in 2003. Dhurjati has been pursuing doctoral degree in Materials Science and Engineering since Jan 2004. Dhurjati’s research interests are in the broad area of tissue engineering and with specific interest in simulating complex physiological and pathological processes using engineered tissue.

Research Accomplishments

Osteogenesis and Osteocytic Transformation In Vitro

Continuous culture of bone forming cells for extended periods up to a year resulted in a specialized bioreactor resulted in growth/maturation of 3D multilayered tissue, osteocytic transformation and macroscopic bone formation.

System in Crisis: Breast Cancer Colonization of Bone In Vitro

In vitro bone tissue surrogate challenged with metastatic breast cancer cells, effectively captured fundamental aspects of breast cancer colonization of bone.

Publications

Ravi D, Liu X, Mastro AM, Gay CV and Vogler EA
Extended-Term Culture of Bone Cells in a Compartmentalized Bioreactor Tissue Engineering, [12], 3045-3054, Nov 2006.

Liu X, Lim JY, Donahue JH, Ravi D, Mastro AM and Vogler EA