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FACTORS AFFECTING BONE CELL GROWTH AND DIFFERENTIATION UNDER DIFFERING CULTURE CONDITIONS

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE MASTER OF ARTS

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ABSTRACT

Marrow stromal cell (MSC) differentiation into osteoblasts is an important part of the bone growth and remodeling process. This process can be exploited to help solve the problem of bone wound healing. Because of problems with bone grafts, implantation of biodegradable 3D scaffolds seeded with MSCs has been suggested. However, differences in osteoblast differentiation in 2D versus 3D cultures remain unclear. In this study, rat marrow stromal cells (MSCs) were grown both on plastic and in 3D polymer scaffolds and their differentiation into osteoblasts studied. MSCs cultured in a synthetic 3D matrix differentiate faster into osteoblasts than those grown on plastic; the osteoblast differentiation markers alkaline phosphatase and osteocalcin peak in mRNA expression first in 3D in vitro cultures.

The culture conditions of MSCs grown in 3D scaffolds were studied to determine the optimal conditions for osteoblastic differentiation. Factors such as cell density, scaffold seeding method, scaffold thickness and secreted soluble factors were investigated. Soluble factors secreted by the differentiated cells into the culture medium were found to be critical for timely differentiation. Lack of such factors promoted cellular proliferation over differentiation. Constant perfusion of cell culture medium through the scaffolds enhanced osteoblastic differentiation.

Mature osteoblasts have been shown to undergo chemotaxis, and it is possible that their progenitor cells (MSCs) could as well. Very little is known about MSC chemotaxis. It is possible that they, like osteoblasts, can be recruited to a site where bone formation is needed. Under agarose chemotaxis assays were performed to investigate MSC
chemotaxis toward osteoblast matrix factors or other cell types. MSCs did not appear to move under any of the conditions studied.
ACKNOWLEDGMENTS

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<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three Dimensional</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>ECL</td>
<td>Western Blot Detection Reagent (Brand Name)</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ERK2</td>
<td>Extracellular Signal-Regulated Kinase 2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-Like Growth Factor</td>
</tr>
<tr>
<td>JNK1</td>
<td>Janus N-Terminal Kinase 1</td>
</tr>
<tr>
<td>MSC</td>
<td>Marrow Stromal Cell</td>
</tr>
<tr>
<td>μεE</td>
<td>Microstrain = 0.0001% Deformation</td>
</tr>
<tr>
<td>NBC</td>
<td>0.5 M Boric Acid, 10 mM Sodium Citrate, 50 mM NaOH, pH 7.5</td>
</tr>
<tr>
<td>OSF2</td>
<td>Osteoblast Specific Factor 2</td>
</tr>
<tr>
<td>p38</td>
<td>p38 MAP Kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PET</td>
<td>Polyvinylpyrrolidone-EGTA-Trypsin</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PPF</td>
<td>Poly(propylene fumarate)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffered Saline-Tween 20</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
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CHAPTER 1
INTRODUCTION

Bone is a complex tissue that is constantly undergoing remodeling to make minor repairs and to maintain homeostasis (Ham and Cormack, 1979). It has long been known that mechanical loading can effect bone remodeling, but the molecular mechanisms behind this remain unclear. Discovering why astronauts during spaceflight and bed-ridden patients lose bone mass and mineral content due to decreased mechanical loading is one of the reasons to investigate these mechanisms. It has been shown that increased mechanical loading up-regulates expression of platelet derived growth factor (PDGF), a mitogenic and chemotactic factor found in the bone extracellular matrix (ECM), in in vitro studies (Wang et al., 1997). Recently, there has been evidence that suggests that bone marrow stromal cells (bone precursor cells) respond to mechanical stress, and this response may regulate their differentiation into osteoblasts, the bone forming cells. Stromal cells from rats whose hind limbs were elevated (unloaded) for 5 days prior to sacrifice were less competent to form osteoblasts, and the osteoblasts derived from these stromal cells underwent impaired proliferation and differentiation (Kostenuik et al., 1997). This suggests that bone marrow stromal cells have the ability to remember the mechanical loading or unloading they experience.

Because of the numerous growth factors and cytokines found in the bone ECM, one theory postulates that increased loading causes up-regulation and release of these factors that will then recruit bone forming cells to a location where bone synthesis is required. It is already known that osteoblasts undergo chemotaxis in response to several
of the factors present in bone matrix, and that stromal cells can remember prior loading and can differentiate into bone forming osteoblasts suggest that stromal cells may undergo chemotaxis as well (Pfeilschifter et al., 1990; Panagakos, 1993; Panagakos, 1994; Godwin and Soltoff, 1997). Thus, increased mechanical loading may cause the release of chemotactic and differentiation factors from the bone matrix that in turn would recruit bone precursor cells to a desired location and cause their increased proliferation and differentiation.

Another problem involving bone growth and, indirectly, mechanical loading is the repair of bone defects. While bone can repair itself, some injuries are so traumatic that even after recovery, the bone is never as strong as it was before. To aid in strengthening and speeding up bone repair, use of three-dimensional biodegradable polymer scaffolds have been proposed (Ishaug et al., 1994; Ishaug et al., 1997). Simply injecting osteoblasts or bone precursor cells at an injury site has not proven to produce satisfactory healing, as bone cells require a 3D matrix on which to attach and proliferate (Bellows et al., 1986). Currently, bone grafts solve the 3D matrix problem, but they are not easily molded to fit the shape of the wound. The possibility of pathogen transfer and the limited availability of graft material also make the use of a synthetic scaffold an attractive proposal. Several biodegradable polymers are being investigated for the proper mechanical properties and the ability to support bone growth so that they might replace bone grafts. These polymers are designed to be very porous to allow cell invasion. Currently, 3D scaffolds mimic the mechanical environment of bone better than any other culture system. By testing what culture and mechanical loading conditions promote the
best bone growth *in vitro*, a possible replacement for bone grafts could be developed while perhaps learning more about bone cell differentiation.
CHAPTER 2

BACKGROUND

A. Bone Cell Types and Functions

Bone is a highly vascularized tissue organized into a skeleton that provides support for the musculature and organs of the body. There are three key cell types that make up mature bone tissue: osteoblasts, osteocytes, and osteoclasts (Ham and Cormack, 1979). Osteoblasts are primarily secretory cells that lie along the surface of growing bone. They are responsible for secreting the extracellular matrix of bone and regulating its calcification. While secreting matrix, osteoblasts move as a front away from the surface of the growing bone. As matrix synthesis occurs, some osteoblasts are left behind within the newly secreted matrix and these cells further differentiate into osteocytes. Osteoblasts at the surface of growing bone extend processes that are in contact with osteocytes embedded within the matrix. Osteoblasts have a large, rounded or column-like morphology and their organelles are arranged in the fashion of a typical secretory cell. An osteoblast’s nucleus is located on the side of the cell opposite of growth front/bone surface. They have a large amount of rough endoplasmic reticulum and their Golgi is located on the side of the nucleus nearer to the growth front. Osteoblasts are capable of secreting material from anywhere along their surface.

Osteocytes are the terminus of the osteoblastic cell lineage and are non-proliferative. A major portion of the bone volume (over 90%) contains osteocytes as
the primary cell type, and they are embedded within the collagen extracellular matrix. Osteocytes are connected to each other by extending cytoplasmic processes through narrow passages in the matrix called canaliculi and forming gap junctions. It is through these canaliculi that osteocytes receive nutrients while embedded in a calcified matrix. The primary function of osteocytes is to maintain both the integrity of the secreted matrix that surrounds them and mineral homeostasis.

Osteoclasts are the last class of mature bone cells. These cells are large, multinucleated, and hematopoietic in origin. Their function is to remodel bone by breaking down the extracellular matrix via a process called resorption. Osteoclasts typically have 5-10 nuclei, but occasionally have as few as two or as many as 100. Resorption occurs when an osteoclast forms a ruffled border attachment to the extracellular matrix and secretes lytic enzymes and acid to degrade the matrix and solubilize calcium salts, respectively. They may also engulf debris or cells in the manner of macrophages.

B. Bone Marrow Stromal Cells and Osteoblastic Differentiation

Within the shaft of a mature bone lies the bone marrow. Bone marrow is a heterogeneous mixture of marrow stromal cells (MSCs), endothelial cells, adipose tissue, and hematopoietic precursor cells. Bone marrow is the site of blood cell and osteoblast generation (See Ham and Cormack, 1979 for full review). Marrow stromal cells are pluripotent mesenchymal cells that may adopt several differing fates depending on their environments. MSCs are able to differentiate in vitro into
osteoblasts, myoblasts, chondrocytes, and adipocytes (Aubin et al., 1993). Figure 2-1 shows possible mesenchymal lineages. Of particular interest is MSCs’ osteoblastic lineage. In response to an unknown signal, MSCs will differentiate into preosteoblasts. These preosteoblasts are located behind the moving front of osteoblasts secreting matrix. Matrix factors then cause preosteoblasts to differentiate into osteoblasts (Aubin et al., 1993). Osteoblasts in turn will then differentiate into osteocytes when embedded within the extracellular matrix. While the signal that initiates osteoblastic differentiation \textit{in vivo} is unknown, \textit{in vitro} studies have shown that the presence of the synthetic glucocorticoid dexamethasone, inorganic phosphate, and ascorbate will promote the differentiation of MSCs into osteoblasts (Bellows et al., 1990). The timing of this differentiation process and the expression of several osteoblastic differentiation markers have been extensively studied \textit{in vitro}. MSC to osteoblast differentiation occurs in three distinct phases: proliferation, matrix maturation, and mineralization (Owen et al., 1990). The timing of these phases is shown in Figure 2-2. In the proliferation phase, it has been shown that dexamethasone (Dex) up-regulates bone morphogenetic protein 6 (BMP6) expression in rat MSCs and currently, BMP6 is the earliest known bone differentiation marker (Boden et al., 1997). Up-regulation of BMP6 in turn causes the expression of osteoblast specific factor 2 (OSF2), a transcription factor both necessary and sufficient for bone formation (Ducy et al., 1997). At this point, the stromal cells have differentiated into preosteoblasts and are starting to synthesize collagen and produce matrix, but are still dividing. As matrix secretion continues, the proliferative capacity of the preosteoblasts decreases, as they are becoming non-dividing osteoblasts.
Collagen synthesis peaks at about day 7 of *in vitro* 2 dimensional cultures (Owen et al., 1990). After the osteoblasts stop dividing and enough matrix has been synthesized, osteoblasts enter a matrix maturation phase. This phase is characterized by a sharp increase in alkaline phosphatase (AP) expression. AP expression peaks at about 14 days after Dex treatment and then falls. Following the peak in AP expression, osteoblasts enter the mineralization phase. At this point, the collagen matrix is calcified and another osteoblast cell marker, osteocalcin, is up-regulated, peaking at around day 28 after Dex treatment (Owen et al., 1990).
Figure 2-1. A schematic showing the possible in vitro cell fates of a marrow stromal cell. As differentiation progresses toward the right, cell proliferation slows down. (Taken from Aubin et al., 1993).
Figure 2-2. A schematic showing the timing of the three stages of osteoblast differentiation as well as when key differentiation markers are expressed. As differentiation progresses, proliferation of the cells slows. Among the markers, collagen (Col I) is expressed during the proliferative phase, alkaline phosphatase (AP) during the matrix maturation stage, and osteocalcin (OC) in the mineralization phase. (Taken from Owen et al, 1990).
C. The Extracellular Matrix of Bone

The ECM of bone is similar to the matrix of cartilage with some important differences. Cartilage’s ECM is much more amorphous in character and lacks an inorganic component and vascularization (Ham and Cormack, 1979). Nevertheless, the ECM of bone is primarily organic with nearly 90% of that organic material consisting of type I collagen. Other types of collagen synthesized for anchoring purposes are present in much smaller amounts. The ECM also contains many growth factors and bone proteins. Many of the growth factors are members of the TGF-β superfamily including the BMPs (Wozney, 1989) and TGF-β (Pfeilschifter et al., 1990) itself. Other factors such as the mitogenic platelet derived growth factors (PDGF) (Andrew et al., 1995; Godwin and Soltoff, 1997), and insulin-like growth factors (IGFs) (Gangji et al., 1998; Panagakos, 1993) are present, and nearly all of these factors have been implicated in osteoblast chemotaxis. Hydroxyapatite, a calcium salt, is the primary inorganic component of the bone ECM, and holds nearly all of the body’s calcium stores.

D. Bone Growth and Remodeling

There are two types of bone growth, intramembranous ossification and endochondral ossification (Buckwalter et al., 1995). The only difference in these types of osteogenesis is the location where it occurs (Ham and Cormack, 1979). The resulting newly formed bone is the same for either process. Intramembranous bone is
formed from mesenchyme where there are no pre-existing cartilage structures. Endochondral ossification occurs in a cartilaginous environment as bone gradually displaces cartilage that is being degraded and vascularized.

Bone growth and bone remodeling are coupled processes. Bone is constantly being synthesized and resorbed in order to make minor repairs or to maintain the body’s ion homeostasis. The first step in remodeling is the resorption of bone by osteoclasts. They acidify, degrade and digest the ECM. Growth factors and mitogens trapped within the ECM are activated and released allowing for additional vascularization and osteoblasts to secrete new matrix and synthesize new bone. Lastly the new matrix is mineralized to produce mature bone. Bone remodeling is also very important in how bone adapts to the mechanical stress it experiences (Ham and Cormack, 1979).

E. How Mechanical Strain Affects Bone

Wolff in 1892 discovered that bone was a tissue that underwent remodeling to adapt to experiencing mechanical loading. Since then, scientists have attempted to explain the mechanism behind this remodeling. Both osteoblasts and osteocytes have been shown to respond to mechanical loading in vitro, but it is osteoblasts’ role in the coupled remodeling process that suggests they are the primary mechanosensors of bone (Klein-Nulend et al., 1994). On a macroscopic level, it is the deformation of bone tissue that initiates the bone remodeling response. Deformation of tissue has been described by the unit microstrain (µE) which is defined as a 0.0001%
deformation of tissue (Turner, 1991). Normal physiological deformation levels range from 200-1500 μE resulting in remodeling that produces no net change in bone mass. Bone experiencing increased mechanical loading (1500-2500 μE) will secrete more matrix and gain bone mass while bone experiencing little or no loading (0-200 μE) will lose mass (Turner, 1991). On a more molecular level, in vitro studies have shown that expression of the bone differentiation markers can be altered drastically with the application or removal of mechanical loading. Several studies have shown that intermittent compressive strain will up-regulate osteoblasts' expression of collagen and alkaline phosphatase resulting in increased matrix production (Harter et al., 1995; Roelofsen et al., 1995). Fluid shear stress has also shown to up-regulate bone formation by osteoblasts (Reich et al., 1990). Unloading of bone progenitor cells causes a decrease in their capacity to become osteoblasts and secrete matrix. Unloaded MSCs that differentiate into osteoblasts have lower expression levels of collagen, alkaline phosphatase, and osteocalcin (Kostenuik et al., 1997).

F. Chemotaxis of Osteoblasts and Marrow Stromal Cells.

Chemotaxis of bone cells and bone remodeling are closely related. It is necessary for osteoblasts to move as they secrete matrix and form bone. Several growth factors present in the ECM, [TGF-β (Pfeilschifter et al., 1990), BMPs (Lind, 1998), PDGF-A (Wang et al., 1997), PDGF-B (Godwin and Soltoff, 1997), IGFs (Panagakos, 1993)] have been shown to cause osteoblasts to chemotax in vitro. The mechanism behind osteoblast chemotaxis is simple, bone matrix is acidified and degraded during
remodeling which releases growth factors (and in the case of TGF-β activates the factor) which can recruit osteoblasts to a location where bone synthesis is needed. Several things about this process still remain unclear. Little is known about what concentrations and combinations of growth factors osteoblasts respond to in vivo. It is also unknown whether marrow stromal cells will respond to any of these growth factors in vivo to be recruited to differentiate into osteoblasts. To date, only BMP2 has been shown to have a chemotactic effect on MSCs, but whether this has any significance in vivo is yet to be determined (Lind, 1998).

G. The Basis Behind Polymer Foam Scaffolds

It has been shown previously that bone requires a three dimensional scaffold to mimic the extracellular matrix it secretes in order to be implanted with any chance of increased healing/proliferation (Bellows et al., 1986). Donor site morbidity and possible pathogen transfer make bone grafts unattractive 3D environments for implanted tissue. Use of 3D biodegradable polymers seeded with MSCs has been suggested to overcome the inherent problems of bone grafts. Poly(lactic-co-glycolic acid) (PLGA) is a semi-elastic, biodegradable co-polymer that has FDA approval for implantation in the human body (Ishaug et al., 1994). The fact that PLGA can be molded into any shape makes it desirable for use in implantation at wound sites as an alternative to bone grafts. A PLGA scaffold may be molded into the shape of the wound, seeded with osteo-progenitor cells obtained from the patient, and then implanted into the patient. The scaffold will slowly hydrolytically biodegrade into
lactic acid and glycolic acid as the MSCs in the scaffold proliferate, differentiate and begin to synthesize new bone. However, PLGA is not without flaws. It is unsuitable for large wounds because of nutrient diffusion limitations with the material itself. Cells near the surface of the scaffold are healthier and more abundant than those at the center. Thus the smaller the wound, the better the healing because in a large wound scenario, the scaffold's center would be sparsely populated with cells and promote only superficial healing at the surface of the scaffold. The other problem with PLGA is that it is not entirely an elastic material, which means that it will experience some plastic deformation when exposed to mechanical stress. Again the size or the location of the wound will play a large role in how much scaffold deformation will take place (Ishaug et al., 1997).
CHAPTER 3

OBJECTIVE

The project outlined in this thesis was developed to investigate three different problems. The first was to elucidate whether marrow stromal cells would undergo chemotaxis toward growth factors that are present in the extracellular matrix of bone. These growth factors are released during bone remodeling and some have been shown to be up-regulated after mechanical loading. The hypothesis driving this project is that these growth factors may recruit MSCs in a similar manner as they do osteoblasts to where bone growth is needed. Once the stromal cells come into contact with the ECM or osteoblasts themselves, they would then proceed to differentiate and synthesize bone. To study this, an under agarose assay was employed to examine possible stromal cell chemotaxis in response to various chemoattractants and cell types. The second project was to see whether the speed of osteoblast differentiation would be different in 3D PLGA scaffolds. Because these scaffolds mimic the 3D environment of bone, it was surmised that MSCs might be able to differentiate faster in 3D cultures than on plastic. The third project was to determine the optimum static cell culture conditions when growing and differentiating MSCs within 3D PLGA scaffolds. It has been shown previously that PLGA will support osteoblastic differentiation, but it is desirable to learn what culture conditions will produce the best cell growth and differentiation. Factors such as cell culture medium, fetal bovine serum concentration, cell seeding density, PLGA porosity, and presence or absence of fluid shear stress have been examined for their effects on stromal cell to osteoblast differentiation. To assess the extent and quality of cell
differentiation. Northern analysis for bone cell markers and alkaline phosphatase and DNA assays were performed.
CHAPTER 4

MATERIALS AND METHODS

PLGA Scaffolds

75:25 PLGA was obtained from Birmingham Polymers (Birmingham, AL). NaCl was run through a mechanical sieve to collect granules 150-300 μm in diameter. The bottom half of several 60mm glass Petri dishes (Fisher) was covered with Teflon/Aluminum overlay (Cole Parmer) to facilitate later polymer removal. 4.5g of NaCl was added to the lined Petri dish bottoms. 0.5g of raw PLGA per Petri bottom was added to glass scintillation vials (Fisher), 0.5g per vial. To each vial containing PLGA, 5ml of dichloromethane was added, and the PLGA was allowed to dissolve for 1 hour. After the PLGA had dissolved completely and the solution was no longer viscous, the PLGA solution from each vial was poured into the Petri dish bottoms containing the NaCl. The Petri dishes were shaken until the salt was uniformly distributed over the Petri dish bottoms. The Petri dishes were covered and placed on a level surface in a fume hood overnight to allow the dichloromethane to evaporate. Then the salt/polymer mixtures were vacuum lyophilized for 24 hours to drive off the last of the solvent. The salt/polymer discs were removed from the Petri bottoms after lyophilization. A 12mm diameter, cylindrical Teflon mold, surrounded by a metal sheath was filled with salt/polymer composite. The mold was heated for 30 minutes at 160°C while maintaining 200-psi pressure on the material using a press (Model#3912, Carver, Wabash, IN). The mold was cooled for 30 minutes, and then the polymer/salt cylinder
was removed. Using a diamond saw (Isomet 11-1180, Buehler, Evanston, IL), the cylinders were sliced into 2 or 4 mm thick scaffolds. If the scaffolds were to be seeded by injection, a hole was drilled through the center of each scaffold. The scaffolds were then placed in MilliQ water for 24 hours to leach out all the salt. The water was changed every 8 hours. The scaffolds were then lyophilized again to dry them completely. Lastly, the scaffolds were sterilized by exposure to ethylene oxide gas and stored in a dessicator for later use.

**Primary Medium**

Primary cell culture medium was prepared by supplementing 450 ml of Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) with 50 ml of fetal bovine serum (FBS) (Atlanta Biologicals) to give a final serum concentration of 10%. 1.25 ml of a 10mg/ml solution of gentamycin sulfate (Gibco) was added to prevent bacterial contamination.

**Complete Medium**

Complete cell culture medium was prepared by dissolving 25 mg of L-ascorbic acid (Sigma) and 1.080g of β-glycerol phosphate (Sigma) in 50 ml of DMEM (Gibco) and then filter sterilized using a .22μm cellulose acetate filter into 400 ml of DMEM. To this mixture, 50 ml of FBS was added as well as 0.4ml of a 10 mg/ml gentamycin sulfate
solution. Lastly, 98.1μl of a 20 μg/ml stock of dexamethasone was added. Without Dex. MSCs will not undergo osteoblastic differentiation.

Cell Culture

Rat marrow stromal cells were harvested from femurs of 6-week-old male Sprague-Dawley rats (Harlan Sprague-Dawley). Rats were euthanized with ether and then their femurs were removed aseptically in accordance with the animal protocol approved by Rice University. Using a pair of sharp scissors, the metaphyseal ends of the femurs were cut off and the marrow flushed from its cavity using a 20-gauge needle attached to a syringe containing 5 ml of primary medium. The marrow was flushed into sterile Petri dishes and the marrow medium mixtures were pipetted gently to break up cell clumps. The cells were centrifuged at 500 x g for 10 minutes and the supernatant (containing adipocytes) was discarded. The cells were resuspended in fresh primary medium and plated into T-75 tissue culture flasks. After two days, the cells were washed with phosphate buffered saline (PBS) to remove any non-adherent cells. The cells were then provided with fresh primary medium every three days until used for experiments.

Cell Passaging and Counting

Polyvinylpyrrolidone-EGTA-Trypsin (PET) solution (0.0625% trypsin (Sigma), 0.0125% EGTA, 0.625% polyvinylpyrrolidone, 26mM HEPES, and 1.125% NaCl) was used to remove adherent cells from the bottom of the T-75 flasks for experimental use.
The cells were first washed with PBS to remove all traces of medium. 1.5 ml of PET was then added to each T-75 flask and then incubated at 37°C for 10 minutes. Then 5 ml of primary medium was added to each flask to inactivate the trypsin. A small sample of the cell suspension was taken (100 µl) to obtain an accurate cell count using a hemacytometer. Cells were centrifuged at 500 x g for 10 minutes and then resuspended in the desired medium at the desired concentration. Cells were passaged at or near confluency.

2D Cultures

Passaged stromal cells were plated at half confluency (26,500 cells/cm²) in 6 well tissue culture plates and given 2 ml of complete medium per well. The cells were washed with PBS and given fresh medium every three days. Every two days for 20 days, cells from a plate were scraped off the surface using a sterile cell scraper and pelleted. Cells were resuspended in 1 ml of denaturation solution (4.0 M guanidinium thiocyanate, Ambion) and frozen at -80°C until RNA extraction.

3D Cultures

Sterilized scaffolds were pre-wetted with complete medium and were seeded with varying concentrations of passaged stromal cells by either injection or by layering a concentrated drop of cells on the scaffold surface. Cells were given 3 hours to attach and then were provided with 4 ml of complete medium. Scaffolds were washed with PBS
and given fresh medium daily (for those scaffolds used in the alkaline phosphatase assays). Every 2 days for 20 days, a scaffold was washed with PBS, then placed in 1 ml of denaturation solution and homogenized with a mechanical grinding device if being used for RNA extraction. Homogenized scaffolds were stored at -80°C until RNA extraction.

**RNA Extraction**

RNA was extracted from 2D or 3D lysates using the Totally RNA isolation kit (Ambion). The RNA was extracted using two successive phenol-chloroform extractions at differing pHs. Chloroform completely dissolves the PLGA of the scaffolds. The second phenol-chloroform extraction was performed under acidic conditions to keep DNA in the organic layer. RNA from the second extraction was precipitated in isopropanol for 30 minutes at -20°C and then pelleted. RNA was resuspended in 50 μl of RNase free water.

**Northern Analysis**

RNA samples of 10-15 μl were combined with 2 μl of 10X NBC (0.5 M boric acid, 10 mM sodium citrate, 50mM NaOH, pH 7.5), 3 μl of 37% formaldehyde and 10 μl of formamide, and incubated for 5 minutes at 65°C. After cooling, 2 μl of ethidium bromide (10 mg/ml) and 1 μl of gel loading buffer (0.025g bromophenol blue, 0.025g xylene cyanol FF, 30% glycerol in 10 ml of H₂O) were added to each sample. Samples
were separated on a 1% formaldehyde-agarose gel and blotted to a nitrocellulose membrane with a Stratagene posiblotter and immobilized with a Stratagene UV autocrosslinker. The membrane was then prehybridized for at least one hour in Church buffer (0.25 M Na₂HPO₄, pH 7.5, with 7% sodium dodecyl sulfate (SDS)) at 65°C and then hybridized overnight at 65°C in the presence of the appropriate probe. Membranes were then washed twice with Church buffer, twice with 0.1 X saline sodium citrate (SSC) (0.8765g NaCl, 0.441g sodium citrate in one liter pH 7.0), 0.1% SDS at 65°C, and placed against a phosphorimaging plate overnight. Developed images were scanned the next day.

**Northern Probe Generation**

Both DNA and RNA probes were used to probe Northern. DNA probes were generated using the DECAprime II Kit (Ambion). Plasmid DNA containing the probe of interest was added to a 10X decamer solution and denatured at 95°C for 5 minutes. The mixture was then snap-frozen in a dry ice/ethanol bath. After thawing, α³²P-dATP and cold dTTP, dCTP, and dGTP in 5X buffer were added along with Klenow fragment. The reaction was incubated for 10 minutes at 37°C. Then 1 µl of 0.5 M EDTA was added to stop the labeling reaction. The probe was again denatured at 95°C for 5 minutes and immediately added to the hybridization solution. RNA probes were generated using the MAXIscript *in vitro* transcription kit (Ambion). Plasmid template DNA containing the probe of interest was linearized by restriction digest. To the template, transcription buffer, cold ribonucleotides, α³²P-ATP (800 Ci/mmol {10 mCi/ml in aqueous
solution}, and T7 RNA Polymerase were added and incubated at 37°C for 1 hour. Then 1 µl of DNase was added to the reaction and incubated for another 15 minutes at 37°C to degrade the template. Unincorporated radiolabel was removed via 2 successive ammonium acetate/ethanol precipitations. Upon removal of the unincorporated nucleotides, the probe was immediately added to the hybridization buffer. The osteocalcin probe was generated from a portion of rat osteocalcin cDNA cloned into the Hind III site of pGEM7Zf(+) (Lian et al., 1989). The AP probe was generated from a portion of rat AP cDNA cloned into the EcoRI-BamHI site of pGEM7Zf(+) (Owen et al., 1990). The GAPDH probe was generated from a portion of GAPDH cDNA cloned into the SacI-BamHI site of pTriplScript (Goldstein et al., 1999).

**Alkaline Phosphatase (AP) Assay**

For analysis of 2D cultures, cells from 1 six well plate were used. To analyze 3D cultures, a scaffold was cut in half; one used in the AP assay and the other half used in the DNA assay. 1 ml of Tris buffer pH 8.0 was added to either the cell pellet or scaffold half. The scaffold half was homogenized with a mechanical grinding device on ice. From this point, the assay proceeded the same for both 2D and 3D samples. Samples were sonicated in a water bath for 10 minutes on ice. The AP substrate reagent (16mM p-nitrophenyl phosphate, Sigma) was prepared by adding 10 ml of water to a vial of substrate and heating it to 30°C. 20 µl of sample was added to 1 ml of substrate in a quartz cuvette and 4 absorbance measurements at 404 nm were taken at 30 sec, 1.5 min, 2.5 min, and 3.5 min using a spectrophotometer. Total AP activity was calculated using
the slope of the spectrophotometer readings plotted versus time in minutes. The units for AP activity represent the conversion of a given amount of substrate to inorganic phosphate per minute per DNA amount or foam depending on the type of culture used.

DNA Assay

For analysis of 2D cultures, cells from 1 six well plate were used. To analyze 3D cultures, a scaffold was cut in half; one used in the AP assay and the other half used in the DNA assay. 1.4 ml of 10 mM EDTA pH 12.3 was added to either the cell pellet or scaffold half. The scaffold half was homogenized with a mechanical grinding device on ice. From this point, the assay proceeded the same for both 2D and 3D samples. Samples were sonicated in a water bath for 10 minutes on ice. The samples were then heated in a 37°C water bath for 20 minutes. Then 200 µl of KH₂PO₄ was added to each sample on ice. A fluorescent Hoescht dye #33258 (Polysciences, Warrington, PA) solution was prepared by adding 0.23g of NaCl and 0.063g of Trizma to 40 ml of water and 40 µl of stock dye solution giving a final dye concentration of 200 ng/ml. The fluorimeter (Amino-Bowman Series 2 Luminescence Spectrophotometer, Urbana, IL) was set to read at an excitation wavelength of 350 nm and an emission wavelength of 455 nm. 1.4 ml of dye solution was added to 1.0 ml of sample right before reading. Readings from no cell or no scaffold samples were used as blanks. DNA standards were used to generate a standard curve that allowed for the calculation of the amount of DNA present in each scaffold or cell pellet. This number was then used to normalize the total AP activity results from the AP assay to DNA content.
Under Agarose Assay

0.5 ml of 1X agarose / 1X DMEM was poured into each well of 4-well Nunc Permanox chamber slides. The chamber slides are essentially microscope slides with a removable set of 4 chambers mounted to the slide. After the agarose had hardened overnight, 2 wells were cut in the agarose in each chamber using a cell cutter template (ICN Radiochemicals) that are 5 mm in diameter and 1.5 mm apart from each other. One of the agarose plugs from each chamber was removed immediately by gentle aspiration, and stromal cells are plated (30,000 cells in 30 μl of primary medium with only 2% FBS). It is vital that this medium contain only 2% FBS because serum contains PDGF (one of the chemoattractants being tested). If normal 10% serum were used, MSCs might not be able to sense a chemotactic gradient due to the large amount of PDGF already present with the cells. Use of less than 2% FBS places the cells in growth arrest. Cells were allowed to attach overnight at 37°C. The chamber slides were placed in the incubator inside Petri dishes with wet Whatman paper on the bottom to keep the small amount of medium in the wells from evaporating. The next day, the second agarose plug was removed from each chamber. This was the chemoattractant well. Either a specific chemical, conditioned medium, or another cell type was added to this well. Figure 4-1 shows the appearance of a typical chamber. The chamber slides were then incubated for 48 hours at 37°C to allow the stromal cells time to undergo chemotaxis. After 48 hours, the medium was removed and the cells fixed in a 4% paraformaldehyde / 12.5% gluteraldehyde cacodylate buffer solution for 30 minutes at room temperature. Then the agarose was removed from the chambers and the cells were stained with 0.1% Coomassie
Blue for 30 minutes at room temperature. The chambers were removed and the cells visualized under a dissecting microscope. The ScionImage program was used to measure the distance of cell movement and to calculate chemotactic index. Chemotactic index is defined as the difference A-B. B is the distance from the back of the MSC well to the clump of at least three cells that has moved farthest away from the well. A is measured in a similar fashion except it is measured in the direction toward the chemoattractant well. Figure 4-2 is a visual representation of how chemotactic index is calculated.
Figure 4-1. A typical under agarose chamber. 1X DMEM/1% agarose is placed in the chamber and allowed to harden. Two 5 mm diameter wells are cut 1.5 mm apart from each other. Stromal cells are plated in the cellular well at 30,000 cells per well. After a day to allow them to attach, chemoattractant in the desired concentration is placed in the chemoattractant well. The cells are cultured for 48 more hours and then the agarose is removed and the cells are fixed and stained with Coomassie Blue.
Figure 4-2. A typical chemotactic index-measurement. A is the distance from the front of the well to the clump of at least three cells closest to the chemoattractant well. B is the distance from the back of the well to the clump of three cells farthest away from the cellular well. Chemotactic index is defined as A-B.
Gelatin Coating of Under Agarose Permanox Chamber Slides

In order for MSCs to properly remain attached to the chamber slides, the slides needed to be pre-coated with gelatin. A 1:1 ratio of 2% gelatin solution and PBS was prepared and enough of this solution was added to just cover the bottom of each chamber of the slides. The slides were then incubated at 37 °C for 30 minutes. Then a solution of 19.6 ml PBS and 400 µl of 25% gluteraldehyde was prepared. This was layered over the gelatin already in the chambers and allowed to incubate at room temperature for 30 minutes. Then the liquid in the chambers was aspirated and the chambers washed with 2 ml of PBS with 0.1 g/L CaCl₂ five times. Slides were stored at 4 °C until agarose was added.

Westerns

Cells from 2D cultures were scraped with a sterile cell scraper under PBS and collected in a centrifuge tube. The cells were pelleted at 400 x g for 10 minutes and the PBS aspirated. 150 µl of lysis buffer pH 7.5 (1% Triton X-100, 25mM HEPES, 130 mM NaCl, 1mM EDTA, 1 mM DTT) was added and the cells resuspended and transferred to a microfuge tube. The cells were sheared with a small gauge needle and then spun at 10,000 x g for 15 minutes to pellet the cell debris. 50 µl of the supernatant was saved for Bradford Assay analysis to ensure equal protein loading. The other 100 µl of the supernatant was added to 100 µl of 2X sample buffer (125mM Tris-HCl pH 6.8, 4.7% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 2 minutes. Samples were stored at –80 °C until needed. Protein samples were resolved on
a 9.6% SDS-PAGE gel. After electrophoresis, the gel was soaked for 15 minutes in 1X Towbin buffer (5 mM Tris pH 7.5, 5 mM glycine) to remove SDS. The proteins were transferred to a nitrocellulose membrane in Towbin buffer using an electroblotting apparatus for 1 hour at 20V. The membrane was blocked in 5% nonfat milk solids in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 hour. The membrane was washed in TBS-T for 15 minutes and then twice again for 5 minutes. Membrane was incubated with properly diluted primary antibody in TBS-T overnight. Following incubation, the membrane was washed in TBS-T for 15 minutes and then twice again for 5 minutes. The membrane was then incubated in secondary antibody (1:2000 dilution) in TBS-T for 15 minutes and then 4 times again for 5 minutes. The membrane was then exposed to a 1:1 mixture of ECL developing reagents (Amersham Pharmacia Biotech) for 1 minute and then the reagents were drained. The membrane was then exposed to film for the desired amount of time.
CHAPTER 5
CHEMOTAXIS STUDIES

A. Experimental Design

Cells of mesenchymal origin, including marrow stromal cells, and osteoblasts have been shown to undergo chemotaxis in response to certain factors. Osteoblasts will migrate toward several chemotactic factors found in the ECM (TGF-β (Pfeilschifter et al., 1990), BMPs (Lind, 1998), PDGF-A (Wang et al., 1997), PDGF-B (Godwin and Soltoff, 1997), IGFs (Panagakos, 1993). Stromal cells’ chemotaxis has not been investigated thoroughly, but some preliminary studies show that they will migrate toward BMP2 (Lind, 1998). Previous experiments involving endothelial cell chemotaxis have shown that mesenchymal stem cells will migrate toward both endothelial cells and factors produced by endothelial cells (Hirschi et al., 1998). Upon making contact with the endothelial cells, the mesenchymal cells begin differentiating into endothelial cells. Because osteoblasts and marrow stromal cells are in an environment where chemotactic factors are present (the ECM), we proposed to examine the effects of these factors on stromal cell movement. We wished to test the hypothesis that either osteoblasts or the factors secreted by osteoblasts into the matrix could recruit stromal cells to a location to where bone formation was needed as well as see whether stromal cells would differentiate upon making contact with osteoblasts. Because the work done to study the mesenchymal cell / endothelial cell
system had employed an under agarose chemotaxis assay, we chose to design a similar set of experiments to study the stromal cell / osteoblast interactions.

The first experiments involving the under agarose assay were done to set up a positive control. The chemotaxis of 10T1/2 mesenchymal cells toward 20 ng/ml of PDGF-BB was used as a positive control. After establishing this control, then MSCs were tested for movement. A variety of substances were placed in the chemoattractant well. Experiments using growth factors (TGF-β, PDGF-BB) over a range of concentrations, conditioned 2% FBS medium from osteoblasts and adipocytes, or adipocytes themselves in the chemoattractant well were attempted. Conditioned medium is the medium from cultured cells that has been left unchanged for three days. It was thought that this medium might contain secreted factors that may be chemotactic. Due to difficulties in passaging fully differentiated osteoblasts, only conditioned medium from osteoblast cultures was used as a chemoattractant. Although a single early experiment showed chemotaxis by MSCs toward PDGF-BB, a long series of subsequent experiments failed to reproduce this promising result.

B. Results

The very first experiment involving stromal cell chemotaxis was very simple. MSCs were platted in a 12 well collagen coated plate and then maintained at 37°C in the presence of 5.0% CO₂ while under a computer-controlled camera. The cells were photographed at 10-minute intervals for 8 hours, but no movement was observed. Because there was no chemotactic factor present in this experiment, it was concluded
that MSCs would not move unless a chemotactic factor is present. Thus, the under agarose assay was employed to provide a chemotactic factor.

During the first few under agarose experiments (refer to Material and Methods page 24 and Figures 4-1 and 4-2) involving MSCs, after the cells were fixed and stained, the slides would be blank aside from some cellular debris found in the original MSC well. At first, it was thought that the cells were not being plated at a high enough density, but subsequent experiments using higher plating densities produced similar results. It was surmised that because these cells can produce matrix, this matrix was adhering to the agarose more tightly than the cells were adhering to the Permanox of the chamber slide. To overcome this difficulty, the chamber slides were pre-coated with gelatin. Slides that were coated with gelatin allowed the stromal cells to adhere properly. Fibronectin or collagen coating was not sufficient to allow the stromal cells to adhere to the Permanox.

Table 5-1 shows chemotactic index measurement data for several but not all of the chemotaxis assays performed. The vast majority of the chemotaxis assays showed no directed movement. (Data not shown.) The table shows 4 separate types of chemotaxis assays: between MSCs and no chemoattractant, MSCs and 20 ng/ml of PDGF-BB, 10T1/2 cells and 20 ng/ml of PDGF-BB, and 10T1/2 cells and 200 ng/ml of PDGF-BB). Four trials were performed for each assay. Table 5-2 presents the average chemotactic index measurements (A-B) from the 4 assays. The p value for the MSC / PDGF-BB assay was calculated using the paired Student's T test. A pictoral example of the cell movement seen in three of the four assay types are shown in Figures 5-1, 5-2, and 5-3. No cell movement was seen in the MSC and no
chemoattractant (negative control) assay (Data not shown). Figure 5-1 shows positive 10T1/2 cell movement toward a source of 20 ng/ml of PDGF-BB. This was established as the positive control. Figure 5-2 shows negative 10T1/2 cell movement away from 200 ng/ml of PDGF-BB. It is not currently understood why these cells move away from 10-fold greater than physiological levels of PDGF-BB, but perhaps it involves the down-regulation of the cells' PDGF receptors. Figure 5-3 shows MSC movement toward a source of 20 ng/ml of PDGF-BB. Aside from the 4 trials involved in this experiment, this result could not be duplicated.
Table 5-1. Chemotaxis Index Measurements for Several Under Agarose Assays

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Chemoattractant</th>
<th>Slide#</th>
<th>A (mm)</th>
<th>B (mm)</th>
<th>A-B (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stromal</td>
<td>none</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stromal</td>
<td>none</td>
<td>2</td>
<td>.20</td>
<td>.20</td>
<td>0</td>
</tr>
<tr>
<td>Stromal</td>
<td>none</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stromal</td>
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<td>4</td>
<td>.35</td>
<td>.30</td>
<td>.05</td>
</tr>
<tr>
<td>Stromal</td>
<td>PDGF-BB (20 ng/ml)</td>
<td>1</td>
<td>.67</td>
<td>.25</td>
<td>.42</td>
</tr>
<tr>
<td>Stromal</td>
<td>PDGF-BB (20 ng/ml)</td>
<td>2</td>
<td>.95</td>
<td>.13</td>
<td>.82</td>
</tr>
<tr>
<td>Stromal</td>
<td>PDGF-BB (20 ng/ml)</td>
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<td>1.1</td>
<td>.42</td>
<td>.68</td>
</tr>
<tr>
<td>Stromal</td>
<td>PDGF-BB (20 ng/ml)</td>
<td>4</td>
<td>.83</td>
<td>.35</td>
<td>.48</td>
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<td>PDGF-BB (200 ng/ml)</td>
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<td>1.03</td>
<td>1.95</td>
<td>-.92</td>
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<td>PDGF-BB (200 ng/ml)</td>
<td>3</td>
<td>1.08</td>
<td>1.22</td>
<td>-.14</td>
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<tr>
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<td>PDGF-BB (200 ng/ml)</td>
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<td>.65</td>
<td>1.13</td>
<td>-.48</td>
</tr>
<tr>
<td>10T1/2</td>
<td>PDGF-BB (20 ng/ml)</td>
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<td>1.33</td>
<td>.77</td>
<td>.56</td>
</tr>
<tr>
<td>10T1/2</td>
<td>PDGF-BB (20 ng/ml)</td>
<td>2</td>
<td>1.25</td>
<td>.65</td>
<td>.60</td>
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<td>PDGF-BB (20 ng/ml)</td>
<td>3</td>
<td>1.17</td>
<td>.93</td>
<td>.24</td>
</tr>
<tr>
<td>10T1/2</td>
<td>PDGF-BB (20 ng/ml)</td>
<td>4</td>
<td>2.34</td>
<td>.48</td>
<td>1.86</td>
</tr>
</tbody>
</table>

Each line shows the result from a separate under agarose assay.
Table 5-2. Average Calculated Chemotactic Indices

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Chemoattractant</th>
<th>A-B (mm) X ± S.D. (n=4)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stromal</td>
<td>None</td>
<td>0.01 ± 0.02</td>
<td>N/A</td>
</tr>
<tr>
<td>Stromal</td>
<td>PDGF-BB (20 ng/ml)</td>
<td>0.60 ± 0.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10T1/2</td>
<td>PDGF-BB (20 ng/ml)</td>
<td>0.82 ± 0.62</td>
<td>N/A</td>
</tr>
<tr>
<td>10T1/2</td>
<td>PDGF-BB (200 ng/ml)</td>
<td>-0.58 ± 0.30</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 5.1. Chemotaxis of 10T1/2 cells toward 20 ng/ml of PDGF-BB. 30,000 10T1/2 cells were plated and cultured in the presence of PDGF-BB for 48 hours before fixation and staining. Chemotactic index was calculated using the ScionImage program. Chemotactic index is 0.56 mm. This experiment was performed four times.
Figure 5.2. Chemotaxis of 10T1/2 cells away from 200 ng/ml of PDGF-BB. 30,000 10T1/2 cells were plated and cultured in the presence of PDGF-BB for 48 hours before fixation and staining. Chemotactic index was calculated using the ScionImage program. Chemotactic index is –0.14 mm. This experiment was performed four times.
Figure 5-3. MSCs' movement toward 20 ng/ml of PDGF-BB. 30,000 MSCs were plated and cultured in the presence of PDGF-BB for 48 hours before fixation and staining. Chemotactic index was calculated using the ScionImage program. In this figure, the chemotactic index is 0.82 mm. The exact measurement is shown in Figure 4-2. This experiment was performed four times.
C. Discussion

Karen Hirsch's results with 10T1/2 cells were reproduced and established as a positive control. Figure 5-1 shows a typical assay. Several assays using conditioned medium from adipocytes or osteoblasts, adipocytes themselves, BMP6 or TGF-β as chemoattractants were performed, but no MSC chemotaxis was seen. (Data not shown.) MSC chemotaxis was observed in only 4 separate trials of a single chemotaxis assay involving MSCs and 20 ng/ml of PDGF-BB. One of these 4 trials is shown in Figure 5-3. Despite much effort, this result could not be duplicated. There are two possible reasons for this. The chemotaxis may have been due to cells that had differentiated into something other than a MSC or osteoblast (i.e. fibroblast). To test this a clonally derived cell line would need to be established. Another reason no MSC chemotaxis was observed could have been due to MSCs not being able to sense the chemical gradient. This is because PDGF is present in the serum. Numerous experiments lowering the amount of serum in the cell culture medium were performed with a minimum FBS concentration of 0.5%. It was found that less than 2% FBS placed the cells in growth arrest and at none of the lower FBS concentrations did chemotaxis occur. Another option to solve this problem would be to immunodeplete all of the PDGF from the serum, but this has not been done yet. It is most likely that MSCs do not move in response to any of the substances that were tested. Since osteoblasts are known to move to where bone formation is required, perhaps MSCs differentiate into osteoblasts before they take part in bone remodeling.
CHAPTER 6
POLYMER SCAFFOLD STUDIES

A. Experimental Design

Marrow stromal cells have been shown to differentiate within PLGA scaffolds in previous studies (Ishaug et al., 1994; Ishaug et al., 1996). They will secrete and calcify matrix within PLGA. Because essentially only one set of culture conditions have been used to grow MSCs in PLGA, experiments were designed to study two aspects of MSC differentiation in PLGA. The first was to compare the timing of osteoblastic differentiation in PLGA (3D) to the already established timing on plastic (2D) (Owen et al., 1990) by looking at the expression of bone differentiation markers such as alkaline phosphatase and osteocalcin. Because the 3D porous PLGA scaffolds mimic the environment of bone, it was suspected that osteoblast differentiation might be altered when comparing 3D to 2D cultures. The other goal was to optimize culture conditions within PLGA cultures to promote the best osteoblast growth from MSCs.

To study the timing of osteoblast differentiation in PLGA, approximately 1 million MSCs suspended in complete medium were seeded by injection into 90% porous PLGA scaffolds 4 mm thick, and 12 mm in diameter. This mimics the high seeding density as reported by Ishaug (Ishaug et al., 1997). Cells were incubated for 3 hours at 37°C to promote cell attachment and then were given 4 ml of complete medium. Medium was changed every 3 days. Starting on the 5th day following
seeded and every 3 days thereafter, a scaffold was washed with PBS, placed in 1 ml of 4M guanidinium thiocyanate, homogenized, and frozen at -80°C until all the samples had been collected. Total RNA was extracted from each sample, and the amount of RNA in each sample was calculated from spectrophotometric measurement. Northern blots for AP and osteocalcin were performed to determine where their peak expression occurred.

The studies to optimize osteoblastic differentiation of MSCs in PLGA involved AP and DNA assays to determine a timecourse of AP activity for the cells under different culture conditions. Marrow stromal cells were seeded onto PLGA scaffolds (90% porosity, 12 mm diameter, 2 or 4 mm thick) with either 500,000 or 1 million cells by either injection or layering of a concentrated drop of cells on just the surface of the scaffold. AP assays were performed to determine the effects of seeding density, seeding method, and scaffold thickness on osteoblastic differentiation. Cell-cell contact was thought to be important in osteoblast differentiation (Ishaug, 1997). Decreasing the thickness of the scaffold, layering a concentrated drop of cells on the scaffold surface, and increasing the number of seeded cells were done to increase the amount of MSC contacts.

Lastly, in conjunction with Aaron Goldstein in the Mikos laboratory, AP assays and Northern were performed to determine whether any of three different fluid convection culture systems: flow chamber, rotary vessel, or spinner flask were able to allow better MSC differentiation compared to static cultures. A picture of the flow chamber device is shown in Figure 6-10. In the flow chamber, recycled cell culture medium was continuously pushed through the PLGA scaffolds to ensure cells
throughout the scaffolds received nutrients. The scaffolds in the spinner flask were immersed in medium being swirled by a magnetic stirrer. The scaffolds were immobilized axially on 22 gauge needles that pierced the stoppered top of the flask and went into the cell medium. The rotary vessel (Synthecon, Houston, TX) is essentially a clinostat that was filled with medium, and the scaffolds were rotated at a constant 20 rpm. Obviously, in all of the culture systems save the static cultures, the cells in the scaffolds experienced some fluid shear stress. The 6 mm thick, 12 mm diameter, 90% porous scaffolds were seeded with 2 million MSCs by syringe injection. Two significant differences in these experiments compared to previous ones were that prior to seeding, the scaffold were all coated with 0.8 ml of a 50 μg/ml fibronectin in PBS solution. This was done to prevent cells from being ripped off the scaffolds in the flow system. The other difference was that only one half of the medium was changed when cells were given new medium in order to allow factors secreted by the cells to accumulate.

B. Results

Initial Northern blot experiments showed that AP expression peaked somewhere between days 5 and 8 post Dex treatment in 3D cultures (Figure 6-1). Previous studies have shown AP expression or activity peaking at around day 14 post Dex treatment for both rat calvarial osteoblasts and MSCs grown in 2D environments (Owen et al., 1990; Peter et al., 1998). My own 2D AP assay showed that AP activity peaked on day 10 post Dex treatment and then decreased after day 10 (Figure 6-5).
Goldstein’s work with 3D scaffolds seed with 2 million MSCs showed a peak of AP activity at day 7 post Dex treatment and decreased after this day (Figure 6-11, Goldstein et al., 1999).

Osteocalcin expression also peaked earlier in 3D cultures. Figure 6-2 shows osteocalcin peaking at day 11 post Dex treatment in PLGA. Both rat calvarial osteoblasts and MSCs grown in 2D environments have peak osteocalcin expression on day 21 post Dex treatment (Owen et al., 1990; Peter et al., 1998).

Comparison between AP assays of 2D and 3D cultures showed a normal AP activity profile for 2D cultures with a peak at day 10 and little normalized AP activity in 3D cultures (Figures 6-3 through 6-5). Total AP activity for the 3D cultures followed a similar trend to the AP activity seen in the 2D cultures. Cell seeding density and scaffold seeding method seemed to have no effect on AP activity in 3D cultures and little normalized activity was seen (Figures 6-6 through 6-9). The DNA assay results that correlate to each AP assay are summarized in Table 6-1. There is a general increasing trend of DNA amount as the 3D timecourse progressed. This is especially evident in the assays corresponding to Figures 6-6 through 6-9. Lastly, Figure 6-11 shows AP activities for the different fluid flow culture conditions in comparison with static 3D cultures. The only significant increase in AP activity was seen in the scaffolds cultured in the flow chamber. Figure 6-12 shows that there was no significant difference in the amount of osteocalcin expression between any of the 4 fluid convection culture methods: fluid flow, rotary vessel, spinner flask, or static cultures.
Table 6-1. DNA Amounts For Each AP Assay

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Alkaline Phosphatase Expression in Stromally Derived Osteoblasts

![Image showing Alk. Phos. and rRNA Bands over time from Day 5 to Day 26.]

Figure 6-1. Alkaline phosphatase mRNA expression in MSCs cultured in 3D PLGA scaffolds. Scaffolds were 90% porous, 4 mm thick, 12 mm in diameter, and were seeded by injection with 1 million MSCs that were resuspended in complete medium. Medium was changed every three days. RNA was collected over 26 days. AP mRNA appears to peak somewhere between days 5 and 8 post seeding. The blot was probed with a portion of rat AP cDNA cloned into the EcoRI-BamHI site of PGEM7Zf(+). rRNA bands are shown as a loading control. This experiment was performed twice.
Figure 6-2. Osteocalcin mRNA expression in MSCs cultured in 3D PLGA scaffolds. Scaffolds were 90% porous, 4 mm thick, 12 mm in diameter, and were seeded by injection with 1 million MSCs that were resuspended in complete medium. Medium was changed every three days. RNA was collected over 26 days. Osteocalcin mRNA appears to peak on day 11 post seeding. The blot was probed with a portion of rat osteocalcin cDNA cloned into the Hind III site of pGEM7Zf(+). rRNA bands are shown as a loading control. This experiment was performed twice.
Figure 6-3. Alkaline phosphatase activity of 3D cultures seeded by injection with 500,000 MSCs resuspended in complete medium into 90% porous, 4 mm thick, 12 mm diameter PLGA scaffolds. Medium was changed daily. After normalization to DNA content, little AP activity was seen over the 20 day timecourse. This experiment was performed once.
Figure 6-4. Alkaline phosphatase activity of 3D cultures seeded by injection with 500,000 MSCs resuspended in complete medium into 90% porous, 4 mm thick, 12 mm diameter PLGA scaffolds. Medium was changed daily. After normalization to DNA content, little AP activity was seen over the 20 day timecourse as compared to the companion 2D assay (Figure 6-5). Total AP activity follows the trend of the normalized activity of the 2D cultures. This experiment was performed once.
Figure 6-5. Alkaline phosphatase activity of 2D cultured MSCs plated at half confluence (26,500 cells/cm²) into 6 well culture plates and given complete medium. Medium was changed every three days. Normalized AP activity shows a clear peak at day 10 post plating after a sharp increase and then falls off more slowly over time. This experiment was performed once.
Figure 6-6. First trial (of two) of alkaline phosphatase activity of 3D cultures seeded by layering with 500,000 MSCs resuspended in complete medium into 90% porous, 2 mm thick, 12 mm diameter PLGA scaffolds. Medium was changed daily. After normalization to DNA content, little AP activity was seen aside from the day 14 timepoint which was still quite low.
Figure 6-7. Second trial (of two) of alkaline phosphatase activity of 3D cultures seeded by layering with 500,000 MSCs resuspended in complete medium into 90% porous, 2 mm thick, 12 mm diameter PLGA scaffolds. Medium was changed daily. After normalization to DNA content, little AP activity was seen aside from the day 14 timepoint which was still quite low.
Figure 6-8. First trial (of two) of alkaline phosphatase activity of 3D cultures seeded by layering with 1,000,000 MSCs resuspended in complete medium into 90% porous, 2 mm thick, 12 mm diameter PLGA scaffolds. Medium was changed daily. After normalization to DNA content, little AP activity was seen aside from the day 14 timepoint which was still quite low.
Figure 6-9. Second trial (of two) of alkaline phosphatase activity of 3D cultures seeded by layering with 1,000,000 MSCs resuspended in complete medium into 90% porous, 2 mm thick, 12 mm diameter PLGA scaffolds. Medium was changed daily. After normalization to DNA content, little AP activity was seen aside from the day 14 timepoint which was still quite low.
Figure 6-10. Schematic of flow chamber apparatus. Complete medium is perfused axially through the foams. The scaffold fits within the cup snugly and prevents medium from flowing around the scaffold. Medium leakage is prevented by the O-rings. Medium is delivered at a constant rate in parallel to three scaffolds per flow chamber device. (Taken from Goldstein et al., 1999).
Figure 6.11. Alkaline phosphatase activity of seeded osteoblasts as a function of culture condition and duration of culture: a) activity per foam, and b) activity per cell. Flow chamber (light stripes, n=6), spinner flask (dark, n=6), rotary vessel (shaded, n=3), and static culture (dark stripes, n=6). Error bars correspond to the standard deviation. An asterisk indicates activity that is statistically different from that for static culture for the same number of days in culture (Taken from Calderpin et al., 1999).
Figure 6-12. Northern blot analysis of 3D cultured MSCs seeded by injection into 90% porous, 6 mm thick, 12 mm in diameter PLGA scaffolds and cultured in either the flow chamber, spinner flask, rotary vessel or statically. RNA was extracted after 14 days in culture. Both GAPDH (Section a) and osteocalcin (Section b) mRNA were probed for. GAPDH served as a loading control. The lane identities are: spinner flask (lanes 1 and 2), flow chamber (lanes 3-5), static culture (lanes 6-8) and the rotary vessel (lanes 9 and 10). Osteocalcin expression seemed to be identical regardless of the culture system used. This experiment was performed twice.
C. Discussion

Comparing the peaks of AP and osteocalcin expression between 2D and 3D MSC cultures suggests that MSCs cultured in a 3D environment will differentiate faster than their 2D counterparts. This is most likely due to the similarity between the PLGA scaffolds and the bone ECM. Because the cells are already in a synthetic 3D matrix, it may not be necessary for the cells to secrete as much matrix as those grown in 2D cultures. Because MSCs cultured in PLGA can enter the matrix maturation stage earlier, they also get an earlier start on matrix mineralization. From these studies, cells grown in scaffolds are predicted to mineralize their matrix about 10 days faster than those cells grown on plastic, although this has not yet been determined.

The results from experiments looking at AP activity in the PLGA scaffolds were surprising. Regardless of cell density, seeding method, or scaffold thickness, AP activity in 3D cultures normalized to DNA content was insubstantial (Figures 6-3, 6-4, 6-6, 6-7, 6-8, 6-9). However, the total AP activities of the 3D cultures were similar to those of 2D cultures. What was different between the 3D and 2D cultures and what caused the normalized AP activities of the 3D cultures to decrease was the fact that in the 3D cultures, cell number continued to increase whereas in the 2D cultures, proliferation slowed down and leveled off by day 10 (Table 6-1). This trend is also seen in the experiments from Figures 6-6, 6-7, 6-8, 6-9 in which total DNA in each scaffold jumps dramatically on day 14 post seeding. Studies performed in the Mikos laboratory have not demonstrated this effect. Work done by Aaron Goldstein has shown in 3D cultures similar to my own that AP activity follows a trend similar to
that of cells grown on plastic with a peak around day 7 (Figure 6-11, Goldstein, et al., 1999). This discrepancy in results was mysterious until it was realized that in my studies, cell culture medium was changed daily while in Goldstein’s experiments, only one half the medium was changed every two days. This strongly suggests that MSCs differentiating into osteoblasts secrete soluble factors into the medium that promote differentiation. This possibility is consistent with previous work showing that osteoblasts secrete bone morphogenetic proteins, and these are required for their differentiation (Boden et al., 1997). Scaffolds that only have half the medium changed every two days will have a much higher soluble differentiation factors to proliferation factors (from the FBS in the medium) ratio than in scaffolds having all the medium changed daily. The lack of normalized AP activity and increased proliferation in my scaffolds are most likely due to the removal of secreted differentiation factors during medium changes. This would explain why AP mRNA expression was seen in Figure 6-1 because medium in those experiments was changed every three days instead of daily. This would also explain why DNA levels shown in Table 6-1 level off at day 10 for the 2D cultures (Figure 6-5) while in the other 3D cultures, DNA values tended to continue to increase over time as cells proliferated instead of differentiated.

Lastly, in the experiments involving comparing static 3D cultures to flow chamber, spinner flask, and rotary vessel culture conditions, AP activity was seen to increase in the scaffold subjected to constant flow (Figure 6-11, Goldstein et al., 1999). The other two systems showed AP activity increases compared to static cultures, but were not statistically significant. AP activity peaked much earlier than
in 2D cultures. The same is true for osteocalcin expression that appeared earlier on day 14 again suggesting differentiation occurs at an accelerated rate in 3D cultures. Northern analysis showed that there was no significant difference between culture conditions on osteocalcin expression by day 14 (Figure 6-12). However, osteocalcin expression could differ between the culture systems at a later point than day 14 and is currently being investigated.
CHAPTER 7
CONCLUSIONS

Bone cells grow within a collagenous three dimensional environment rich in growth and differentiation factors. The use of polymer scaffolds on which to grow bone in order to mimic this 3D environment is gaining popularity. Clinically, bone growth is more efficient if there is a 3D matrix on which bone cells can attach. Already the polymer used in these studies, PLGA, has been FDA approved for implantation. The scaffold's biodegradability and its ability to be molded make it an attractive alternative to bone grafts.

In these studies, differences between osteoblastic differentiation of marrow stromal cells cultured in 3D versus 2D environments were studied. Both systems supported osteoblastic differentiation of MSCs well into the mineralization phase. However, differentiation occurred much faster in the 3D cultures. Alkaline phosphatase mRNA expression peaked between days 5 and 8 in 3D culture while peaking at about day 15 in 2D cultures. These data suggest that cells cultured in 3D scaffolds enter the matrix maturation phase several days before cells grown on plastic. A similar trend was seen in osteocalcin mRNA expression. In 3D cultures, osteocalcin expression peaked at day 11 while in 2D cultures, it peaked at day 21. This suggests that cells cultured in 3D scaffolds are able to mineralize the matrix much sooner than in 2D systems.
Culture conditions were also altered in the 3D cultures to determine optimal conditions for osteoblastic growth. Although not witnessed in these experiments, previous work has suggested lower cell density promotes faster differentiation (Ishaug, 1996). Presence of osteoblast secreted differentiation factors in the medium appear to be critical for proper cell differentiation. Little differentiation was seen in scaffolds that had their medium changed daily, as shown by the little AP activity in these cultures. However, identical scaffold cultures that had medium changed every three days or half the medium exchanged every other day had a normal AP activity profile. These data suggest that it is critical that the ratio of differentiation factors (secreted by osteoblasts) to growth factors (present in medium) remain high in order for differentiation to take place.

Future work with 3D cultures should take into account some of the limitations of PLGA. It is not an entirely elastic material, which means if it is implanted, some deformation will occur over time as it experiences mechanical loading. The larger the scaffold implant, the more serious this problem becomes. This is also true for the problem of nutrient exchange in the scaffolds. Cell culture medium constantly flowing though the scaffolds enhanced differentiation (an increase in AP activity was seen). In large PLGA implants, nutrients may have difficulty reaching cells that are not near the periphery. While PLGA is biodegradable, the breakdown products glycolic acid and lactic acid are acidic and this may cause difficulties with cell growth and differentiation. Currently a new polymer material, poly(propylene fumarate) (PPF), is being investigated as a possible material for 3D scaffolds. PPF is a biodegradable, injectable polymer that can be crosslinked into scaffold (like PLGA)
or hydrogels (Peter et al., 1999). The breakdown product of PPF is fumaric acid, which is taken up in the citric acid cycle. Preliminary studies of PPF's mechanical properties suggest that it is stronger than PLGA (Peter et al., 1999). It would be interesting to compare and contrast osteoblastic differentiation in the two polymers. The clinical applications and benefits would be enormous were PPF to perform better than PLGA.

It has been suggested that marrow stromal cells could be induced to move to a site where bone synthesis was occurring due to the release and activation of growth/differentiation factors during the remodeling process. An under agarose assay was employed to investigate stromal cell chemotaxis. A number of different experiments were designed to test MSC chemotaxis. Growth factors (TGF-β, PDGF-BB), conditioned medium from osteoblasts and adipocytes, and different cell types were all tested as chemoattractants. Only one experiment suggested that stromal cells would move toward 20 ng/ml of PDGF-BB, but this result could not be reproduced. Two further experiments could be performed to determine whether MSCs would truly respond to PDGF-BB. The PDGF found in calf serum could be immunodepleted and then used in the under agarose assay to ensure that the only PDGF the cells could sense would be in the chemoattractant well. The other experiment is more difficult. Because the femoral rat prep provides a heterogeneous cell population, not every cell in the chemotaxis assay wells will be a stromal cell. To test that the movement seen in Figure 5-3 is due solely to MSCs, a clonally derived stromal cell line would need to be developed. This has been successfully done for fetal rat calvarial cells (Aubin et
However, this process is labor intensive and it appears that MSCs do not move in response to any of the chemotactic agents tested.
APPENDIX A

MAP KINASES IN OSTEOBLASTS

A. Experimental Design

Although previous studies have shown that osteoblasts could be the potential mechanosensor of bone, little is known about their expression of MAP kinases that could be involved in signal transduction from a mechanical stimulus. Experiments were designed to determine whether three major mammalian MAP kinases (ERK2, JNK1, and p38) are expressed and if so at what point during osteoblastic differentiation.

Passaged MSCs were plated in 2D cultures (6-well culture plates) and provided with complete medium. Cells were washed with PBS and medium changed every 3 days. Starting on day 2 following passaging continuing every three days until 26 days post-passaging, cells from one 6-well plate were scraped off the plate with a sterile cell scraper and collected. The cells were lysed and a protein lysate was obtained. Protein samples were normalized by Bradford assay to ensure equal gel loading. Once the 26-day timecourse was complete, Western blots were performed. An SDS-PAGE gel was run for each MAP kinase being investigated. The proteins were transferred to nitrocellulose and then incubated with the proper primary and secondary antibodies then developed.
B. Results

1) ERK2

A 10-minute exposure (Figure A-1) showed that ERK2 protein was present in all days tested with about equal expression in all days except for Day 2 in which only a small amount of ERK2 protein was present.

2) p38

A 10-minute exposure (Figure A-2) showed that p38 protein was present throughout the entire 26-day timecourse with slightly higher protein expression on Days 5, 8, and Days 17 on.

3) JNK1

A 60-minute exposure (Figure A-3) showed that JNK1 protein was expressed only on Days 5, 8, and 11 in very small amounts.
Figure A-1. ERK2 Western blot. MSCs were subcultured after 14 days and grown on plastic for a period of 26 days in complete medium. Protein was extracted from cells every 3 days starting at day 2 post subculture. Protein amount was assayed via Bradford assays to ensure equal loading per lane. This experiment was performed three times.
Figure A-2. p38 Western blot. MSCs were subcultured after 14 days and grown on plastic for a period of 26 days in complete medium. Protein was extracted from cells every 3 days starting at day 2 post subculture. Protein amount was assayed via Bradford assays to ensure equal loading per lane. This experiment was performed three times.
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JNK1 Mar. 28, 1997 60 min exp.

**Figure A-3.** JNK1 Western blot. MSCs were subcultured after 14 days and grown on plastic for a period of 26 days in complete medium. Protein was extracted from cells every 3 days starting at day 2 post subculture. Protein amount was assayed via Bradford assays to ensure equal loading per lane. This experiment was performed three times.
C. Discussion

Cells used in these Western blots were all from the same original culture. MSCs that had been plated at the beginning of the timecourse had indeed differentiated into osteoblasts because matrix secretion in the 2D cultures began at about Day 8 and mineralized bony nodules were visible by about Day 20. This suggests that the matrix maturation phase begins on or a little after day 8 and matrix mineralization on or a little after Day 20 post-passaging. Aside from Day 2, ERK2 protein is expressed at about equal levels throughout osteoblastic differentiation. The fact that not much ERK2 protein is expressed on Day 2 suggests that MSCs may not produce much or any ERK2 and that it is only after MSCs start to undergo osteoblastic differentiation that ERK2 will be expressed.

p38 was expressed throughout the entire differentiation timecourse with slightly increased protein expression during the mineralization phase (Day 17 through Day 26) and the end of the proliferation phase (Days 5 and 8). Unlike ERK2, p38 protein is present in Day 2 cells. Further experiments with earlier timepoints will need to be performed to determine whether p38 is expressed in MSCs as well as osteoblasts.

Lastly, JNK1 protein expression was very low, and protein was only present on Days 5, 8, and 11. This suggests that JNK1 protein’s role in osteoblastic differentiation is minor and that it is limited to the proliferation phase.

All three of the MAP kinases investigated have been implicated in transducing mechanical loading in other cell types. In endothelial cells, JNK1 and ERK2 both
have been shown to activate AP-1, a transcription factor involved in adapting to fluid shear stress (Li et al., 1996). p38 has been shown to be involved in the transduction of a compressive stress signal (Wang and Ron, 1996). Because ERK2 and p38 are both expressed in differentiating osteoblasts, it may be through these MAP kinases that the cell perceives mechanical loading. Further studies need to be performed in order to determine whether downstream targets of these MAP kinases are activated in osteoblasts following the application of compressive or fluid shear stress.
APPENDIX B

DEVELOPMENT OF OSTEOCALCIN PROMOTER CONSTRUCT

A. Experimental Design

To develop an osteocalcin promoter vector appropriate for cell transfection, the rat osteocalcin promoter was PCR amplified from rat genomic DNA using rat osteocalcin specific primers. The forward primer's sequence was 5' AAGAACAACCTTCACCTTTAATATTATGATAACAT3'. Its melting temperature was 64.2°C. The reverse primer's sequence was 5' AGGTCTGCACCCGAGTTGCTGTGGGTACCTTCATG3'. Its melting temperature was 83.2°C. PCR performed at an annealing temperature of 61°C produced the correct sized product of ~1100 bp. Sequencing confirmed its identity as the rat osteocalcin promoter. The PCR product was made blunt ended by incubating it at 72°C for 30 minutes in the presence of dNTPs and Pfu DNA Polymerase. The promoter was then ligated into the Promega Basic β-gal Vector. A simple map of this vector is shown in Figure B-1. This was done by performing a Sma I blunt end digest and ligation with T4 DNA ligase in the same reaction tube. Once the vector is cut with Sma I, either vector religation or insert ligation into the Sma I site of the vector (upstream of the lacZ gene) could occur. If insert ligation takes place, then the vector's Sma I site is destroyed and thus the promoter construct is created. Of course, the osteocalcin promoter could enter the β-gal vector in either orientation. E. coli were transformed and colonies mini-prepped. Recovered vector DNA was digested
with EcoRV to determine the insert orientation. This digestion showed that 4 prepped colonies had the osteocalcin promoter in the correct orientation.

B. Results and Discussion

The construction of the osteocalcin promoter β-gal reporter gene construct was successful. The osteocalcin promoter was cloned into the Sma I site of pβgal-Basic upstream of the lacZ gene. The promoter construct allows for the visualization of the osteocalcin gene’s transcriptional regulation using chemiluminescent detection of β-galactosidase activity. MSCs could be transfected with this construct, and culture conditions altered, to study culture conditions’ effects on osteocalcin’s transcriptional regulation. For example, MSCs could be transfected with this construct, and cells could be mechanically loaded or their medium supplemented with a factor up-regulated by mechanical loading (such as PDGF). Then these treatments’ effects on osteocalcin gene transcription could be investigated. This construct could also be used to compare osteocalcin transcriptional activity between MSCs grown in 2D culture and 3D polymer scaffolds. Before this construct is used, studies would need to be performed to determine the optimal conditions for MSC transfection.
Figure B-1. Map of the pβgal-Basic vector. A 1100 bp fragment of the rat osteocalcin promoter was cloned into the Sma I site of this vector upstream of the lacZ gene. The SV-40 intron and poly A sequences are present to ensure proper transcript processing. (Taken from the 1999 Promega Catalog).
REFERENCES


Gangji, V., Rydziel, S., Gabbitas, B., and Canalis, E., “Insulin-Like Growth Factor II
Promoter Expression in Cultured Rodent Osteoblasts and Adult Rat Bone,”

Factor Promote Receptor-mediated Chemotaxis in Osteoblasts through Different

Convection on Osteoblastic Cell Growth and Function in Biodegradable Polymer


Mechanical Strain with Increased Bone Matrix Protein Production Independent of

Cell-Cell Interactions Mediate Endothelial Cell-induced Recruitment of 10T1/2
Cells and Their Differentiation to a Smooth Muscle Fate,” J Cell Biol 141, 805-14


