Surface Microtopography Modulation of Biomaterials for Bone Tissue Engineering Applications

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SURFACE MICROTOPOGRAPHY MODULATION
OF BIOMATERIALS
FOR BONE TISSUE ENGINEERING APPLICATIONS

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This work is dedicated to my best friend, my mother, for giving me the strength, support, and unconditional love to complete this.
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SURFACE MICROTOPOGRAPHY MODULATION OF BIOMATERIALS FOR
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ABSTRACT
The ultimate goal of this project is to develop a biodegradable and implantable scaffold with precise surface topographies that can provide osteoconductive stimuli to connective tissue progenitor cells (CTPs), and subsequently, enhance bone regeneration applications without the complications of autogenous cancellous bone grafts. This dissertation presents the modification of surface microtextures to provide osteoconductive stimuli to CTPs for bone regeneration applications. First, the effect of surface topography on cell proliferation and osteogenic differentiation was validated through experiments using surface post microtextures and CTPs. Post microtextures accelerated CTP growth behaviors compared to smooth polydimethylsiloxane (PDMS) and standard cell culture dishes. Second, soft lithographic techniques were used to develop PDMS post microtextures with varying geometry and arrangement. 10 µm diameter post microtextures with various inter-spaces (5, 10, 20, and 40 µm) and post heights (5, 10, 20, and 40 µm) were developed, and cultured with CTPs to establish optimal and precise surface post microtextures that can provide CTPs with an osteoconductive environment. Cells on post microtextures with 10 µm height and 10 µm inter-space exhibited higher cell number than other micro-posts with different
heights or inter-spaces, and smooth surfaces. The results demonstrate a significant response of CTPs to topography, and suggest a practical role for optimal post size on textured materials in modifying CTP behavior. Third, substrate stiffness of various PDMS formulations was analyzed to investigate the effects on morphology, proliferation, and osteogenic differentiation of CTPs. Stiffer PDMS substrates with surface microtextures provided an enhanced osteoconductive microenvironment to CTPs relative to softer PDMS substrates. Finally, soft lithography techniques were successfully applied to biodegradable materials, including cellulose acetate (CA) and poly lactic-co-glycolic acid (PLGA). More specifically, CTPs on CA and PLGA post microtextures exhibited accelerated cell proliferation rates compared to those on PDMS post microtextures.
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Figure 6.1
Alternate biomaterials such as cellulose acetate (CA), poly octanediol citrate (POC), poly lactic-co-glycolic acid (PLGA), and cyclic acetal monomer-5-ethyl-5-dimethyl-1,3-dioxane-2-ethanol diacrylate (EH-Network) are potential candidates for bone tissue engineering applications.

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changes for 30 days.
CHAPTER I

INTRODUCTION

In suboptimal bone fracture repair conditions where attachment, migration, proliferation, and differentiation of bone forming cells is hindered, bone grafts play an important role in promoting an environment in which bone formation is enhanced. Autogenous cancellous bone is widely considered to be the most successful graft material, and is used in ~50% of the bone grafting procedures performed annually in the United States. However, autografts bring a number of related complications such as prolonged rehabilitation and infection risk. Consequently, there is a need for a desirable alternative that can provide the bone forming properties of autografts while avoiding its related complications. The current state-of-the-art approach to enhance bone regeneration has been the transplantation of adult stem cells derived from bone marrow onto an implantable scaffold. These connective tissue progenitor cells (CTPs) represent a small population (~1:20,000 cells) in normal bone marrow, and the quantity of these cells in early fracture repair is suboptimal. Therefore, an optimal microenvironment is essential
to stimulate a large number of progenitor cells by allowing them to migrate and proliferate on the scaffold.

The scaffold surface should allow transplanted cells to retain their differentiated function and promote cell growth. Recently, surface topography has been shown to affect and selectively stimulate cell behavior such as attachment, orientation, migration, proliferation, gene expression, protein production, and differentiation on a variety of cells \textit{in vitro} (Table 1.1). Therefore, the incorporation of engineered surface topography at the cell-scaffold interface might provide an attractive approach to selectively enhance specific and desirable CTP behavior without destabilizing the optimum biochemical conditions during bone fracture healing.

Numerous research groups have focused on polydimethylsiloxane (PDMS) as the material of choice for producing surfaces with microtopography. PDMS is chemically inert, simple to handle and manipulate, and exhibits homogeneous properties as well as relatively low cost, and can conform to molds to produced precisely defined microstructures. However, it is not biodegradable, and therefore, not a suitable material for bone tissue engineering applications. Consequently, strategies are required to translate the PDMS-based approaches to other candidate biodegradable materials, such as cellulose acetate (CA), poly 1,8-octanediol-\textit{co}-citric acid (POC), poly lactic-co-glycolic acid (PLGA) and cyclic acetal monomer-5-ethyl-5-dimethyl-1,3-dioxane-2-ethanol diacrylate (EH-Network).

The ultimate goal of the research project is to develop a biodegradable and implantable scaffold that can modulate CTP growth characteristics, and subsequently, enhance bone regeneration without the complications of autogenous cancellous bone
grafts. The immediate objective of this dissertation research is to evaluate the hypothesis that biocompatible and biodegradable scaffolds can be patterned with surface microtopographies, which, in turn, can modulate CTP growth characteristics, and subsequently, enhance bone regeneration. The underlying hypothesis is that surface microtextures can selectively stimulate CTP behavior.

1.1. SPECIFIC AIMS

Specific aim 1:

**Investigate enhancement in CTP growth characteristics by varying geometry and arrangement of surface microtextures.**

The PDMS post microtextures with varying height and inter-space parameters will be selected and fabricated using soft lithography techniques. Consequently, the CTP growth characteristics, such as attachment, proliferation, and osteogenic differentiation, by varying geometry and arrangement of surface post microtextures will be investigated. The optimal size of geometry and arrangement of surface microtextures will be used to complement current microfabrication related techniques to fabricate microstructures and enhance their effect on cell growth.

Specific aim 2:

**Investigate CTP behavior on PDMS substrate with varying stiffness.**

This relationship between surface microtextures and varying stiffness levels on CTP growth behavior will be explored for potential application to bone tissue engineering scaffolds. PDMS substrates with optimal surface microtextures and stiffness that can
provide CTPs with an osteoconductive environment will be developed using soft lithography. CTP growth characteristics, such as proliferation and osteogenic differentiation, on PDMS surface post microtextures with varying stiffness levels will be investigated.

Specific aim 3:
Explore the feasibility of microtextured biodegradable materials as an alternative to PDMS.

The need for a biocompatible and biodegradable material as a substitute for PDMS motivates an examination of alternate scaffold biomaterials such as CA, PLGA, POC and EH-Network. Biocompatible and biodegradable materials that can provide CTPs with an osteoconductive environment similar to that provided by autogenous bone grafts will be selected and developed surface microtopographies. CTP behaviors, such as proliferation and osteogenic differentiation, on biodegradable materials comprised of surface microtopographies will be experimentally determined and compared.

1.2 OUTLINE
This dissertation presents the development of a biodegradable and implantable scaffold with precise surface topographies that can provide osteoconductive stimuli to CTPs, and subsequently, enhance bone regeneration applications as a replacement for that of autogenous cancellous bone grafts. In order to achieve this goal, various engineering and biology-related parameters are considered. First, the effect of surface topography on human bone marrow derived CTP behavior is investigated for long term
cultivation. This information is important to confirm the potential of surface microtextures to stimulate and sustain specific and desirable cell behavior on later stages of cell growth characteristics that are critical to bone tissue regeneration. Next, topographical and mechanical properties of PDMS are characterized followed by the comparison of CTP growth behavior. This work is essential to guide scaffold design and enable the acceleration of osteogenesis of progenitor cells in scaffolds with an optimal microenvironment. Finally, the effect of biodegradable materials relevant to bone tissue engineering applications is explored. In vitro culture experiments with human CTPs are performed to compare the effect of the biodegradable substrates on these cells’ behavior.

In the first part of this dissertation, Chapter II introduces the fundamental concepts that are the cornerstones of this work. In addition, it provides the background information that defines the problem, identifies the essential parameters of the problem, and motivates possible solutions. The second part of this dissertation presents investigations into the effect of microarchitecture and surface microtextures on the behavior of CTPs. Chapter III presents the effect of surface post microtextures on human CTP behavior. First, soft lithography techniques are used to create surface post microtextures on PDMS substrates. Freshly harvested human CTPs are then cultured on microtextured surfaces and smooth PDMS to investigate their effect on cell attachment, proliferation, and osteogenic differentiation for up to 60 days. The chemical inertness of PDMS permits the isolation of surface microtexture effects on cell behavior from those due to surface chemistry. Chapter IV presents the fabrication of the SU-8 photoresist molds to develop post microtextures with varying height and inter-space parameters. In this chapter, protocols for designing masks with varying inter-space of post microtextures
and controlling thickness of SU-8 photoresist using spin coat process are established. CTPs are cultured on these post microtextures with varying geometry and arrangement and their cell growth characteristics compared. Chapter V investigates the effect of stiffness of post microtextures on CTP growth characteristics. PDMS is used to fabricate the scaffolds for tissue engineering because, in addition to its chemical inertness, it is biocompatible, amenable to microtexturing, relatively low cost, and widely used within the biomedical microelectromechanical systems (BioMEMS) community. First, various formulations of PDMS are mixed and analyzed using tensile testing to determine changes in mechanical properties produced by the varying the weight ratios of PDMS components. Then, PDMS post microtextures and smooth PDMS surfaces with various weight ratios are used to culture CTPs and compare their growth characteristics. Chapter VI presents the microfabrication of biocompatible and biodegradable materials as a substitute for PDMS. First, CA and POC are poured onto a suitable patterned SU-8 mold and other biodegradable materials, PLGA and EH-Network, are poured onto a similar patterned PDMS mold. PDMS will be used as the mold instead of SU-8 to facilitate easier de-molding of the materials. Next, human CTPs are cultured on both smooth surfaces and post microtextures of biodegradable scaffolds, and cell growth characteristics are analyzed. Finally, Chapter VII presents conclusions drawn from the technical work presented in this dissertation as well as a discussion of limitations. Ideas and strategies are also offered for the future direction of the project.
Table 1.1 Effect of surface microtexture on cells

<table>
<thead>
<tr>
<th>Reference</th>
<th>Shape</th>
<th>Diameter</th>
<th>Height</th>
<th>Inter-space</th>
<th>Investigation/Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steinberg T</td>
<td>Pillar</td>
<td>5 μm</td>
<td>15 μm</td>
<td>8, 11, 14 μm</td>
<td>Early keratinocyte differentiation on micropillar interfaces</td>
</tr>
<tr>
<td>Lemmon A</td>
<td>posts</td>
<td>3 μm</td>
<td>11 μm</td>
<td>9 μm</td>
<td>Shear force at the cell-matrix interface: Enhanced analysis for microfabricated post array detectors</td>
</tr>
<tr>
<td>Tan J</td>
<td>posts</td>
<td>3 μm</td>
<td>11 μm</td>
<td>6 μm</td>
<td>Cells lying on a bed of microneedles: an approach to isolate mechanical force</td>
</tr>
<tr>
<td>Tymchenko N</td>
<td>Pillar</td>
<td>2 μm</td>
<td>51 μm</td>
<td>6, 8 μm</td>
<td>A novel cell force sensor for quantification of traction during cell spreading and contact guidance</td>
</tr>
<tr>
<td>Deutsch J</td>
<td>pegs</td>
<td>10 μm</td>
<td>5 μm</td>
<td>10 μm</td>
<td>Fabrication of microtextured membranes for cardiac myocyte attachment and orientation</td>
</tr>
<tr>
<td>Craighead G</td>
<td>column</td>
<td>0.5 μm</td>
<td>1-2.7 μm</td>
<td>1 μm</td>
<td>Chemical and topographical surface modification for control of central nervous system cell adhesion</td>
</tr>
<tr>
<td>Berry CC</td>
<td>pits</td>
<td>7, 15, 25 μm</td>
<td>4.8 μm</td>
<td>20, 40 μm</td>
<td>The influence of micro-scale topography on fibroblast attachment and motility</td>
</tr>
<tr>
<td>Tan J</td>
<td>Pillars/holes</td>
<td>2 μm</td>
<td>4 μm</td>
<td>10 μm</td>
<td>Controlling human polymorphonuclear motility using microfabrication technology</td>
</tr>
<tr>
<td>Tan J</td>
<td>Holes</td>
<td>2 x 2 μm</td>
<td>210 nm</td>
<td>6, 10, 14 μm</td>
<td>Micron-scale positioning of features influences the rate of polymorphonuclear leukocyte migration</td>
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<td>Milner K</td>
<td>pillar</td>
<td>400, 700 nm</td>
<td>1 μm</td>
<td>400, 700 nm</td>
<td>Submicron poly(L-lactic acid) pillars affect fibroblast adhesion and proliferation</td>
</tr>
<tr>
<td>Li B</td>
<td>posts</td>
<td>2 μm</td>
<td>6 μm</td>
<td>2 μm</td>
<td>Development of micropost force sensor array with culture experiments for determination of cell traction forces</td>
</tr>
<tr>
<td>Mata A</td>
<td>posts</td>
<td>5, 10, 20, 40 μm</td>
<td>6 μm</td>
<td>5, 10, 20, 40 μm</td>
<td>Growth of connective tissue progenitor cells on microtextured polydimethylsiloxane surfaces</td>
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<td>Fewster D</td>
<td>Pillars</td>
<td>1, 5, 10, 50 μm</td>
<td>2, 5, 10 μm</td>
<td>5 μm</td>
<td>Precise ultrafine surface texturing of implant materials to improve cellular adhesion and biocompatibility</td>
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<td>Green AM</td>
<td>Pillars/wells</td>
<td>5 μm</td>
<td>5 μm</td>
<td>5 μm</td>
<td>Fibroblast response to microtextured silicone surfaces: Texture orientation into or out of the surface</td>
</tr>
<tr>
<td>Lim JM</td>
<td>Pillar</td>
<td>5 μm</td>
<td>5 μm</td>
<td>5 μm</td>
<td>Retinal pigment epithelial cell behavior is modulated by alterations in focal cell–substrate contacts</td>
</tr>
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<td>Russo AP</td>
<td>Pillars</td>
<td>0.5-5.2 μm</td>
<td>6-20 μm</td>
<td>1-20 μm</td>
<td>Microfabricated plastic devices from silicon using soft intermediates</td>
</tr>
<tr>
<td>Turner AP</td>
<td>Pillars/wells</td>
<td>0.5-2.0 μm</td>
<td>0.5 μm</td>
<td>1 μm</td>
<td>Attachment of astroglial cells to microfabricated pillar arrays of different geometries</td>
</tr>
<tr>
<td>Desai SP</td>
<td>Pillars</td>
<td>15, 80 μm</td>
<td>20, 45 μm</td>
<td>1 μm</td>
<td>A photo patternable silicone for biological applications</td>
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<tr>
<td>Pirone M</td>
<td>Posts</td>
<td>3 μm</td>
<td>11 μm</td>
<td>6 μm</td>
<td>Strategies for engineering the adhesive microenvironment</td>
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</table>
CHAPTER II
BACKGROUND

Substantial portions of this Chapter have been published in Biomaterials 2009;30:4610-4617.

2.1 BONE TISSUE ENGINEERING

The field of tissue engineering combines cells and biomaterials to repair or replace physiological functions of diseased or damaged organs.\(^1\) In the last decade, there have been significant advances in tissue and organ replacement, which have led to the development of biological scaffolds that restore, maintain, or improve tissue formation.\(^2\) The scaffold organizes the cells on a microscopic level and provides cues to stimulate growth, migration, and differentiation. Although this process appears to be somewhat straightforward, complex fully functional organs have not yet been engineered. In order to meet the goal of engineering organs and improving organ function, further investigations into cell-material interactions are needed to determine what scaffold properties induce certain cellular behavior.
2.1.1 BONE GRAFTS

The ultimate goal of bone tissue engineering is to create a bone-healing response in a precise anatomic area where bone is desired. Bone grafting is a tissue engineering therapy that is increasingly used worldwide. It is a surgical procedure by which new bone or a replacement material is placed into spaces between or around bones to: (a) stimulate healing of fractures that have failed to heal; (b) promote healing between two bones across a diseased joint; (c) regenerate bone that is lost due to trauma, infection, or disease; and (d) improve bone formation around surgically implanted devices. In the United States, 74% of bone grafts are used for orthopedic procedures of the spine; 16% for trauma reconstruction, 6% for non-union fractures, and 4% for maxillofacial procedures. The bone graft itself is the overall implant that is used to stimulate bone healing in the different regions.

An ideal bone graft would provide three forms of stimulation that should lead to bone formation and ultimately determine the effectiveness of the implant. First, osteogenic stimulation requires the presence of sufficient bone forming cells at the grafting site. These cells can be either transplanted into the graft or arise from neighboring tissues. Second, osteoconductive stimulation refers to the ability of the graft to permit migration and attachment of osteoprogenitor cells from surrounding tissues into the grafting site. Third, osteoinductive stimulation refers to the biochemical bone forming stimuli of growth factors and cytokines to promote cells to migrate, proliferate, and differentiate into bone forming cells.

Bone graft materials are divided into autografts (tissue harvested from the host), allografts (tissue from donor of same species), xenografts (tissue from donor of different
species), and other synthetic or natural materials (e.g. hydroxyapatite and collagen)\textsuperscript{3,8}, of which allografts and autografts are more widely used.\textsuperscript{4} Currently, there are a number of allograft materials that surgeons can choose for bone-grafting procedures. These material choices include allograft products such as VertiGraft\textsuperscript{TM} (LifeNet, Virginia Beach, VA) and BioD\textsuperscript{TM} (Osteotech, Edison, NJ); demineralized bone matrix products such as Grafton\textsuperscript{®} (Musculoskeletal Transplant Foundation, Edison NJ), Dyna Graft\textsuperscript{TM} (Gensci Regeneration Laboratories, Irvine, CA), and Allomatrix\textsuperscript{TM} (Wright Medical Technology, Arlington, TN); or other bone substitute materials such as coralline and hydroxyapatite (Pro-Osteon\textsuperscript{®}, Interpore Cross, Irvine, CA) and collagen with hydroxyapatite and tricalcium phosphate (Collagraft\textsuperscript{®}, Zimmer, Warsaw, IN).\textsuperscript{4} Allografts are used in ~43\% of the bone graft procedures in the United States\textsuperscript{4}, but exhibit a higher degree of immune reaction and lower osteogenic properties than autografts.

Cancellous bone autografts are still considered to be the most effective bone graft because they provide the best combination of osteoactive and low-immunorejection characteristics than the other alternatives,\textsuperscript{9,10} and have been shown to achieve optimum skeletal incorporation in bone defects, and fracture repair.\textsuperscript{5,6,11} The three dimensional structure of cancellous bone provides a more porous material than cortical bone. Cancellous bone autografts are used in ~50\% of the 500,000 bone grafting procedures performed annually in the United States.\textsuperscript{4,6,11} The number of bone graft procedures using autogenous cancellous bone is predicted to rise as procedures requiring bone grafts continue to increase with ageing of the population.\textsuperscript{8} However, autografts bring a number of related problems such as blood loss, pain, prolonged surgical time, prolonged rehabilitation, increased exposure to blood products, and infection risk.\textsuperscript{1,5,6} Consequently,
there is a need for an alternative bone graft materials that contain a sufficient quantity of osteoblastic progenitor cells, and provide an effective osteoconductive scaffold that would enhance their migration, proliferation, and osteogenic differentiation (Figure 2.1).\textsuperscript{5,12}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_1.png}
\caption{Illustration of an alternative approach to enhance bone regeneration provided by transplantation of bone marrow derived cells with a 3D scaffold.\textsuperscript{12}}
\end{figure}

2.1.2 BONE TISSUE, CELLS AND EXTRACELLULAR MATRIX

Bones shape, support, and protect body structures. They also aid body movements, house tissues that produce blood cells, and store various inorganic salts. Bone tissues are complex structures made of cells, extracellular matrix (ECM) and intracellular signaling systems. Bone tissue is divided in cortical bone and cancellous bone. The composition of the ECM is the same in both types of bone, but the three-dimensional structure is different.\textsuperscript{6} Cortical bone consists of aligned osteons, which are concentric layers of matrix-embedded osteocytes around a central canal that encloses blood vessels. The
alignment of these osteons provides the high strength of cortical bone along the direction of the osteons. Cancellous bone does not comprise osteons and exhibits 3D structure similar to a sponge, which provides some shock absorbance properties. Nutrients reach osteocytes within cancellous bone by diffusion.

The maintenance of bone takes place through the interaction of three cell types: osteoblasts, osteocytes, and osteoclasts. Osteoblasts synthesize bone matrix and assist in elevating local concentrations of calcium phosphate. Osteocytes are matured osteoblasts that are entrapped within the produced matrix and maintain bone tissue. These cells also sense changes in the ECM and respond by recycling calcium within the ECM. Osteoclasts resorb bone matrix that is replaced with new matrix laid down by osteoblasts. The interaction of all three cell types is essential for maintaining a healthy bone tissue.

The ECM provides the mechanical stability and signaling to create an environment in which cells can develop into bone tissues. The ECM is a complex network of proteins and polysaccharides that are secreted locally and organized into a meshwork by the cells that are in close association with it. The ECM provides the substrate upon which cells attach and determines the physical properties of bone tissue. The relative amounts and types of matrix macromolecules and their organization gives rise to many forms of the matrix, each adapted to the functional requirements of a specific tissue. The ECM is comprised of several classes of macromolecules and two of them are: (1) polysaccharide chains of the class called glycosaminoglycans (GAGs), and (2) fibrous proteins. Polysaccharide occupies most of the extracellular space and provides the compressive mechanical support to the tissue,
and fibrous proteins provide either more structural support or play an adhesive role. The majority of fibrous proteins in the ECM are collagens (e.g., type I, II, IV, and V). The relative amount and type of collagen is determined by tissue demands. For example, collagen type I (Col I) is the main fibrous protein in the ECM of bone. The rope-like structure of this collagen provides a structural framework upon which the cells can organize and tensile strength to the tissue.\textsuperscript{11,13}

ECM molecules interact with cell surface receptors which transmit signals across the cell membrane to molecules in the cytoplasm. A cascade of signaling events transfer the signal through the cytoskeleton into the cell nucleus. As a result, the expression of specific genes is altered and various proteins are produced which can affect the ECM.\textsuperscript{10,11,14} Integrins are a key adhesive proteins that function as transmembrane linkers mediating communication between the ECM and the cytoskeleton as well as functioning as signal transducers.\textsuperscript{13,15,16} The integrin-mediated attachments affect cell behavior such as adhesion, migration, proliferation, and differentiation.\textsuperscript{10,17} Integrin $\alpha$1$\beta$1 and $\alpha$2$\beta$1 bind to Col I, which is the dominant bone matrix protein, and this binding has been reported to regulate osteoblastic differentiation.\textsuperscript{10} Binding of integrin $\alpha$5$\beta$1 to ECM is known to regulate osteoblast survival, proliferation, bone specific-gene expression, and matrix mineralization.\textsuperscript{11}

Osteoblastic differentiation is particularly relevant to clinical bone repair strategies. Figure 2.2 illustrates a generally accepted pattern of gene expression that reflects three major periods of cell and tissue development in bone formation.\textsuperscript{11,14} The differentiated osteoblast is typically characterized by a specific pattern of gene expression such as alkaline phosphatase (AP), collagen, osteocalcin (OC), and \textit{in vitro} mineralization.
capacity.\textsuperscript{11,14,18} At the early stage, osteoblastic progenitor cells synthesize significant levels of Col I to support matrix formation. Although Col I levels are highest during the proliferation stage, matrix deposition continues to increase throughout the entire culture period. The termination of growth and accumulated ECM accelerates upregulation of AP, an early stage marker of osteogenesis. OC is expressed towards the end of the proliferation stage during development of the osteoblast phenotype and reaches peak levels during mineralization.

\textbf{Figure 2.2} Relationship between cell growth and differentiation-related gene expression during the \textit{in vitro} cultivation of osteoblastic progenitor cells.\textsuperscript{11,14}

\subsection*{2.1.3 CONNECTIVE TISSUE PROGENITOR CELLS (CTPs)}

A novel approach to enhance bone regeneration provided by transplantation of bone marrow derived cells involves rapid concentration and selection of the osteoblastic progenitor population in the graft using selective attachment to the matrix surface. Concentration of these cells known as CTPs has recently been demonstrated to improve graft efficacy.\textsuperscript{19,20} CTPs are acknowledged to be derived from a small pool of
undifferentiated, resting, and pluripotent connective tissue stem cells, which are activated by some signal to leave their normal resting state, migrate into the fracture or diseased site, divide, and eventually differentiate into osteoblasts.\textsuperscript{9,19} These cells are present in small numbers in bone marrow, periosteum, bone trabecula, and within haversian canals of cortical bone, adipose tissue, and muscle.\textsuperscript{19-21} A characteristic of marrow derived CTPs is their ability to give rise to progeny that are capable of differentiating along a number of mesenchymal lineages including bone, cartilage, muscle, and fat.\textsuperscript{20}

**Figure 2.3** Bone grafting surgical procedure developed by Dr. George Muschler at the Cleveland Clinic consisting of aspiration of patient’s bone marrow (usually from the iliac crest) (a); CTP concentration using the Cellect\textsuperscript{®} system (Depuy Acromed, Raynham, MA) (b); loading of CTPs onto a 3D scaffold (normally collagen matrix, allograft, or bone chips) (c, d); and implantation of the CTP loaded scaffold into the fracture site (e, f).\textsuperscript{20-22}

Recently, Muschler et al.\textsuperscript{22} have developed a patented method for selection, concentration, and delivery of CTPs into a 3D scaffold for use within the operating room.
during bone graft procedures. They have identified an optimum bone marrow aspirate volume to increase CTP concentration, as well as correlations between sex, age, and CTP count. This group has also reported animal testing results that demonstrate an improved quality of bone fracture union (spinal fusion) when CTP concentration was increased, as well as similar bone growth characteristics as those of an autograft cancellous bone model. The current surgical procedure used by this group consists of: (a) aspirating bone marrow from the patient (usually from the iliac crest); (b,c,d) concentration and loading of CTPs into a 3D scaffold; and (e,f) implantation of the CTP loaded scaffold into the fracture site (Figure 2.3).

During normal bone fracture healing, CTPs give rise to osteoblasts that form the new bone matrix. However, it has been experimentally shown that they represent a small population (~1:20,000 cells) in normal bone marrow, and the abundance of these cells in early fracture repair is suboptimal during bone fracture repair. This fact is especially true in older patients and women after menopause. Therefore, an optimal microenvironment within the scaffold is crucial to enhance CTP numbers by allowing them to migrate into the fracture site, proliferate, and differentiate into osteoblasts.

### 2.1.4 CTPS AND MESENCHYMAL STEM CELLS (MSCS)

Bone and bone marrow tissues are a particularly abundant reservoir of CTPs and mesenchymal stem cells (MSCs), and often used in therapeutic applications. Like CTPs, bone marrow derived MSCs are multi-potent stem cells that are capable of proliferating to produce progeny that can differentiate into one or more connective tissue phenotypes (bone, cartilage, adipose, muscle, blood etc.). According to Dr. Muschler’s
group, a MSC is a culture expanded cell that has the advantage of being far more homogeneous than a primary CTP cell, resulting in experiments less burdened with batch to batch and inter-subject variation. However, culture expanded populations change rapidly in vitro and take on a phenotype that is different than the founding CTP.

Recently, the proliferation rate of CTPs and MSCs on PDMS substrates with both post microtextures with 10 μm diameter, 6 μm height and 10 μm inter-spaces, and smooth surfaces was compared. CTP and MSC cultures from all three donors were expanded for up to 30 days (Figure 2.4). The number of both types of cells initially attached (Day 1) on the PDMS substrates were almost identical (Figure 2.4(a)). MSCs on both surfaces exhibited log phase growth, and there were more cells on PDMS post microtextures than smooth surfaces throughout the culture period. Furthermore, MSC numbers were higher compared with those of CTPs for 30 days (Figure 2.4(b)). In contrast, CTPs on both surfaces exhibited both lag and log phase growth. There were minimal changes in daily CTP numbers for both substrates through day 4. On day 5, CTPs on PDMS post microtextures transitioned to the log phase as demonstrated by a 3.6-fold increase in cell number. The lag phase of CTPs on smooth surfaces lasted longer, with a 2.1-fold increase in cell number occurring on day 7 (Figure 2.4(a)). After exiting from the lag phase, the rate of increase in cell number in the log phase was similar for both PDMS post microtextures and smooth surfaces (Figure 2.4(b)). From this study, the increased number in expanded MSC was greater compared with those of primary CTP throughout the culture period because MSC entered directly into log phase without lag phase.
Figure 2.4  CTP and MSC cultures from all three donors were expanded for up to 10 days (a) and 30 days (b). The proliferation rate of CTPs and MSCs on PDMS substrates with post microtextures (10 μm diameter and 6 μm high posts) and smooth surfaces was compared.
2.2 SCAFFOLDS

2.2.1 CELL-SURFACE INTERACTION

Cell adhesion is critical to ensure subsequent migration, proliferation, and differentiation into osteoblasts, which would populate the scaffold and ultimately form bone tissue. Cell adhesion is primarily determined by the interaction of cell membrane receptors with specific ECM proteins.\(^\text{24}\) The presence of an adhesive ECM protein, either contributed from an exogenous source from culture media or produced by the cell itself, is a prerequisite for the formation of cell-matrix contacts (focal adhesions).\(^\text{25}\) Classic examples of these types of proteins are fibronectin and Col I, both of which have arginine-glycine-aspartic acid (RGD) sequences that bind to specific cell surface integrin receptor complexes.\(^\text{11}\) These high affinity interactions allow cells to anchor to their substrate through the focal adhesions, and influence subsequent cellular behavior.\(^\text{11,25}\)

The first step after exposure of any biomaterial to a biological environment results in the rapid adsorption of proteins to its surface.\(^\text{25}\) The composition, type, amount, and conformation of adsorbed proteins regulate the secondary phenomena such as cellular adherence, and also, the cellular reactions, such as migration, proliferation, and differentiation. The potency for a biomaterial to adsorb proteins is influenced by its physiochemical characteristics such as surface energy or hydrophobicity, and is also dependent on the local environment (pH, composition and functional groups of proteins, temperature) (Figure 2.5).\(^\text{13-15}\)
Recent studies\textsuperscript{13-15} investigating the response of adherent cells to scaffold surfaces indicate that different cell phenotypes have different levels of sensitivities. Moreover, the qualitative and quantitative kinetics in gene and protein expression is strongly influenced by physiochemistry of a defined surface. There is evidence that surfaces of biomaterials such as hydroxyapatite can activate early intracellular signaling pathways as shown by expression of relevant molecules such as $\alpha$- and $\beta$1-integrin, focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK) followed by c-jun and c-fos genes for proliferation and AP for differentiation.\textsuperscript{14,26,27}
2.2.2 SURFACE TOPOGRAPHY

Investigations of cell-substrate interactions have been undertaken to determine which surface properties can elicit specific cell responses in order to optimize the scaffold for its particular application. It is well known that mechanical loading such as shear, compression, and stretch forces affect cell behavior (Table 2.1). Like mechanical loading, physical stimuli provided by the scaffold architecture and surface textures (Table 1.1) play a crucial role in stimulating cell growth and directing cells to produce bone tissue in a desirable manner.

Surface topography, such as roughness and microtextures, have been extensively investigated and identified as one of the classic factors that can control cell behavior on a surface.\textsuperscript{28-30} Both surface roughness and microtextures have been reported to affect cell attachment, orientation, migration, proliferation, gene expression, protein production, protein deposition, and differentiation on a variety of cells such as CTPs, chondrocytes, fibroblasts, osteocytes, osteoblasts, macrophages, leucocytes, lymphocytes, neurons, and myoblasts \textit{in vitro}.\textsuperscript{29,30} Surface roughness may be defined as surface topography with random size and distribution, while surface microtextures are defined as topography with defined dimensions and distribution. There are a number of technologies available for the fabrication of surface topographies.\textsuperscript{29-35}
### Table 2.1 *In vitro* model systems used in the investigation of mechanical loading on bone generation

<table>
<thead>
<tr>
<th>Reference</th>
<th>Investigation/Results</th>
</tr>
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<tr>
<td><strong>1. Fluid shear</strong></td>
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<td>Forwood M[37]</td>
<td>Inducible cyclo-oxygenase (cox-2) mediates the induction of bone formation by mechanical loading <em>in vivo</em></td>
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<tr>
<td>Koslow A[37]</td>
<td>A flow system for the study of shear forces upon cultured endothelial cells</td>
</tr>
<tr>
<td>Li Y[38]</td>
<td>The Ras-JNK pathway is involved in shear-induced gene expression</td>
</tr>
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The generation of surface roughness at the cellular level influences cell behavior. In the early 1912, the work of Harisson demonstrated the growth of embryonic cells on spider webs. Since then, other techniques to achieve surface roughness for cell behavior studies have included mechanical polishing, sandblasting, and grit blasting (Figure 2.6 a-c). Published literature suggests that roughened surfaces in the dimensions of 1–50 μm show better early adhesion of cells, increased osteoblastic function and functional gene expression, reduced fibrous encapsulation, and enhanced integration of subcutaneous implants, compared with smooth surface and grooved surfaces. However, a consensus on what geometry and magnitude of surface roughness is ideal for different cell types has not been established.
One potential reason for the discrepancy is the lack of precisely quantified surface topographies. In general, only qualitative descriptions of the surface based on scanning electron and optical micrographs are reported, and therefore, it is not possible to compare surfaces accurately from study to study. Secondly, in most investigations there is no precise range of roughness reported; only comparison between smooth and rough surfaces, which can be orders of magnitude different. Thus, a more systematic approach to surface roughness investigations would provide scientists with more definitive insight into the effect of surface roughness on cell behavior.

With technological advancements, newer processes with higher precision capabilities were created and applied to the study of cell-surface topography interactions. Micromachining, the process used to produce silicon microelectronic components, was one of the first techniques employed to produce surfaces with tightly controlled surface parameters with high reproducibility and is still used frequently today. In the 1980’s, microfabrication related techniques were first used to create surfaces with more precise topographies for cell studies. These techniques revolutionized the study of cell-surface interactions by providing micron-size textures (microtextures) with defined dimensions and distributions (Figure 2.6 d,e).\textsuperscript{38,39}

Microelectromechanical systems (MEMS) technology has existed for more than 30 years. However, it is only within the last fifteen years that applications in biology have emerged.\textsuperscript{46} Although initial exploration of these technologies for biomedical applications had been focused on the development of diagnostic tools such as electrophoretic, chromatographic, and cell micromanipulation systems, recent investigations have increasingly concentrated on studying cellular behavior.\textsuperscript{89}
Microfabrication and micromachining have been a key factor in the rapid progress of MEMS. These techniques can provide cheaper, faster and more precise processing of scaffolds.\textsuperscript{89,90} Photolithography is the workhorse of microfabrication\textsuperscript{91} and the key process in the development of microtextures for the study of cell behavior \textit{in vitro}. This technique allows the fabrication of features that closely parallel the size scale of living cells, and therefore may be exploited to provide tissue engineering scaffolds that possess topographical, spatial, and chemical properties to optimize control over cell behavior.\textsuperscript{90} However, the development of these applications through established MEMS manufacturing approaches is hindered by inherent characteristics of traditional silicon-based processes such as expensive equipment, limited access to clean room environments, and limited 3D fabrication capabilities.\textsuperscript{89}

Some of the new microfabrication and micromachining techniques provide cheaper and faster processing of biocompatible polymers.\textsuperscript{72,89,92,93} Polymers are particularly attractive to the biomedical microelectromechanical systems (BioMEMS) community because of their lower cost compared to silicon and glass, as well as the practicability for fabrication with rapid prototyping techniques.\textsuperscript{89} Lately, numerous research groups have focused on polydimethylsiloxane (PDMS) as the material of choice for manufacturing surfaces with precise microtextures. PDMS is chemically inert, thermally stable, permeable to gases, simple to handle and manipulate exhibits isotropic and homogeneous properties as well as lower cost than silicon, and can conform to sub 0.1 \textmu m features to develop microstructures. In addition, it is transparent, non-fluorescent, biocompatible and nontoxic, and has been traditionally used as a biomaterial in catheters,
drainage tubing, insulation for pace makers, membrane oxygenators, and ear and nose implants.\textsuperscript{72,92,93}

The use of PDMS has been particularly driven by the development of Soft Lithography techniques such as micro-contact printing (\(\mu\)CP), replica molding (REM), micro-transfer molding (\(\mu\)TM), micromolding in capillaries (MIMIC), and solvent-assisted micromolding (SAMIM).\textsuperscript{92} All these processes utilize an elastomeric stamp or mold that incorporates microstructures, usually created out of PDMS, that transfers micropatterns onto a subsequent substrate.\textsuperscript{92} Soft Lithography readily enables the fabrication of surface microtextures on 2D substrates since photolithography is used to define mold geometry. Consequently, this technique has had a tremendous impact on the study of cell behavior by providing a highly reproducible, controlled, and precise scaffold surface topography. However, investigations of cells on 2D substrates have little resemblance to the majority of 3D tissues in the body.\textsuperscript{72,93} Therefore, a strategy that would allow the design of 3D scaffolds with microarchitectures and surface microtextures optimized to maximize osteoconductive stimuli and subsequent bone formation is highly desirable.
2.2.3. MICROARCHITECTURE

Microarchitecture is defined the micro-size architecture (porosity, pore size and geometry) of 3D scaffolds while microtexture is surface topography with defined dimensions and distribution. Ideal bone scaffold microarchitecture should be highly porous, exhibit a high surface area to volume ration, and allow high rates of mass transfer cell ingrowth and vascularization.\textsuperscript{94} The scaffold microarchitecture significantly affects the development and function of specific tissues, by providing a 3D space that defines the spatial organization and nutritional conditions of cells.\textsuperscript{95} The 3D scaffold should stimulate CTPs osteoconductively by allowing them to migrate and proliferate within the scaffold and osteoinductively by differentiating them into bone forming cells.\textsuperscript{6} An ideal bone scaffold microarchitecture should be highly porous with ~200-900 μm diameter interconnected pores, and exhibit high surface area-to-volume ratio to allow high rates of mass transfer, and cell in-growth.\textsuperscript{95}

Various research groups have compared different microarchitectures that are achieved through traditional and novel scaffold fabrication methods.\textsuperscript{94,96,97} Traditional scaffold fabrication methods include techniques such as phase separation, fiber bonding, solvent casting and particulate leaching, freeze drying, melt molding, and electrospining.\textsuperscript{98,99} Although these techniques provide high porosity, they rely on processes such as solvent evaporation and phase separation conditions to determine the geometry and dimensions of the scaffold microarchitecture, and limited in their ability to provide well-controlled, precise, and reproducible microarchitectures.\textsuperscript{97} These limitations are important because it is well known that specific micro- and macro-scale features
within a 3D scaffold have important effects on multicellular structures that are required for complex tissue function.\textsuperscript{94,96}

More recently, novel techniques have been developed based on rapid prototyping or solid free-form fabrication to fabricate microarchitectures with more precision and reproducibility.\textsuperscript{97} Some of the most currently popular techniques include fused deposition modeling (FDM), selective laser sintering (SLS), 3D printing (3-DP), and microstereolithography (\(\mu\text{SL}\)).\textsuperscript{6,94,100} These techniques form 3D objects through layer-by-layer construction via the processing of solid sheet, liquid, or powder material stocks. Other advantages of these techniques include the potential for customized design for patient specific data, computer-controlled fabrication, anisotropic scaffold microarchitecture, and room temperature processing. Of the various novel processing techniques, 3-DP has been possibly the most successful in the manufacturing of bone scaffolds. This method was originally developed at Massachusetts Institute of Technology and consists of using a printer head to print a liquid binder onto a thin layer of powder following a computer assisted design file.\textsuperscript{6} The subsequent stacking of printed powder recreates the full 3D scaffold. This technique is currently used by Therics, Inc. (Princeton, NJ) for commercial production of scaffolds for bone graft applications with \(~100 \mu\text{m}\) resolution.\textsuperscript{101,102}

Another novel approach for scaffold manufacturing has been to use the miniaturization and reproducibility characteristics of microfabrication related techniques to realize precise and reproducible scaffold microarchitectures.\textsuperscript{89,103,104} This approach is still at an early stage, but has achieved promising results. Vozzi et al.\textsuperscript{103,104} have developed poly (DL-lactide-co-glycolide) (PLGA) scaffolds using a microsyringe, a
computer controlled micropositioner, and soft lithographic techniques including PDMS molds. Folch et al.\textsuperscript{89} have also developed scaffolds using a similar soft lithographic technique using PDMS as a mold and microfluidic molding and stacking methods.

Recently, an innovative technique to fabricate 3D scaffolds with both precise microarchitecture and surface microtextures designed to provide osteoconductive stimuli to CTPs and subsequent bone regeneration has been developed.\textsuperscript{105} This process allowed the fabrication of a 3D PDMS texture scaffold with 66% porosity by volume that consisted of 300 \(\mu\)m diameter meandering vertical pores, 200 \(\mu\)m X 400 \(\mu\)m horizontal pores, and 71\% of the surfaces within the scaffold covered with 10 \(\mu\)m diameter and 10 \(\mu\)m high posts (Figure 2.7). The scaffold microarchitecture allowed cells to migrate in three dimensions, while 10 \(\mu\)m diameter posts increased the cell number on 3D PDMS Texture scaffolds compared to 3D PDMS Smooth scaffolds (Figure 2.8). These results support the scaffold microarchitecture and surface microtextures can significantly influence bone tissue engineering. Consequently, the advantage of using these engineered 3D scaffolds with optimum microarchitecture and surface microtextures could lead to the production of synthetic bone scaffolds with a higher degree of osteoconduction than currently possible.
Figure 2.7  SEM images show (a) the resulting SU-8 mold with a cross-section (inset), and (b) the dual-sided molded PDMS layer with 300 µm diameter and 100 µm deep through holes, 200 µm diameter and 200 µm high columns, and 10 µm diameter and 10 µm high posts on both sides of the layer (inset). SEM images exemplify (c) a five-layer PDMS scaffold on a penny, (d) a closer view of the cross-section showing the alignment between adjacent layers that resulted in a meandering pore geometry, and (e) 10 µm diameter and 10 µm high posts present on all horizontal surfaces. (f) Scaffold height was increased by adding more PDMS layers.\textsuperscript{105}
Tissue engineering scaffolds are designed to be a surrogate ECM onto which cells can be seeded to grow and organize into tissue. The scaffold should ultimately guide the cells into organizing themselves like the natural tissue and provide the cells with the necessary environments to proliferate and differentiate on the correct time frame. Material selection for scaffolds is extremely important. Scaffolds materials as well as their degradation products must be biocompatible, in that it does not elicit an inflammatory response or act as a toxin to the cells. Furthermore, scaffold materials should permit cell adhesion, promote cell growth, and allow retention of differentiated cell function. The materials should be capable of reproducible processing and exhibit sufficient mechanical strength. A number of different materials have been used as...
scaffolds from bio-polymers like collagen to hydroxyapatite, and come in a range of architectures from fiber matrices to foamed structures. While metals and ceramics have been quite successful, in the medical field especially in orthopedics, their amenability to wide range of processing conditions and methods is limited. As a result, polymeric materials have received the most attention in tissue engineering applications due to the ease of control over their processing.

Many polymer materials are known to be relatively biocompatible, which allows them to be used either as implant materials or for in vitro applications with a minimal damaging effect on the host tissue or cells. Polymers also have a wide range of properties with respect to bulk chemistry, surface chemistry, and mechanical and physical properties. This broad range of properties provides a large materials selection pool and allows materials to be chosen based on the appropriate behavior in a specific application. Polymer processing can offer some distinct advantages in minimizing both materials and processing costs. Many polymer materials have significantly lower cost relative to traditional materials used in microdevices. Much of the processing can also be performed outside of a cleanroom environment without expensive equipment, significantly reducing processing costs.

2.3.1 POLYDIMETHYLSILOXANE (PDMS)

PDMS is a silicone elastomer that has been used extensively in medical implants and biomedical devices because of its biocompatibility, low toxicity, and thermal stability. The practicability of PDMS for fabrication with both rapid prototyping and mass production techniques as well as lower cost relative to silicon and glass make it
particularly attractive for the development of bioMEMS.\textsuperscript{90,92,110} It is nontoxic, transparent, chemically inert, simple to handle and manipulate, exhibits isotropic and homogeneous properties, is less expensive than silicon, and can conform to submicron features to develop microstructures. In addition, PDMS has been used as a biomaterial in a number of bioMEMS applications including biosensors, tissue engineering scaffolds, cell sorting and analysis devices, and various microfluidic devices for biological applications.\textsuperscript{89,111}

The most widely used form of PDMS derives from a two part polymer: a pre-polymer and a cross-linker, and the different proportions of these components affect the stiffness properties of the PDMS.\textsuperscript{112-115} Recently, several groups\textsuperscript{114,115} have reported that alterations of stiffness can influence cell proliferation directly. These characteristics of PDMS, tunable stiffness and precise surface topographies, makes it feasible to investigate the synergetic or independent effects of substrate condition on cell behavior.

PDMS, however, is not a universally suitable biomaterial for all tissue engineering systems, because it is not biodegradable.\textsuperscript{116} The desire for a biocompatible and biodegradable material as a substitute for PDMS motivates an examination of alternate scaffold biomaterials such as cellulose acetate, poly octanediol citrate, poly lactic-co-glycolic acid, and polycaprolactone.

\subsection*{2.3.2 CELLULOSE ACETATE (CA)}

Cellulose acetate (CA), one of the cellulose derivatives, is not only a biocompatible and biodegradable material, but it lends itself to the molding of very fine (nano- and micro-scale structures) and intricate features.\textsuperscript{117} CA also known to be nontoxic, transparent, inexpensive, non-immunogenic and, upon biodegradation, yields
glucose as its the final product. CA has been reported to exhibit biocompatibility
and CA scaffolds used for cell growth were found to boost cell growth and help increase
their connectivity. Its cell adhesion properties are better than that of other polymeric
artificial scaffolds, and the very low water solubility of CA is an advantage during
scaffold fabrication.

In typical processing of CA, acetone is used as a solvent. Afterwards, the acetone is
evaporated and CA is then treated with NaOH or water. This deacetylation reaction
allows for deep penetration of water into CA and removes the remaining traces of acetone.

Several investigators have used CA or cellulose-based materials for tissue
eering studies and have shown that these materials are well suited for optimization
and control of cell adhesion. More specifically, CA scaffolds exhibit a favorable bony
response and rapidly formed initial ‘connective tissue’ around scaffolds. On a more
general level, it is conceivable that MEMS and related Soft Lithography techniques can
be used to produce CA scaffolds that incorporate precision topographical cues to
modulate cell growth for tissue engineering applications.

2.3.3 POLY (LACTIC-CO-GLYCOLIC ACID) (PLGA)

PLGA is a Food and Drug Administration (FDA) approved copolymer that is used
extensively as a host of therapeutic devices and scaffolds for engineering bone, cartilage
and the meniscus. PLGA has been successful as a biodegradable polymer because it
undergoes hydrolysis in the body to produce the original monomers, which are lactic acid
and glycolic acid. PLGA is synthesized by means of co-polymerization of two different
monomers: lactic acid and glycolic acid. Depending on the ratio of lactide to glycolide
used for the polymerization, different forms of PLGA can be obtained. For example, PLGA 75:25 identifies a copolymer whose composition is 75% lactic acid and 25% glycolic acid. It has been shown that the time required for degradation of PLGA is related to the monomers' ratio used in production.\textsuperscript{127,128}

PLGA is currently being investigated as biomaterials for tissue engineering applications, as resorbable sutures, as bone plates and screws, and in drug delivery devices.\textsuperscript{129-131} Many research groups have attempted to pattern surface topographies using PLGA. The reported surfaces include nanofibers, grooves, and textures of PLGA with irregular feature sizes.\textsuperscript{127,128}

### 2.3.4 POLY (1,8-OCTANEDIOL-CO-CITRIC ACID) (POC)

Poly (1,8-octanediol-co-citric acid) (POC), which is a crosslinker of equimolar amounts of citric acid and 1,8-octanediol, was reported to be a biodegradable and hydrophilic “cell-friendly” material for bone, blood vessel and cartilage tissue engineering.\textsuperscript{132-135} This material’s advantages appear to be that it forms from a non-toxic monomer via a relatively simple synthesis that can be carried out under mild conditions without addition of toxic catalysts or crosslinking reagents, controllable mechanical and biodegradation properties, easy processing, and inherent surface affinity for various cell types. POC has been shown to be compatible (i.e., cell adhesion, proliferation, and differentiation) with several cell types including pig endothelial cells, human osteoblasts, bovine chondrocytes, and bovine fibroblasts.\textsuperscript{133-135} Qiu H et al.\textsuperscript{135} reported the successful synthesis of POC–HA composites that were molded and machined into bone screws.
Compared to existing biodegradable elastomers in tissue engineering, POC is inexpensive and easy to synthesize and process. The presented biocompatibility of POC\textsuperscript{133-135} support the potential use of POC in tissue engineering applications as well as other clinical procedures that may require a biodegradable elastomeric implant.

2.3.5 CYCLIC ACETAL MONOMER-5-ETHYL-5-(HYDROXYMETHYL)-DIMETHYL-1,3-DIOXANE-2-ETHANOL DIACRYLATE (EH-NETWORK)

EH network is the hydrolytically degradable biomaterials based upon a cyclic acetal unit and it is made from the cyclic acetal monomer 5-ethyl-5-(hydroxymethyl)–\(\beta,\beta\)-dimethyl–1,3–dioxane–2–ethanol diacrylate. One of novel properties of EH network is that it lacks the ester groups present in many other biodegradable polymers and, therefore, EH produces less acidic byproducts as it degrades. Thus, premature scaffold degradation due to high acidity is prevented and the inflammatory response of skeletal muscle tissue surrounding the implant is decreased.\textsuperscript{136,137}

EH network is commercially available and reacted with the initiator benzoyl peroxide (BP) crosslink to form EHD networks, which present themselves as a rigid plastic material.\textsuperscript{136} It is known that concentration of BP initiator solution used during EH network fabrication alters the mechanical property of these networks, and thus, EH networks have a higher mechanical modulus compared to other polymeric materials, such as PDMS.\textsuperscript{137} It is reported that EH scaffold would be a favorable material to facilitate myoblast attachment and proliferation.\textsuperscript{136} In comparison to various available biomaterials, EH network appears to be a promising scaffold for tissue engineering due to its ease of fabrication and superior degradation properties.
CHAPTER III
EFFECT OF SURFACE POST MICROTEXTURES ON PROLIFERATION AND OSTEOGENESIS OF CTPS

Substantial portions of this Chapter have been published in Acta Biomaterialia 2010;6(1):160-169.

3.1 INTRODUCTION

The response of cells to a substrate surface is strongly influenced by its properties such as chemical composition and physical topography. These surface characteristics have important implications in the rational design and optimization of biological implants and bioreactors.\textsuperscript{109,110,138-140} Recent advances in MEMS (microelectromechanical systems) technology provide new opportunities for the investigation of a variety of biological phenomena.\textsuperscript{141} For example, soft lithography has been used to pattern the distribution of various chemistries on a material surface to explore selective cellular responses to specific biomolecular species.\textsuperscript{110} Microfabrication and related MEMS techniques also enable precise production of surface topographical features to investigate the effects of physical cues on cellular behavior.\textsuperscript{109,110,141,142} For tissue engineering applications, these
techniques could be conceptually combined with conventional scaffold processing strategies to ultimately provide scaffolds that possess precise topographical, spatial, and chemical properties to optimize control over cellular behavior.

Numerous studies have been conducted to show that different surface topographies influence bone cell behavior.\textsuperscript{68,109,110,139-144} For example, Hamilton et al.\textsuperscript{142} reported increased cell proliferation and enhanced osteoblast differentiation on discontinuous-edge surfaces compared to smooth ones; and Mata et al.\textsuperscript{68} reported an apparent increase in the proliferation of the progeny of bone marrow derived connective tissue progenitors (CTPs) on polydimethylsiloxane (PDMS) microposts compared to identical cells cultured on smooth PDMS surfaces. CTPs refer to a heterogeneous population of stem and progenitor cells that are resident in native tissue. These cells are capable of proliferating and giving rise to progeny, which contribute directly to the formation of one or more connective tissues.\textsuperscript{23} Harvest and transplantation, and even concentration, of CTPs from native bone marrow have been known to improve bone graft efficiency.\textsuperscript{22,90} A characteristic of many marrow derived CTPs is their ability to give rise to progeny that are capable of differentiating along a number of mesenchymal lineages including bone, cartilage, muscle and fat.\textsuperscript{22,23,90}

Osteoblastic differentiation is particularly relevant to clinical bone repair strategies. Figure 2.2 illustrates a generally accepted pattern of gene expression that reflects three major periods of cell and tissue development in bone formation.\textsuperscript{145,146} The differentiated osteoblast is typically characterized by a specific pattern of gene expression such as alkaline phosphatase (AP), collagen, osteocalcin (OC), and \textit{in vitro} mineralization capacity.\textsuperscript{138,145,146}
In a previous study\textsuperscript{68}, human bone marrow derived CTPs were cultured for 9 days on smooth PDMS surfaces and on PDMS post microtextures that were 6 \textmu m high and 5, 10, 20 and 40 \textmu m in diameter, respectively. It was discovered that cells on PDMS post microtextures exhibited different morphology and increased cell count relative to those on smooth PDMS surface. In particular, the investigations showed that 10 \textmu m diameter post textures significantly enhanced CTP growth. This result suggested that despite identical surface chemistry, the substrate topography had a significant effect on the biological performance of CTPs, and suggested a potential role for microtextured materials in bone tissue engineering applications. In the current study, the influence of microtextured surfaces on proliferation and osteogenic differentiation of CTP progeny in long-term culture is investigated. Human marrow-derived CTPs are plated in primary culture on PDMS substrates presenting either post microtextures or a control (smooth) surface for up to 60 days, as an \textit{in vitro} model to investigate the potential response of CTPs to different surface textures in a bone healing environment \textit{in vivo}.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 SUBSTRATE PREPARATION

The microfabricated PDMS substrate was produced by soft lithography techniques.\textsuperscript{68} Briefly, a 6 \textmu m thick layer of SU-8 2010 negative photoresist was first coated on top of a silicon (Si) wafer. By using an ultraviolet (UV) photolithography process, the 10 \textmu m diameter and 6 \textmu m high micropost pattern, with 10 \textmu m separation between posts, was then transferred from a photomask into the photoresist. Afterwards, this SU-8 mold was coated with 1H,1H,2H,2H-perfluorodecyltrichlorosilane (Lancaster
Synthesis; Pelham, NH) to aid the release of PDMS in the final step of substrate production. The liquid PDMS base and curing agent (Sylgard 184; Dow Corning; Midland, MI) components were subsequently mixed at a ratio of 10:1, degassed for 20 min, and then poured uniformly on top of the patterned SU-8 mold. After additional degassing for 10 min, the PDMS was cured at 85°C for 2 h or at room temperature for 1 day (Figure 3.1(a)). The cured PDMS cast was finally released from the mold and sectioned into 2 cm X 2 cm samples. Representative samples were inspected by scanning electron microscopy (SEM; JSM-5310; JEOL USA; Peabody, MA). An unpatterned SU-8 mold was used to produce the smooth PDMS surfaces, which served as the control substrates for the study (Fig. 3.1(b)).

Figure 3.1 SEM images of PDMS post microtextures, and control surfaces. The PDMS substrates were produced by soft lithography techniques. (a) PDMS post microtextures that are 6 µm in height, 10 µm in diameter, and 10 µm separation between posts; and (b) control (smooth PDMS) surface.
3.2.2 CELL CULTURE

As described by Muschler et al.\textsuperscript{21}, bone marrow aspirates were harvested from the anterior iliac crest with informed consent from three patients immediately prior to elective orthopedic procedures. Briefly, a 2 mL sample of bone marrow was aspirated from the anterior iliac crest into 1 mL of saline containing 1000 units of heparin (Vector Labs; Burlingame, CA). The heparinized marrow sample was suspended into 20 mL of heparinized carrier media ($\alpha$-minimal essential medium (MEM) + 2 units/mL of Na-heparin; Gibco; Grand Island, NY) and centrifuged at 1500 rpm (400 X) for 10 min. The buffy coat was collected, resuspended in 20 mL of 0.3% bovine serum albumin-MEM (Gibco), and the number of nucleated cells was counted. The PDMS substrates were sterilized for 30 min with 70% ethanol and placed inside a standard tissue culture dishes (Lab-Tek Chamber Slide System; Naperville, IL). Cells were then plated on day 0 at a seeding concentration of $1 \times 10^6$ cells per well (2 cm X 2 cm), and cultured for up to 60 days under conditions promoting osteoblastic differentiation.\textsuperscript{21}

In previous studies\textsuperscript{21,23}, it was established that CTPs exhibit osteoblastic phenotype after 9 days of culture. In order to determine possible effects of surface topography on the transition of cells from the lag to the log phases, cell counts were performed daily for the first 10 days, and subsequently on days 30 and 60.

3.2.3 CELL MORPHOLOGY

On days 9, 30 and 60, the media was removed and the plated substrates were placed in a solution containing 2% glutaraldehyde (Electron Microscopy Sciences; Fort Washington, PA), 3% sucrose (Sigma-Aldrich Co.; Irvine, UK) and 0.1 M of phosphate
buffered saline (PBS; Cambrex BioScience; Walkersville, MD) at 4°C and pH 7.4. After 1 h, the substrates were rinsed twice with the PBS for 30 min at 4°C and washed with distilled water for 5 min. Dehydration was achieved by placing the plated substrates in 50% ethanol for 15 min while increasing the concentration of ethanol sequentially to 60, 70, 80, 90 and finally 100%. Dehydrated samples were then mounted on aluminum stubs, sputter-coated with gold-palladium, and examined using scanning electron microscopy (SEM) (JSM-5310; JEOL USA).

3.2.4 CELL PROLIFERATION AND DIFFERENTIATION

PicoGreen DNA quantification

CTP progeny-seeded substrates were resuspended with a 50 μl of lysis buffer (1% sodium dodecyl sulfate, 10 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM Tris-HCl, pH 8.1) (Sigma-Aldrich Co.) to lyse the membrane of adherent CTP progeny. After 60 min, the samples were centrifuged at 14000 rpm for 5 min and the supernatant was removed for analysis. A 40 μL sample of aqueous supernatant containing DNA was added to 0.96 mL TE buffer (10 mM Tris adjusted to pH 7.0 with HCl, 1 mM EDTA) (Molecular Probes; Eugene, OR). As per the manufacturer’s instructions (Molecular Probes), stock PicoGreen reagent was diluted 1:200 in TE buffer and 1 ml of that was added to each DNA containing sample. The tubes were capped, vortexed, and incubated at room temperature in the dark room for 3 min. The fluorescence was measured with the SpectraMax Gemini fluorescence microplate reader (Molecular Devices Co.; Sunnyvale, CA), with excitation and emission wavelengths of 480 and 520 nm, respectively. All calibration samples were assayed four times and a fresh calibration curve was generated.
for each 96 well plate. Baseline fluorescence was determined with a TE blank, the average of which was subtracted from the averaged fluorescence of other samples. Using this analysis, it was determined that ~4.5 μg of DNA was obtained from $1 \times 10^6$ adherent CTPs. Thus, it is possible to estimate total cell number by assuming that 4.5 pg of DNA represents one cell. Also, a calculation was performed to determine that, for identical projected surface areas, the actual surface area of post microtextures was 1.47 times greater than that of the smooth (control) surface. Consequently, the total cell number (estimated via DNA quantification) from the post microtextures was divided by 1.47 to enable a meaningful comparison with the cell number from smooth surfaces.

**DAPI stain**

Cell nuclei were stained with 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI). Ethanol-fixed cells were rinsed three times with PBS, and then a 10 μL drop of DAPI-containing Vectashield mounting media (Vector Labs) was placed on the substrates. Immediately thereafter, the edges of the coverslips were sealed with three coats of clear nail polish and viewed under a fluorescent microscope (Olympus BX50F; Olympus Optical Co.; Japan).

**Alkaline Phosphatase stain**

Cells were stained for AP, using the Vector Red substrate working solution (5 ml of 100 mM Tris-HCl adding 2 drops of Reagent 1, 2 and 3) (Vector Labs) for 30 min at room temperature in the dark, and then, washed in distilled water. The positively stained cells with AP activity appeared red when viewed under a fluorescent microscope.
von Kossa stain

Cells were rinsed with PBS and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 1 h. They were then incubated in 5% silver nitrate (Sigma-Aldrich Co.) for 30 min in the dark room, rinsed with distilled water, and exposed to UV light for 1 h. Secretion of calcified extracellular matrix (ECM) was confirmed as deep blue-purple nodules with von Kossa staining under a phase contrast microscope.

Integrin expression

Integrin expressions were confirmed by Western blot analysis. Cell extracts were prepared by adding lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 10% glycerol and a cocktail of protease inhibitors (Sigma-Aldrich Co.). Lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C and the protein contents of supernatants were determined using a modified Bradford assay (Bio-Rad; Hercules, CA). Diluted 15 µg protein samples were loaded onto a 10% Tris-HCl ready gel (Bio-Rad) and electrophoresed in SDS running buffer at 130 V for 2 h. The proteins were electrophoretically transferred onto a Hybond-P membrane (Amersham Pharmacia Biotech; Piscataway, NJ) in a 1X transfer buffer at 100 V for 2 h in cold room (4°C). Membranes were blocked with 5% w/v skimmed milk and PBS containing 0.1% Tween (TPBS) at room temperature for 1 h. Primary antibodies (integrin α1, α2, α5, β1 and GAPDH) (Chemicon; Temecula, CA) were diluted 1/200 - 1/1000 in 5% skimmed milk and incubated with the membranes overnight. After washing the membranes three times in TPBS, the membranes were incubated for 1 h with the 1/1000 horseradish peroxidase (HRP) - conjugated secondary
antibody (Chemicon). The membrane was washed again three times in TPBS, and signals were visualized using ECL detection reagents (Amersham Pharmacia) for 3 min and exposed to radiographic film (Eastman Kodak; Rochester, NY) for 30 sec to 10 min. The intensity of exposed bands were measured by Gel-Pro program (Gel-Pro Analyzer Version 3.1.; Silver Spring, MD) to quantify the protein expression.

**Real time reverse transcript - polymerase chain reaction (Real time RT-PCR)**

Expression of osteoblast specific genes, such as AP, type I collagen (Col I) and OC were detected by real time RT-PCR. Total cellular RNA was isolated with RNeasy kit (Qiagen Inc.; Valentia, CA) and reverse transcribed by conventional protocols with a Sensiscript Reverse Transcription kit (Qiagen Inc.). Expression of the AP, Col I, OC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using real time RT-PCR analysis with Power SYBR® Green PCR Master Mix kit (Applied Biosystems; Foster City, CA). GAPDH is an enzyme utilized in cellular metabolism and is assumed to be expressed at the same level in most cells; therefore, gene expression of GAPDH was used as an internal control to normalize out any differences in amount of total RNA isolated.\(^{21}\) Primer sequences are presented in Table 3.1. Real Time quantitative PCR was performed on a 7500 Real Time PCR system (Applied Biosystems). Data analysis was carried out using the 7500 System Sequence Detection software (Applied Biosystems).\(^{147}\)
Table 3.1 List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase</td>
<td>5’ ACA GAT GCC AAC TTC CCA CAC G 3’</td>
</tr>
<tr>
<td></td>
<td>3’ GAG GCA CCT TGT AAG ACC TAG AC 5’</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>5’ CTC CAC TCC TTC CCA AAT CTG TC 3’</td>
</tr>
<tr>
<td></td>
<td>3’ CTT TGT AGC CTA AAC CCC TTG C 5’</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>5’ AGG TGC AGC CTT TGT GTC CAA G 3’</td>
</tr>
<tr>
<td></td>
<td>3’ GGG AAG AAA GGA GAA GGG GAA C 5’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ GGG CTG CTT TTS SCT CTG GT 3’</td>
</tr>
<tr>
<td></td>
<td>3’ TGG CAG GTT TTT CTA GAC GG 5’</td>
</tr>
</tbody>
</table>

Statistical analysis

The PicoGreen DNA quantification and real time RT-PCR analyses were each repeated a total of 9 times (3 replicates for each of the 3 patients; or n = 9 for each substrate) as per standard laboratory protocols. The mean and standard deviation (SD) values were subsequently calculated using the data of all groups. Statistical analysis (n = 9) was performed by a one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test (SPSS Version 10.0.; SPSS Inc.; Chicago, IL) to a significance level of p < 0.05 (the 95% confidence interval).
3.3 RESULTS

3.3.1 CELL MORPHOLOGY

The SEM images revealed that human CTPs attached to PDMS substrate surfaces with varying cell morphologies (Figure 3.2(a)). CTP progeny on the smooth surfaces exhibited arbitrary flattened shapes and migrated without any preferred orientation for up to 60 days. In contrast, on day 9 CTP progeny on PDMS post microtextures mostly tended to attach next to the posts and spread between them while directing their processes towards other posts and cells. On day 30, increased cell growth was observed on the post microtexture substrates, and cells attached to the posts, especially in contact with the flat substrate underneath, and spread between them (Figure 3.2(b)).

By day 60, numerous cells grew and spread over the top of the post microtextures, and covered the most of surface (Figure 3.2(a)). Interestingly, most of cells do not make contact with the smooth floor between posts (Figure 3.2(b)). Craighead et al.\(^2\) and Turner et al.\(^3\) similarly observed rat astrocytes contacting only the tops of columns. The influence of the cytoskeleton or other mechanical effects may be responsible for preventing the cells from sagging down onto the smooth floor. It is also possible that this behavior may be due to either the greater availability of nutrients from the medium when cells remain elevated or an aversion to the smooth surface.\(^3\) Even though the mechanisms of this preferred adhesion are not yet clear, the locomotion of cells on the top of posts may provide physical foot prints appropriate for biological functions, especially osteogenic differentiation.
Figure 3.2 (a) SEM images of CTPs on PDMS post microtextures and control surfaces on day 9, 30, and 60. CTPs attached to post microtextures and control surfaces with varying cell morphology. On post microtextures, CTPs mostly tended to attach next to the posts and spread between them while directing their processes towards posts and other cells on day 9. On day 30, increased cell growth on the post microtexture scaffolds can be observed. By day 60, numerous cells grew and spread over the top of the post microtextures and covered the most of surface with ECM. In contrast, cells on the control surfaces exhibited arbitrary flattened shapes and migrated without any preferred orientation for up to 60 days.
Figure 3.2 (b) On day 30, increased cell growth was observed on the post microtexture substrates and cells attached to the posts, especially in contact with the flat substrate underneath and spread between them. On day 60, however, numerous cells are growing on the tops of the posts and not making contact with the flat substrate underneath.
Figure 3.3 Time dependent (a) growth curves and (b) fold changes of CTPs to PDMS substrates. DNA quantification analysis revealed that the number of CTPs attached initially on PDMS substrates are almost identical. However, the greatest fold change was observed on between day 4 and day 5 as the cells on PDMS post microtextures were about to exit from the lag phase. Cells on control surfaces were also about to exit from the lag phase on day 6 and 7. After exit from the lag phase, the slopes of cell growth showed similar on PDMS post microtextures and control surfaces.
3.3.2 EARLY CELL GROWTH

CTP cultures from all three donors expanded with characteristic lag and log phases (Figure 3.3(a)). DNA quantification analysis revealed that the number of cells initially attached (Day 1) on the PDMS substrates were almost identical (391 cells/cm² for smooth, and 361 cells/cm² for post microtextures). Furthermore, there were minimal changes in daily cell numbers for both substrates through day 4. On day 5, CTPs on PDMS post microtextures transitioned to the log phase as demonstrated by a 3.6-fold increase in cell number. In contrast, the lag phase of CTPs on smooth surfaces lasted longer, with a 2.1-fold increase in cell number occurring on day 7 (Figure 3.3(b)). After exiting from the lag phase, the rate of increase in cell number in the log phase was similar for both PDMS post microtextures and smooth surfaces.

3.3.3 CELL PROLIFERATION

The CTPs on substrates were viewed in situ on days 9, 30, and 60 by DAPI staining (Figure 3.4). The stain revealed that there were qualitatively more cells on PDMS post microtextures than smooth surfaces throughout the culture period. This observation was confirmed by the DNA quantification analysis (Figure 3.4). On day 9, PDMS post microtextures supported more than twice the number of cells supported on smooth surfaces. Furthermore, on day 30, the PDMS post microtextures supported over three times the number of cells supported on control surfaces (p < 0.05). Finally, on day 60, PDMS post microtextures continued to support almost twice the number of cells compared to control surfaces.
Figure 3.4  CTP number on PDMS post microtextures and corresponding control surfaces. On day 9, CTPs on the post microtextures was greater than that exhibited on the control surfaces. Post microtextures exhibited a significant increase of CTPs on day 30. On day 60, the number of CTPs increased on post microtextures compared to the control surfaces. Fluorescence images showed cell nuclei were stained with DAPI and revealed more cells on post microtextures than control surfaces. The PicoGreen DNA quantification was repeated a total of 9 times (replicates of 3 times of the each 3 patients; n=9/substrate) as per standard lab protocols (n = 9/group; mean + SD) and the original color fluorescence images of DAPI were reversed to enhance visual clarity. * denotes statistical significance compared to control surfaces on day 30 ($p < 0.05$).
3.3.4 INTEGRIN EXPRESSION

On days 30 and 60, the Western blot analysis revealed that integrins α1, α2, α5 and β1 were expressed by the cells on all surfaces, though integrin α1 was expressed at generally lower levels (Figure 3.5). On day 30, integrins α1, α2, and β1 demonstrated comparable expression levels between post microtextures and smooth surfaces. In contrast, integrin α5 exhibited an almost 3-fold greater expression level on the PDMS post microtextures. On day 60, integrin α5 still exhibited increased expression on PDMS post microtextures compared to smooth surfaces, though the difference had decreased to 1.5-fold.

<table>
<thead>
<tr>
<th>Day 30</th>
<th>Day 60</th>
</tr>
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<tbody>
<tr>
<td>P</td>
<td>C</td>
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<tr>
<td>Integrin α1</td>
<td>![Image]</td>
</tr>
<tr>
<td>Integrin α2</td>
<td>![Image]</td>
</tr>
<tr>
<td>Integrin α5</td>
<td>![Image]</td>
</tr>
<tr>
<td>Integrin β1</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>![Image]</td>
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</tbody>
</table>

Figure 3.5 Integrins and GAPDH expression in CTPs after 9, 30, and 60 days culture on PDMS post microtextures, and control surfaces. The expressions of integrins on day 9 were low, most probably due to the relative low number of cells. On day 30 and 60, integrins α1, α2, and β1 demonstrated comparable expression levels between post microtextures and smooth surfaces. On day 60, compared to day 30, the actual expression level for α2 had increased over 2-fold, while decreasing very slightly for integrin α1, and increasing slightly for β1. Integrin α5 exhibited an almost 3-fold greater expression level on the PDMS post microtextures on day 30 and still exhibited increased expression on day 60 compared to smooth surfaces, though the difference had decreased to 1.5-fold. However, compared to day 30, the actual expression of integrin α5 on smooth surfaces had increased by almost 2.5 fold, while increasing very slightly for the post microtextures.
On day 60, integrins $\alpha_1$, $\alpha_2$, and $\beta_1$ again demonstrated comparable expression levels between substrates. However, compared to day 30, the actual expression level for $\alpha_2$ had increased over 2-fold, while decreasing very slightly for integrin $\alpha_1$, and increasing slightly for $\beta_1$. Integrin $\alpha_5$ still exhibited a greater expression level (1.5-fold) on the PDMS post microtextures compared to smooth surfaces. However, compared to day 30, the actual expression on smooth surfaces had increased by almost 2.5 fold, while increasing very slightly for the post microtextures.

3.3.5 AP EXPRESSION AND MINERALIZATION

CTPs on the various PDMS substrates were also viewed *in situ* on days 9, 30, and 60 using AP and von Kossa staining. In general, cells on the PDMS post microtextures stained more intensely for AP compared to smooth surfaces on day 9 (Figure 3.6(a)). Almost all cells expressed AP on both substrates on days 30 and day 60. In contrast, the von Kossa staining was minimal on all substrates on day 9, but increased on day 30, and even further more on day 60 (Figure 3.6(b)). Furthermore, the spatial distribution and intensity of the von Kossa stain was appeared to be consistently greater on PDMS post microtextures than on the smooth surfaces.

3.3.6 GENE EXPRESSION

Expression of the key osteoblastic bone markers, such as AP, Col I, and OC was assessed in the CTP progeny on substrates using real time RT-PCR. The results revealed that AP expression was higher on PDMS post microtextures on day 9, but decreased on day 30 and 60. (Figure 3.6(a)). Col I expression increased over time on both
microtextured and control surfaces, but was consistently greater on microtextured surfaces than on control surfaces (Figure 3.6(c)).

Figure 3.6 Gene expression of (a) AP, (b) collagen type I (Col I), and (c) osteocalcin (OC) from CTPs after 9, 30 and 60 day on PDMS post microtextures, and control surface. (a) AP mRNA expressed during development, with highest levels present on day 9 on post microtextures, while it expressed more highly on the control surface on day 60. Fluorescent images showed cells on the post microtextures stained more intensely for AP compared to control surfaces on day 9 and AP increased on all surfaces by day 30 and day 60.
Trends in OC expression were similar to Col I, with consistently greater expression on microtextured surfaces, particularly on day 60 (Figure 3.6(b)).

**Figure 3.6** (b) *(Continued)* Compared to day 9, OC mRNA expression showed a significant increase in cells grown on post microtextures on day 60 compared to cells grown on the control surface. Phase contrast images show von Kossa stain and the intensity of this stain on post microtextures increased with time compared to the control surfaces.
Figure 3.6 (c) (Continued) mRNA of Col I expressed at all time points with slightly higher expression on day 30 and significantly higher on day 60. * denotes statistical significance compared to control surfaces on same day (p < 0.05).

3.4 DISCUSSION

Understanding and optimizing of cell–ECM–substrate surface interactions is critical to the rational design of biological implant materials and tissue engineering scaffolds, particularly for bone healing applications, where the concentration and prevalence of local osteogenic CTPs in native tissues are generally suboptimal. It was hypothesized that the biological performance of CTPs and their progeny with respect to proliferation, migration, and osteoblastic differentiation would be significantly modified by interaction with PDMS post microtextures when compared to control.
(smooth PDMS) surfaces, as assessed by cell retention, expression of integrins, AP activity, matrix deposition, and mineralization associated with osteoblastic differentiation.

This study shows that the transition of CTP progeny growth from lag to the log phase, and associated indicators such AP expression, collagen and osteocalcin synthesis, mineralization, as well as expression of integrin α5 were all increased or accelerated on PDMS post microtextures when compared to the smooth surfaces. The choice of PDMS as the construction material for both substrates, and the similarities in processing and handing and identical culture conditions, allow us to gain insights into the role of surface topography on cell growth since the underlying surface chemistry is identical.

Cellular responses are generally attributed to the surface-adsorbed ECM, which comes from surrounding media and also can be produced by cells themselves.\textsuperscript{149-151} Cells are known to attach to ECM via integrin receptors, and the integrin-mediated attachments affect cell behavior such as adhesion, migration, proliferation, and differentiation.\textsuperscript{152} Integrin α1β1 and α2β1 bind to type I collagen, which is the dominant bone matrix protein, and this binding has been reported to regulate osteoblastic differentiation.\textsuperscript{153} Binding of integrin α5β1 to ECM is known to regulate osteoblast survival, proliferation, bone specific-gene expression, and matrix mineralization.\textsuperscript{153,154} In the Western blot results, integrin α1 and β1 expressed similarly over time, while the expression of integrin α5 was greater on the PDMS post microtextures on both days 30 and 60 (Figure 3.5). Thus, this data suggests that the increased integrin α5 expression of CTPs on post microtextures resulted in increasing ECM production, and subsequent osteoblast specific gene expression of cells on post microtextures (Figure 3.6).
Figure 3.4 shows that the cell number on PDMS post microtextures were consistently greater than on the smooth surfaces. The reported cell numbers per unit area have already been adjusted to account for the difference in actual surface areas between post microtextures and smooth surfaces. Furthermore, according to previous experiments and Figure 3.3, the numbers of CTPs attached initially to the PDMS post microtextures and smooth surfaces were almost identical. Therefore, it appears that the enhanced cell growth on post microtextures resulted primarily from the shortened lag phase, which is evident in Figure 3.3. The earlier onset of proliferation would lead to increased cell number on days 9, 30 and 60. Presumably, the higher cell number resulted in their early confluence and stimulation of osteogenic differentiation (ECM production, osteoblast-ECM related integrins expression and subsequent osteoblast specific gene expression) on post microtextures.

Larson et al. have investigated the growth of passaged human bone marrow-derived multipotent stromal cells in low density (50 cells/cm²) cultures on standard tissue culture substrates for up to 10 days. In this time frame, their cultures expanded with characteristic lag, log and near stationary phases, and that the greatest fold change was observed on days 2 and 3, when the cells transitioned to the log phase. In contrast, the current CTPs remained in the lag phase for 4 and 6 days for smooth and post microtextures, respectively. Furthermore, the CTPs continued to proliferate for over 30 days without achieving the stationary phase. Possible reasons for the differences in the present results from the previous reports could arise from current choice of substrate material (PDMS), culture conditions (high density), and the heterogeneity of the CTP population. Additional investigations will be required to gain further insights on the
differences in growth characteristics between CTPs and conventional mesenchymal stem cells.

Numerous groups have examined the effect of surface topographies on cell behavior.\textsuperscript{142,156-165} Table 3.2 presents a summary of results from previous work and a comparison to the current study. In general, current results are consistent with previous reports which suggest that surface microtopography influences osteogenesis. However, the current study has focused on CTPs, which have not been examined similarly in the past. In addition, it was demonstrated that, in spite of the identical surface chemistry, cells cultured on post microtextures appear to exhibit a shortened lag phase relative to smooth surfaces. Thus, even when the initial numbers of cells attached to the substrates are similar, post microtextures ultimately result in increased cell number and appear to accelerate osteogenesis.

There are many factors contributing to the increased cell numbers and expression of osteogenic markers on different surface topographies. The incorporation of micro- and nano-scale topographies at the cell–substrate interface might provide an attractive approach to enhancing specific cell behaviors without destabilizing the delicate biochemical environment. However, the mechanisms by which cell behaviors changes in response to different geometrical and biochemical stimuli remain unclear. Further investigation is needed to elucidate all of the possible factors and establish definitive mechanistic links between cell-surface interactions and cell differentiation.

Moreover, the feasibility demonstration of CTP-surface topography constructions for bone tissue formation will require experiments in an animal model for performance assessment \textit{in vivo}. These experiments could be initially performed with the 3
dimensional (3D) scaffolds with surface topographies to verify enhanced CTP growth characteristics. Afterwards, the performance of 3D scaffold should be investigated in the animal model to provide insight into the behavior of these for human bone graft applications.

3.5 CONCLUSION

Post microtextures accelerates entry into proliferation and osteogenic differentiation of CTP cultures compared to those on control (smooth) surfaces. This difference in overall cell growth is attributed to reduction in lag time between initial cell contact with the substrate surface and cell spreading. Cells proliferate to greater numbers and express higher amount of ECM genes and minerals on the post microtextures than on the control surfaces after long-term culture. The earlier onset of cell proliferation on microtextures results in increased cell numbers, their early confluence, and stimulation of osteogenic differentiation. This study demonstrates a valuable in vitro model, based on the precise and reproducible patterning capabilities of microfabrication and related MEMS techniques, in which to explore the relationship between topographical features of bone tissue engineering scaffolds and the likely response of human adult stem cells and progenitor cells in the settings of bone repair in vivo.
Table 3.2 Effect of surface topography on cells

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cell Type</th>
<th>Time</th>
<th>Surface Topography</th>
<th>Investigation/Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>156</td>
<td>Mg63 osteoblast-like cells</td>
<td>1 week</td>
<td>rough Ti surfaces with different morphologies (SLA, Ra = 3.3 μm and TPS, Ra = 0.22 μm)</td>
<td>Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition</td>
</tr>
<tr>
<td>157</td>
<td>Mg63 osteoblast-like cells</td>
<td>1 week</td>
<td>Ti disks also were sandblasted (SB), and acid etched (CA), or plasma sprayed with Ti particles (PS)</td>
<td>Bone cell response to systemic hormones is modified by surface roughness and that surface roughness increases the responsiveness of MG63 cells to 1α,25-(OH)2D3</td>
</tr>
<tr>
<td>158</td>
<td>Mg63 osteoblast-like cells</td>
<td>5 days</td>
<td>rough Ti surfaces with different morphologies (SLA, Ra = 3.97 μm and TPS, Ra = 5.21 μm)</td>
<td>Both cyclooxygenase-1 and cyclooxygenase-2 mediate osteoblast response to titanium surface roughness</td>
</tr>
<tr>
<td>159</td>
<td>Human osteoblasts</td>
<td>1 week</td>
<td>rough Ti surfaces with different morphologies (SLA, Ra = 3.97 μm and TPS, Ra = 5.21 μm)</td>
<td>Osteoblast gene expression and mineralization are affected by roughened implant surface microtopographies during osseointegration of dental implants.</td>
</tr>
<tr>
<td>160</td>
<td>Osteoblasts</td>
<td>3 weeks</td>
<td>600-grit (grooved) or sandblasted (roughened) cpTi implant discs</td>
<td>Preosteoblast cell differentiation is affected by implant surface microtopographies during osseointegration of dental implants.</td>
</tr>
<tr>
<td>161</td>
<td>Rat osteoblasts</td>
<td>6 weeks</td>
<td>discontinuous-edge surfaces (DESs): 34 x 34 μm to 65 x 65 μm in width and 4 and 10 μm in depth</td>
<td>DESs alter adhesion, migration, and proliferative responses from osteoblasts at early time points and promote multilayering, matrix deposition, and mineral deposition at later times.</td>
</tr>
<tr>
<td>162</td>
<td>Rat osteoblasts</td>
<td>6 weeks</td>
<td>(A) 30 μm deep grooves with a 45 μm pitch, (B) 10 μm deep gap cornered boxes, and (C), 30 μm deep-tapered pits.</td>
<td>The effect of substratum topography on osteoblast adhesion mediated signal transduction and phosphorylation</td>
</tr>
<tr>
<td>163</td>
<td>Epithelial cells and osteoblasts</td>
<td>4 weeks</td>
<td>TPs and inverted pyramids within the range of 30–120 μm in depth</td>
<td>Tapered pits stimulate osteoblast mineral deposition in vitro and in vivo, but do not prevent epithelial sheet migration</td>
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<tr>
<td>164</td>
<td>Human mesenchymal stem cell and osteoprogenitor cells</td>
<td>4 weeks</td>
<td>PMMA 120 nm diameter pits (100nm deep, absolute or average 300nm centre-centre spacing) with displaced square 50 (±50 nm from true centre)</td>
<td>Topographically treated MSCs have a distinct differentiation profile compared with those treated with osteogenic media.</td>
</tr>
<tr>
<td>165</td>
<td>Human mesenchymal stem cell</td>
<td>3 weeks</td>
<td>PMMA pit (width:deep=30μm:300nm or 40μm:400nm) and groove (width:deep=5μm:500nm or 50μm:300nm)</td>
<td>The nanotopographies allowed control of cell adhesion, cytoskeleton, growth and production of the osteoblastic markers.</td>
</tr>
<tr>
<td>166</td>
<td>Human bone marrow derived CTPs</td>
<td>60 days</td>
<td>PDMS post microtextures that has 10 μm diameter and 6 μm height (effective Ra=3 μm)</td>
<td>PDMS post microtextures accelerate proliferation and osteogenic differentiation of CTPs</td>
</tr>
</tbody>
</table>
CHAPTER IV

CTP GROWTH ON SURFACE MICROTEXTURES WITH VARYING GEOMETRY AND ARRANGEMENT

4.1 INTRODUCTION

Successful bone healing requires the presence of a sufficient number of CTPs to adhere, proliferate, and organize extracellular matrix molecules into a functional tissue. A state of the art approach, combining CTPs with a scaffold to enhance regeneration in bone grafting procedures, involves rapid concentration and selection of this cell population from the bone marrow into the graft using selective attachment to a matrix surface. At the cell-surface interface, both an appropriate physicochemical environment and the surface topography profoundly affect the overall behavior of the engineered tissue. The surface topographical characteristics have important implications in the rational design and optimization of biological implants. Recent advances in microfabrication and related MEMS (microelectromechanical systems) techniques enable
precise production of surface topography features to investigate the effects of physical
cues on cellular behavior. \cite{109,110,141,142} For example, soft lithography has been used to
pattern the distribution of various chemicals on a material surface to explore selective
cellular responses to specific biomolecular species. \cite{110} For tissue engineering applications,
this technique could be conceptually combined with conventional scaffold processing
strategies to ultimately provide scaffolds that possess precise topographical, spatial, and
chemical properties to optimize control over cellular behavior.

It is important to select the optimal size geometry (e.g. dimension, height and
inter-space) of microposts when designing surface post microtextures. A number of
research groups (Table 2.2) have selected microposts that were of particular diameter-
height ratios (e.g. 1:1, 2:1, 1:2, and 1:4) and dimension-inter space ratios (e.g. 1:1, 1:2,
and 1:4) and showed that different sizes of micropost geometries influence cell behavior.
In a previous study \cite{68}, human bone marrow-derived CTPs were cultured for 9 days on
smooth PDMS surfaces and on PDMS post microtextures that were 6 \( \mu \)m high and 5, 10,
20 and 40 \( \mu \)m in diameter. It was discovered that cells on PDMS post microtextures
exhibited different morphology and increased cell count relative to those on smooth
PDMS surface. In particular, these investigations showed that 10 \( \mu \)m diameter post
textures significantly enhanced CTP growth. \cite{68} This result suggested that despite identical
surface chemistry, the optimal size of microposts on the substrate surface had a
significant effect on the biological performance of CTPs, and suggested a potential role
for microtextured materials in bone tissue engineering applications. In this current study,
the 10 \( \mu \)m diameter micropost with varying height and inter-space parameters were
fabricated using soft lithography techniques, and human bone marrow cells containing
CTPs were cultured on these PDMS substrates in order to establish optimal micropost dimensions for cell growth.

4.2 MATERIALS AND METHODS

4.2.1 EXPERIMENTAL DESIGN

Bone marrow derived CTPs were cultured on PDMS substrates comprising unpatterned smooth surfaces and cylindrical post microtextures that were 10 μm in diameter, 4 different heights (5, 10, 20 and 40 μm) and 3 different inter-spaces (10, 20, and 40 μm) (Figure 4.1). The microtextured PDMS substrates were realized by soft lithography, which has been derived from microfabrication and micromachining techniques commonly used to produce MEMS devices. CTPs were plated on the substrates, cultured for 9 days, fixed, analyzed using fluorescent and scanning electron microscopy, and tested for proliferation using PicoGreen DNA quantification. Each experiment was repeated three times and the results were compared to those from cells grown on smooth surfaces of PDMS.
Figure 4.1  PDMS post microtextures with varying geometry and arrangement. Cylindrical post microtextures that were 10 μm diameter, 4 different heights (5, 10, 20 and 40 μm) and 3 different inter-spaces (10, 20, and 40 μm) were (a) selected, and (b) designed, respectively. The microtextured substrates were produced using soft lithography.
4.2.2 SUBSTRATE PREPARATION

Control the post heights

To obtain appropriate micropost heights (5, 10, 20 and 40 μm), SU-8 2010 was spin coated onto a silicon wafer at different speeds (Figure 4.2) using a Karl Suss RC8 spinner (Suss MicroTec, Waterbury, VT), then, soft baked, exposed, and post exposure baked. Thickness measurements of the SU-8 layers were verified using scanning electron microscopy (SEM) (JSM-5310, JEOL USA, Peabody, MA).

Figure 4.2 (a) To obtain appropriate micropost heights (5, 10, 20 and 40 μm), SU-8 2010 was spin coated onto a silicon wafer with an optimal speed.
Figure 4.2 (b) (Continued) Cross-section of SU-8 which was spin coated onto silicon wafer. Thickness of the SU-8 layers was verified using SEM.
Control the inter-space between posts

To design the patterns with varying inter-space (10, 20 and 40 μm) dimensions between posts, a computer-aided design program (MEMS Pro V5.1; L-Edit, SoftMEMS LLC) was used (Figure 4.3). Substrate patterns were designed to study cell growth preference on different arrangement of post microtextures.

Figure 4.3 (a) Substrate pattern designed using MEMS Pro V5.1 computer program. Substrate patterns were designed to study cell growth preference on different arrangement of post microtextures.
Top view of Mask
(Diameter=10 μm, 3 different sizes of inter-space)

<table>
<thead>
<tr>
<th>10 μm</th>
<th>20 μm</th>
<th>40 μm</th>
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Figure 4.3 (b) Pattern layout on the photomask for the pattern with varying inter-space (10, 20 and 40 μm) dimensions between posts. Dark regions correspond to chrome (opaque) regions on the photomask, and light regions correspond to regions transparent to UV exposure.

Microfabrication of PDMS post microtextures and smooth substrates

The microfabricated PDMS substrates were fabricated by soft lithography techniques (Figure 4.4). Briefly, various film thicknesses (5, 10, 20 and 40 μm) of SU-8 2010 photoresist were spin coated on top of silicon wafers at different optimal speeds using a spinner. By using ultraviolet (UV) exposure, the post microtexture patterns with varying inter-space (10, 20 and 40 μm) dimensions were transferred from a photomask onto the photoresist, and then developed. The liquid PDMS base and curing agent (Sylgard 184; Dow Corning) components were mixed at a ratio of 10:1, degassed for 20 min, and then poured uniformly on top of the patterned mold. The PDMS substrates were cured at 85°C for 2 h. Unpatterned SU-8 was used to produce the smooth PDMS substrates. The cured
PDMS casts were released from the mold and sectioned into 2 cm X 2 cm samples. Representative samples were inspected by SEM.

**Figure 4.4** Fabrication of PDMS post microtextures by soft lithography. Briefly, various film thicknesses (5, 10, 20 and 40 μm) of SU-8 2010 photoresist were spin coated on top of silicon wafers at different optimal speeds. By using ultraviolet (UV) exposure, the post microtexture patterns with varying inter-space (10, 20 and 40 μm) dimensions were transferred from a photomask onto the photoresist, and then developed. The liquid PDMS were mixed at a ratio of 10:1, degassed for 20 min, and then poured uniformly on top of the patterned mold. The PDMS substrates were cured at 85°C for 2 h.

### 4.2.3 CELL CULTURE AND ANALYSES

As described by Muschler et al., bone marrow aspirates were harvested from the anterior iliac crest with informed consent from four patients immediately prior to elective
orthopedic procedures. Briefly, 2 mL samples of bone marrow were aspirated from the anterior iliac crest into 1 mL of saline containing 1000 units of heparin (Vector, Burlingame, CA). The heparinized marrow sample was suspended into 20 mL of heparinized carrier media (α-minimal essential medium (α-MEM) + 2 units/mL of Na-heparin; Gibco, Grand Island, NY) and centrifuged at 1500 rpm (400 X) for 10 min. The buffy coat was collected, resuspended in 20 mL of 0.3% bovine serum albumin-MEM (Gibco), and the number of nucleated cells was counted. The PDMS substrates were sterilized for 30 min with 70% ethanol. Cells were then plated on day 0 at a seeding concentration of $1 \times 10^6$ cells per well and were cultured for 9 days under conditions promoting osteoblastic differentiation.\textsuperscript{21} Cell characteristics on all substrates were investigated using SEM, PicoGreen DNA quantification, and fluorescent stains. In this study, the PicoGreen DNA quantification was repeated 3 times.

**Scanning Electron Microscopy (SEM)**

After the cells were cultivated for 9 days, the media was removed and the plated substrates were placed in a solution containing 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA), 3% sucrose (Sigma-Aldrich Co., Irvine, UK) and 0.1 M of PBS at 4°C and pH 7.4. After 1 h, the substrates were rinsed twice with PBS for 30 min at 4°C and washed with distilled water for 5 min. Dehydration was achieved by placing the plated substrates in 50% ethanol for 15 min while increasing the concentration of ethanol to 60, 70, 80, 90 and finally 100%. Dehydrated samples were then mounted on aluminum stubs, sputter-coated with gold-palladium, and examined using SEM.
**PicoGreen DNA Quantification**

CTP-seeded PDMS substrates were resuspended in 50 µl of lysis buffer (1% sodium dodecyl sulfate, 10 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM Tris-HCl, pH 8.1) to lyse the membranes of adherent CTP progeny. After 60 min, the samples were centrifuged at 14,000 rpm for 5 min and the supernatant was removed for analysis. A 40 µL sample of aqueous supernatant containing DNA was added to 0.96 mL TE buffer (10 mM Tris adjusted to pH 7.0 with HCl, 1 mM EDTA). As per the manufacturer’s instructions (Molecular Probes, Eugene OR), stock PicoGreen reagent was diluted 1:200 in TE buffer and 1 ml of that was added to each DNA containing sample. The tubes were capped, vortexed, and incubated at room temperature in the dark room for 3 min. The fluorescence was measured with a SpectraMax Gemini fluorescence microplate reader (Molecular Devices Co., Sunnyvale, CA) at excitation and emission wavelengths of 480 and 520 nm, respectively. All calibration samples were assayed four times and a fresh calibration curve was generated for each 96 well plate. Baseline fluorescence was determined with a TE blank, the average of which was subtracted from the averaged fluorescence of other samples. Using this analysis, we determined that ~4.5 µg of DNA in 1 X 10^6 adherent CTPs. Thus, we assumed that one cell has ~4.5 pg of DNA, and estimated the number of cells for each sample. Because individual donors differed with respect to the initial prevalence of CTPs, the cell count on the substrates were normalized to the control surfaces for each donor within the particular experiment. The PicoGreen DNA quantification repeated a total of 9 times (replicates of 3 times of the each 3 patients; n=9/substrate) as per our standard lab protocols (n = 9/group; mean + SE).
**DAPI Stain**

Cell nuclei were stained with 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI). Ethanol-fixed cells were rinsed three times with phosphate buffered saline (PBS), and then a 10 µL drop of DAPI-containing Vectashield mounting media (Vector Labs, Burlingame, CA) was placed on the scaffolds. Immediately thereafter, the edges of the coverslips were sealed with three coats of clear nail polish and viewed under a fluorescent microscope (Olympus BX50F, Olympus Optical Co., Japan).

**Alkaline Phosphatase Stain**

After DAPI staining, the same samples were again stained *in situ* for AP, using the Vector Red substrate, working solution (5 ml of 100 mM Tris-HCl adding 2 drops of Reagent 1, 2 and 3) for 30 min at room temperature in the dark, and then, washed in distilled water. The positively stained cells with AP activity appeared red when viewed under a fluorescent microscope.

**Statistical Analysis**

The mean and standard deviation values were calculated using the data of all groups. All data was subjected to analysis of variance (ANOVA) and Tukey testing where appropriate (SPSS Version 10.0., SPSS INC., Chicago, IL). Significance levels were set at $p < 0.05$. 
4.3 RESULTS

4.3.1 CELL MORPHOLOGY ON PDMS SUBSTRATES

SEM examinations confirmed that the PDMS post microtextures are 10 µm diameter, and 5 µm, 10 µm, 20 µm, and 40 µm heights with the 10 µm, 20 µm, and 40 µm separation (inter-spaces) between the posts (Figure 4.5).

Figure 4.5    SEM images of PDMS post microtextures of 10 µm diameter, and 5 µm, 10 µm, 20 µm, and 40 µm heights with the 10 µm, 20 µm, and 40 µm separation (IS=inter-spaces) between the posts.
Figure 4.5 (a) (Continued) PDMS post microtextures of 10 µm diameter, 5 µm height with the 10 µm, 20 µm, and 40 µm separation between the posts.

Figure 4.5 (b) (Continued) PDMS post microtextures of 10 µm diameter, 10 µm height with the 10 µm, 20 µm, and 40 µm separation between the posts.
Figure 4.5 (c) (Continued) PDMS post microtextures of 10 µm diameter, **20 µm height** with the 10 µm, 20 µm, and 40 µm separation between the posts.

Figure 4.5 (d) (Continued) PDMS post microtextures of 10 µm diameter, **40 µm height** with the 10 µm, 20 µm, and 40 µm separation distances between the posts.
CTPs attached and proliferated on all PDMS post microtextures and smooth surfaces (Figure 4.6). On post microtextures, cells migrated within the posts exhibiting narrower shapes and higher contours than cells on smooth surfaces. The cells on post microtextures with 10 µm and 20 µm inter-spaces tended to attach next to the posts and spread between and top of them, exhibited highly contoured morphology, and directed their long processes towards posts and other cells. However, the morphology of cells grown on the post microtextures with 40 µm heights was different from those on the other post microtextures (Figure 4.6(d)). Cells on post microtextures with 40 µm heights pulled and bent posts around cells, and cell morphology grown on post microtextures with 40 µm heights and 40 µm inter-spaces exhibited similar shape to cells on smooth surfaces. Cells on smooth surfaces appeared to anchor to random locations on the surface as they migrated and cell bodies adopted a broad flattened shape (Figure 4.6(a)).

At the interface between post microtextures and smooth surfaces, cells were observed to attach to the posts and stay within the posts. This behavior is confirmed in Figure 4.7 where fixed cells, stained with DAPI and AP, clearly shows the preference of cells to stay and grow on the post microtextures that have 10 µm and 20 µm inter-spaces compared to the post microtextures that have 40 µm inter-space and smooth surfaces.

Cells on all substrates stained positive for alkaline phosphatase (AP), which is used as an early marker of osteoblastic differentiation (Figure 4.7). In general, cells on the post microtextures stained more intensely for AP compared to smooth substrates on day 9, but more especially so, on the post microtextures with 10 µm and 20 µm inter-spaces.
Figure 4.6  SEM images showing morphology of cells grown on the PDMS post microtextures that are 10 µm diameter, (a) 5 µm, (b) 10 µm, (c) 20 µm, and (d) 40 µm heights with the 10 µm, 20 µm, and 40 µm separation (IS=inter-spaces) between the posts. The cells on post microtextures tended to attach next to the posts and spread between them while directing their processes towards posts and other cells (white arrows). Regardless post heights, the narrowest processes were observed on cells cultured on the post microtextures with 10 µm IS, in which individual cells tended to grow between and along the array of posts. On the smooth surfaces and post microtextures with 40 µm IS, cell bodies adopted a broad flattened shape and appeared to anchor to random locations on the surface as they migrated. Cells were cultured for 9 days.
Figure 4.6 (Continued)
Figure 4.7 (a) Fluorescent microscopy images of CTPs near the interface between a smooth surface and PDMS post microtextures that are 10 µm diameter, 5 µm height with the 10 µm, 20 µm, and 40 µm separation between the posts (IS). More cells stained intensely for DAPI (blue) and AP (red) on the post microtextures with 20 µm inter-spaces. Cells were cultured for 9 days.

Figure 4.7 (b) Fluorescent microscopy images of CTPs near the interface between a smooth surface and PDMS post microtextures that are 10 µm diameter, 10 µm height with the 10 µm, 20 µm, and 40 µm separation distances between the posts. More cells stained intensely for DAPI and AP on the post microtextures with 10 µm inter-spaces. Cells were cultured for 9 days.
Figure 4.7 (c) Fluorescent microscopy images of CTPs near the interface between a smooth surface and PDMS post microtextures that are 10 µm diameter, **20 µm height** with the 10 µm, 20 µm, and 40 µm separation distances between the posts. More cells stained intensely for DAPI and AP on the post microtextures with 10 µm and 20 µm inter-spaces. Cells were cultured for 9 days.

Figure 4.7 (d) Fluorescent microscopy images of CTPs near the interface between a smooth surface and PDMS post microtextures that are 10 µm diameter, **40 µm height** with the 10 µm, 20 µm, and 40 µm separation distances between the posts. More cells stained intensely for DAPI and AP on the post microtextures compared to smooth surfaces. Cells were cultured for 9 days.
### 4.3.2 CELL PROLIFERATION

CTPs attached, and proliferated on all PDMS substrates. All post microtextures had increased numbers of cells compared to the smooth surfaces, as illustrated in Figure 4.8(a). The maximum number of cells was observed on the post microtextures with 20 μm height and 10 μm inter-space (20H-10IS), which exhibited around seven times (675%) compared with the number of those on the smooth surfaces (100%) ($p < 0.05$). Among the post microtextures with same height and different inter-spaces, 10 μm inter-space (10IS) showed highest cell numbers. Among the post microtextures with same inter-space and varying heights, post with 20 μm height (20H) showed maximum cell numbers.

![Figure 4.8](image)

**Figure 4.8** CTP proliferation on PDMS post microtextures and corresponding smooth surfaces after 9 days of culture. (a) All post microtextures had increased numbers of cells compared to the smooth surfaces. The maximum number of cells was observed on the post microtextures with 20 μm height and 10 μm inter-space (20H-10IS), compared with the number of those on the smooth surfaces ($p < 0.05$). For the same height, post microtextures with 10 μm inter-space have shown highest cell numbers. For the same inter-space, post microtextures with 20 μm height have shown highest cell numbers. * denotes statistical significance compared to other surfaces ($p < 0.05$).
Figure 4.8 (b) Post microtextures with 10 μm height and 10 μm inter-space (10H-10IS) have shown cell numbers normalized to surface area that are nearly three times greater than that on the control surface, while 40 μm height and 20 μm inter-space (40H-20IS) post microtextures exhibited the lowest cell number/unit area. For the same height, post microtextures with 10 μm and 40 μm inter-spaces have shown higher cell numbers while 20 μm inter-space have shown lowest cell numbers. For the same inter-space, post microtextures with 40 μm height have shown lowest cell numbers.

The cell number normalized to surface area on the post microtextures with 10 μm heights and 10 μm inter-spaces (10H-10IS) was more than three times (330%) greater than that on the control smooth surface (100%), while 40 μm height and 20 μm inter-space (40H-20IS) post microtextures exhibited the lowest normalized cell number (62%) (Figure 4.8(b)). For the same height, post microtextures with 10 μm inter-space (10IS) showed higher normalized cell numbers while 20 μm inter-space (20IS) showed lowest normalized cell numbers. For the same inter-space, post microtextures with 10 μm height (10H) exhibited highest normalized cell numbers while post microtextures with 40 μm height (40H) showed lowest normalized cell numbers.
4.4 DISCUSSION AND CONCLUSIONS

CTP culture on post microtextures with varying geometry and arrangement can result in the modification of cell morphology, proliferation, and has shown potential to enhance specific cell differentiation (AP stain). Specifically, cells on post microtextures with 10 μm height and 10 μm inter-space exhibited higher cell number compared to other microposts with different size of heights or inter-spaces, and smooth surfaces. The optimal aspect-ratio of microposts on a substrate surface could be utilized to investigate the effect of the geometric control on many other cellular activities, such as cell motility, cell proliferation, and stem cell differentiation. Thus, the criterion to determine the optimal aspect-ratio of the microposts is important, and therefore should be considered very carefully.

Surface post microtextures should provide a large number of extracellular matrix (ECM) contacts to cells to perform tissue-specific function. The Chen group has investigated human mesenchymal stem cells on the different sizes of micropatterns, and found that cells occupying the larger size of ECM contacts underwent osteogenesis, while cells on small ECM contacts appeared more adipogenic. In general, post microtextures have larger surface areas and adhesions with more ECMs compared to smooth surface. For example, the surface area of post microtextures with 10 μm diameter and 10 μm height, with 10 μm inter-space between posts was 1.8 times greater than that of the smooth surface. However, too large inter-space separation causes small surface adhesions with ECM. For example, surface area of post microtextures with 10 μm diameter and 10 μm height, with 40 μm inter-space between posts was only 1.1 times greater than that of the smooth surface. Moreover, in our study, the morphology of cells grown on the post
microtextures with 40 μm inter-space was different from those on the post microtextures with 10 μm and 20 μm inter-spaces, but exhibited similar cell morphology as on smooth surfaces (Figure 4.6(d)). Similarly, Turner et al.\textsuperscript{73} reported that the larger microarray features than an optimal size stimulated the same response as the smooth surfaces. Not only large inter-spaces, but also inter-space smaller than the cell size causes less ECM adhesions to cells. This is because cells cannot readily occupy the spaces between microposts, and therefore, tend to remain only on top of posts.\textsuperscript{62,73}

It is known that changing micropost height effectively varies surface stiffness without altering the bulk mechanical properties or the surface chemistry of the material used to fabricate the substrate.\textsuperscript{59,73,168} More specifically, Tan et al.\textsuperscript{59} demonstrated that PDMS microposts of different dimensions (diameter and height) expressed a range of stiffness values. Because the stiffness of microposts varies as the inverse cube of their height, decreasing the height by half caused a local change in stiffness by 8-fold.\textsuperscript{59} Theoretically, shorter microposts cause higher stiffness, and higher stiffness allows for the acceleration of cell growth. However, decreasing post heights than the optimal size causes fewer adhesions with ECM, and subsequent poor cell growth behavior.\textsuperscript{73,168} Thus, this present study demonstrates the applicability of microposts on substrates for modifying CTP behavior. These findings are of importance in that biomaterials for tissue engineering scaffolds should be designed to control the spatial organization of attached cells in order to direct specific tissue structures.

The incorporation of micro-scale topographies at the cell–substrate interface can provide an attractive approach to enhance specific cell behavior without destabilizing the delicate biochemical environment. However, the mechanisms by which cell behavior
changes in response to different geometrical surface topography stimuli remain unclear. Further investigation is needed to elucidate all of the possible factors and establish definitive mechanistic links between cell-surface interactions and cell growth characteristics.\textsuperscript{110}

The present study is unique not only in designing post microtextures with varying geometry and arrangement, but also in comparing CTP growth behaviors on these microposts and in establishing optimal micropost sizes (10 μm diameter, 10 μm height and 10 μm inter-space). Knowledge of CTP response to surface stimuli could lead to the incorporation of specific size post microtextures into surfaces of bone implants to achieve surface-textured 3D scaffold materials for bone grafting or augmenting fracture healing. The cell quantification on post microtextures demonstrates that a small change in geometry of surface post microtextures can have either small or dramatic effects on cell behavior. Therefore, there is great value in using microfabrication techniques in the production of scaffolds to obtain precise and optimal surface post microtextures that can selectively stimulate specific cell behavior.
CHAPTER V

EFFECT OF SUBSTRATE STIFFNESS AND SURFACE POST MICROTEXTURES ON CTP BEHAVIOR

5.1 INTRODUCTION

Understanding and optimizing cell-substrate interactions is critical to the rational design of implant materials and tissue engineering scaffolds, particularly for bone healing applications. Successful bone fracture healing depends upon having an osteoactive environment. The surface of bone grafts should allow transplanted cells to retain their differentiated function and promote cell growth. Also, proper mechanical property range of graft material should stimulate cell growth. In bone tissue engineering, however, the number of materials used to investigate the effects of surface topography or mechanical properties, such as stiffness, on cell response has been limited.

Polydimethylsiloxane (PDMS) is a silicone elastomer with desirable properties that make it attractive for the development of a microelectromechanical system (MEMS) for biomedical applications. In addition, PDMS has been used as a
biomaterial in a number of biomedical MEMS (bioMEMS) applications including biosensors, tissue engineering scaffolds, cell sorting and analysis devices, and various microfluidic devices for biological applications. PDMS is nontoxic, transparent, chemically inert, simple to handle and manipulate, less expensive than silicone, and can conform to submicron features to develop microstructures. PDMS has a more physiologically relevant range of stiffness (~MPa), while the range of other polymeric biomaterials such as polyacrylamide, alginate and agarose is much lower (~kPa) than the stiffness of many human tissues.

The applications of MEMS-based devices in the biomedical arena that use PDMS as a biomaterial has been largely driven by the development of soft lithography techniques such as microtransfer molding, microcontact printing, replica molding, and solvent-assisted micromolding. These techniques usually require the use of PDMS to create an elastomeric stamp or mold that incorporates microstructures to transfer patterns onto a subsequent substrate to explore selective cellular responses to specific substrate characteristics. Numerous studies have demonstrated that the biological performance of cells with respect to proliferation, migration, and differentiation can be significantly modified by different topographical features on a PDMS scaffold surface.

Recently, several research groups have reported that alterations of substrate stiffness can influence cell proliferation directly. Rowlands et al. reported that stiffer substrates encouraged up to a 10-fold increase in mesenchymal stem cell numbers over soft substrates. Tzvetkova-Chevolleau et al. demonstrated that fibroblast cells exhibit differential morphology and motility responses to changes in
substrate stiffness. Moreover, Chen et al.\textsuperscript{110,170} used unoptimized substrates that were patterned with two levels of stiffness (PDMS with a base:cure ratio of 50:1 and 10:1) and demonstrated that fibroblasts and endothelial cells accumulated preferentially on stiffer regions of PDMS substrates. However, comparatively little is known about the cellular effects of the extended range of substrate stiffness, and no studies have presented a systematic analysis of the combined effects of the surface microtopography and varying stiffness on stem cell behavior. Indeed, it remains largely unclear how cells sense combinations of these two different types of structural factors.\textsuperscript{113-115,171}

In this study, a novel approach is used to systematically investigate the effects of PDMS surface topography and optimized stiffness on the behavior of connective tissue progenitor cells (CTPs). The PDMS post microtextures were developed using soft lithography techniques and were correlated to preferential CTP behavior as a function of varying stiffness.

### 5.2 MATERIALS AND METHODS

#### 5.2.1 SUBSTRATE PREPARATION

In this study, PDMS pre-polymer and cross-linker were combined at various weight ratios designated as PDMS1, PDMS2, PDMS3, PDMS4, and PDMS5, corresponding to 5.7, 10.0, 14.3, 21.4, and 42.9 wt.% cross-linker, respectively.\textsuperscript{112} The PDMS microtextures were manufactured by the soft lithography technique (Figure 5.1(a)). Briefly, a 6 µm thick layer of SU-8 2010 photoresist was coated on top of a silicon wafer. By using UV photolithography, the 10 µm diameter texture pattern was
transferred from a photomask onto the photoresist, and then developed and cured at 120°C. PDMS Sylgard ® 184 (Dow Corning Corp., Midland, MI) pre-polymer and cross-linker mixtures with various weight ratios were poured on top of the patterned master and cured at 85°C for 2 h. Unpatterned (smooth) PDMS substrates were served as the control surfaces for cell growth experiments, and also used to determine storage modulus values, which represented stiffness of the various PDMS formulations.

Figure 5.1 (a) Fabrication of PDMS post microtextures and smooth PDMS by soft lithography.
Figure 5.1  (b) 5 different types of PDMS microtextures and smooth PDMS were bonded together in place to make a Square- or a Circular-shaped substrate with varying stiffness regions.

To investigate the preferential cell proliferation and osteogenic differentiation, two different shaped substrates, which combine 5 different types of PDMS microtextures and smooth PDMS, were developed. Each PDMS was cut and bonded together in place to make a square-\textsuperscript{171} or a circular-shaped topographical map of PDMS substrate (Figure 5.1(b)). To bond the PDMS sections, a small amount of PDMS pre-
polymer was injected into the gap between the samples. The composition of the PDMS matched the composition of one of the adjacent samples. For example, either PDMS4 or PDMS5 pre-polymer was used to bond the PDMS4 and PDMS5 samples, PDMS4 or PDMS3 pre-polymer was used to bond the PDMS3 and PDMS4 sample together, and so on. In the circular shaped substrate, the duplicity of the regions should serve as a check and balance system for filtering out errant results. Results that appear in one region should reoccur in the sister region, thus identifying that characteristic as possibly being preferentially induced due to the specific topographical region on the substrate.

5.2.2 ELASTIC MODULUS OF PDMS

Stiffness of a polymer refers to the resistance of the viscoelastic material to deformation by an applied force.\textsuperscript{113} Elastic modulus is directly related to stiffness; therefore, the terms stiffness and elastic modulus are used interchangeably.\textsuperscript{113,169} The elastic modulus of the PDMS substrates was determined using a tensile test method using an MTS Alliance\textsuperscript{TM} RT/5 material testing system (MTS Corp., Oak Ridge, TN).\textsuperscript{113} Substrates were cut to a dumbbell shape, sterilized, and tested. Testing was carried out according to the ASTM D 412 standard for rubber and thermoplastic elastomers with the modification that the dumbbell-shaped test specimens were made one-fourth the standard size.\textsuperscript{112} Figure 5.2 presents a schematic illustration of the sample geometry prior to testing. The design of the custom grips was selected because it allows for maximum contact surface area with the specimen, while reducing the stress concentration on the specimen near the edges of the grip.\textsuperscript{112}
5.2.3 CELL CULTURE

As described by Muschler et al., bone marrow aspirates were harvested from the anterior iliac crest with informed consent from four patients immediately prior to elective orthopedic procedures. Briefly, 2 mL samples of bone marrow were aspirated from the anterior iliac crest into 1 mL of saline containing 1000 units of heparin (Vector, Burlingame, CA). The heparinized marrow sample was suspended into 20 mL of heparinized carrier media (α-minimal essential medium (α-MEM) + 2 units/mL of Na-heparin; Gibco, Grand Island, NY) and centrifuged at 1500 rpm (400 X) for 10 min. The buffy coat was collected, resuspended in 20 mL of 0.3% bovine serum albumin-MEM (Gibco), and the number of nucleated cells was counted. The PDMS substrates were sterilized for 30 min with 70% ethanol. Cells were then plated on day 0 at a seeding concentration of 1 X 10⁶ cells per well and were cultured for 10 and 30 days.
under conditions promoting osteoblastic differentiation. Cell characteristics on PDMS substrates were investigated using scanning electron microscopy (SEM), PicoGreen DNA quantification, fluorescent stains, and real time reverse transcript-polymerase chain reaction (real time RT-PCR). In this study, the PicoGreen DNA quantification was repeated 3 times and the real time RT-PCR was also repeated 3 times.

5.2.4 CELL CULTURE ANALYSES

Scanning Electron Microscopy (SEM)

After the cells were cultivated for 30 days, the media was removed and the plated substrates were placed in a solution containing 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA), 3% sucrose (Sigma-Aldrich Co., Irvine, UK) and 0.1 M of PBS at 4°C and pH 7.4. After 1 h, the substrates were rinsed twice with PBS for 30 min at 4°C and washed with distilled water for 5 min. Dehydration was achieved by placing the plated substrates in 50% ethanol for 15 min while increasing the concentration of ethanol to 60, 70, 80, 90 and finally 100%. Dehydrated samples were then mounted on aluminum stubs, sputter-coated with gold-palladium, and examined using SEM.

PicoGreen DNA Quantification

The bonded PDMS substrates were cut into separate sections with varying stiffness and resuspended with 50 μl of lysis buffer (1% sodium dodecyl sulfate, 10 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM Tris-HCl, pH 8.1) to lyse the
membranes of adherent CTP progeny. After 60 min, the samples were centrifuged at 14,000 rpm for 5 min and the supernatant was removed for analysis. A 40 μL sample of aqueous supernatant containing DNA was added to 0.96 mL TE buffer (10 mM Tris adjusted to pH 7.0 with HCl, 1 mM EDTA). As per the manufacturer’s instructions (Molecular Probes, Eugene OR), stock PicoGreen reagent was diluted 1:200 in TE buffer and 1 ml of that was added to each DNA containing sample. The tubes were capped, vortexed, and incubated at room temperature in the dark room for 3 min. The fluorescence was measured with a SpectraMax Gemini fluorescence microplate reader (Molecular Devices Co., Sunnyvale, CA) at excitation and emission wavelengths of 480 and 520 nm, respectively. All calibration samples were assayed four times and a fresh calibration curve was generated for each 96 well plate. Baseline fluorescence was determined with a TE blank, the average of which was subtracted from the averaged fluorescence of other samples. Using this analysis, we determined that ~4.5 μg of DNA in 1 X 10^6 adherent CTPs. Thus, we assumed that one cell has ~4.5 pg of DNA, and estimated the number of cells for each sample. Because individual donors differed with respect to the initial prevalence of CTPs, the cell count on the substrates was normalized to the control surfaces for each donor within the particular experiment.

**DAPI Stain**

Cell nuclei were stained with 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI). Ethanol-fixed cells were rinsed three times with phosphate buffered saline (PBS), and then a 10 μL drop of DAPI-containing Vectashield mounting media (Vector Labs, Burlingame, CA) was placed on the scaffolds. Immediately thereafter,
the edges of the coverslips were sealed with three coats of clear nail polish and viewed under a fluorescent microscope (Olympus BX50F, Olympus Optical Co., Japan).

**Alkaline Phosphatase (AP) Stain**

After DAPI staining, the same samples were again stained *in situ* for AP, using the Vector Red substrate, working solution (5 ml of 100 mM Tris-HCl adding 2 drops of Reagent 1, 2 and 3) for 30 min at room temperature in the dark, and then washed in distilled water. The positively stained cells with AP activity appeared red when viewed under a fluorescent microscope.

**Osteocalcin (OC) immunohistochemistry (IHC)**

The cells were rinsed with PBS and fixed in 1% hydrogen peroxide for 10 min at room temperature followed by incubation in 1.5% blocking serum (rabbit ABC staining system, Santa Cruz Biotech, CA) 60 min to block nonspecific binding. Osteocalcin (OC) primary antibody (Santa Cruz Biotech) was diluted 1/100 in PBS and incubated with cells overnight at 4°C. After washing cells three times in PBS, they were incubated for 1 h with Biotinylated secondary antibody, 30 min with AB enzyme reagent, and 10 min with Peroxidase substrate (ABC staining system). Between each step, cells were rinsed three times with PBS. Secretion of OC was confirmed visually under a phase contrast microscope.
Real Time Reverse Transcript – Polymerase Chain Reaction (Real Time RT-PCR)

The expression of osteoblast specific genes, such as AP, OC and collagen type I (Col I), were detected by real time RT-PCR. Total cellular RNA was isolated using an RNeasy kit (Qiagen Inc., Valencia, CA) and reverse transcribed by conventional protocols with a Sensiscript Reverse Transcription kit (Qiagen Inc). The expression of the AP, OC, Col I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using real time RT-PCR analysis with a Power SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA). GAPDH is an enzyme utilized in cellular metabolism and is assumed to be expressed at the same level in most cells; therefore, gene expression of GAPDH was used as an internal control to normalize out any differences in the amount of total isolated RNA. Primer sequences are presented in Table 3.1. Real Time quantitative PCR was performed on a 7500 Real Time PCR system (Applied Biosystems). Data analysis was carried out using the 7500 System Sequence Detection software (Applied Biosystems).147

Statistical Analysis

The mean and standard deviation values were calculated using the data of all groups. All data was subjected to analysis of variance (ANOVA) and Tukey testing where appropriate (SPSS Version 10.0., SPSS Inc., Chicago, IL). Significance levels were set at $p < 0.05$. 
5.3 RESULTS

5.3.1 ELASTIC MODULUS (STIFFNESS) OF PDMS

The elastic modulus of five PDMS formulations was evaluated using the tensile test method. The elastic modulus was calculated from the slope of the plot of strain versus stress. Elastic modulus increased from 0.78 MPa (PDMS1) to 2.83 MPa (PDMS3), and decreased down to 1.66 MPa (PDMS5) (Figure 5.3). Even though the manufacturer’s recommended optimum formulation is PDMS2, our observation suggests that the highest elastic modulus was for PDMS3.

Figure 5.3 The elastic modulus (σ) of 5 different PDMS samples. Data is obtained from tensile tests on PDMS1, PDMS2, PDMS3, PDMS4, and PDMS5. Elastic modulus increased from PDMS1 to PDMS3, and decreased from PDMS3 to PDMS5. The test repeated a total of 6 times (n=6 for each substrate). * denotes statistical significance compared to other substrates ($p < 0.05$).

5.3.2 CELL MORPHOLOGY AND PROLIFERATION

The PDMS sections remained bonded together successfully for up to 30 days without failure. This approach enabled cells to preferentially migrate and grow on the
different PDMS substrates. Figure 5.4 presents images of cells stained with DAPI, which clearly shows the preference of cells to stay and grow on the PDMS2, PDMS3 and PDMS4 post microtextures compared to the PDMS1 and PDMS5 post microtextures and smooth surfaces. Cells were observed to spread and grow at the interface between PDMS sections, excluding PDMS1 and PDMS5. The bonded PDMS substrates with varying stiffness were cut for the following SEM (Figure 5.5) and staining (Figure 5.7) investigations to get clear images.

![Figure 5.4](image)

**Figure 5.4** The DAPI fluorescence images of CTP progeny on (a) post microtextures and (b) smooth surfaces of PDMS 1-5 on day 10. The number of CTPs on PDMS2, PDMS3, and PDMS4 were greater than cell numbers exhibited on the PDMS1 and PDMS5. The variation in the focal distance for the two sections results from the difference in the overall heights of the PDMS substrates.
The various PDMS cross-linker weight ratios did not change the morphology of cells on post or smooth surfaces. However, the morphology of cells grown on the post microtextures was different from those on the corresponding smooth surfaces (Figure 5.5). Cells on the smooth surfaces exhibited arbitrary shapes and migrated without any preferred orientation for up to 30 days. In contrast, CTPs on post microtextures on day 10 mostly tended to attach next to the posts and spread between them while directing their processes toward posts and other cells. By day 30, numerous cells had spread over the top of the post microtextures and covered nearly the entire surface with ECM.

![SEM images of CTP progeny on post microtextures and smooth surfaces of PDMS1-5 on day 10, and 30. The various PDMS cross-linker weight ratios did not change the morphology of cells on both surfaces. However, CTPs attached to post microtextures, and smooth surfaces with different cell morphology. Cells on the smooth surfaces exhibited arbitrary shapes and migrated without any preferred orientation for up to 30 days. In contrast, CTPs on post microtextures on day 10 mostly tended to attach next to the posts and spread between them while directing their processes toward posts and other cells. By day 30, numerous cells had spread over the top of the post microtextures and covered nearly the entire surface with ECM.](image)
Cell numbers calculated using PicoGreen DNA quantification analysis also revealed that the CTP proliferation was proportional to the PDMS stiffness level of post microtextures and smooth PDMS (Figure 5.6). On both substrate surfaces, the number of CTPs on PDMS2, PDMS3, and PDMS4 was greater than cell numbers exhibited on the PDMS1, and PDMS5 on day 30. PDMS post microtextures exhibited a greater number of CTPs compared to smooth PDMS \( (p < 0.05) \) at 30 days. These results demonstrated that the combination effects of surface microtextures and stiffness substrate (PDMS) accelerate cell growth.

![Figure 5.6](image-url)  
**Figure 5.6**  
CTP proliferation on PDMS post microtextures and corresponding smooth surfaces. Cell numbers calculated using PicoGreen DNA quantification analysis revealed that PDMS post microtextures exhibited a greater number of CTPs compared to smooth PDMS \( (p < 0.05) \) for 30 days. On day 20, the CTP proliferation was proportional to the PDMS stiffness level on both post microtextures and smooth PDMS. On both substrate surfaces, the number of CTPs on PDMS2, PDMS3, and PDMS4 were greater than cell numbers exhibited on the PDMS1, and PDMS5 on day 30. \( n = 9/\text{group}; \text{mean} \pm \text{SD} \).
5.3.3 OSTEOGENIC DIFFERENTIATION

Figure 5.7 (a) AP mRNA expressed with higher levels on post microtextures compared to smooth surfaces. On post microtextures, the AP expression had increased by day 20, and then, decreased by day 30, except PDMS2 microtextures. The cells on PDMS3 and PDMS4 showed the highest levels of AP expression on day 20 ($p < 0.05$). In contrast, AP expression on all smooth surfaces had increased by day 30, except on PDMS5. Cells on the post microtextures stained more intensely for AP compared to smooth surfaces on day 10 and AP increased on all surfaces by day 30, especially on the PDMS2 and PDMS3 post microtextures. * denotes statistical significance compared to other substrates on day 20 ($p < 0.05$).
Figure 5.7 (b) The mRNA expression of OC increased greatly from day 10 to day 20 on each substrate, with consistently greater expression for 30 days. The highest level of OC expression was on PDMS2 smooth surface and post microtextures on day 30. Phase contrast images show OC immunohistochemistry (IHC) stain and the intensity of this stain on PDMS2 and PDMS3 post microtextures on day 30 greatly increased compared to the other substrates.
Figure 5.7 (c) Col I expression generally increased over time on both surfaces excluding the PDMS5 smooth surfaces. On day 30, post microtextures generally expressed consistently greater than smooth surfaces excluding PDMS1. Cells on smooth surfaces of PDMS2 and PDMS3 expressed the higher levels of Col I mRNA on day 30 compared to other smooth surfaces. For post microtextures, cells on PDMS2 expressed the highest levels on day 30.

Gene expression

The results of real time RT-PCR revealed that AP mRNA expression was higher on post microtextures compared to smooth surfaces for 30 days (Figure 5.7(a)). On post microtextures, the mRNA expression of AP had increased by day 20, and then decreased by day 30, except PDMS2, which decreased from day 10 to day 20, and increased again by day 30. The cells on PDMS3 and PDMS4 showed the highest levels of AP expression on day 20 ($p < 0.05$). In contrast, AP expression on all smooth surfaces had increased by day 30, except on PDMS5. The mRNA expression of OC significantly increased from day 10 to day 20 ($p < 0.05$) on both substrates, with consistently greater expression for 30 days (Figure 5.7(b)). However, there was no significant difference in OC mRNA expression between smooth substrates and post
microtextures for all days. These results indicate that osteogenesis, especially OC expression, had started by day 10 and increased immensely over time, and it is likely to be proportional to the stiffness level. Col I expression increased over time on both surfaces, but was generally greater on post microtextures than on smooth surfaces excluding PDMS5 smooth surfaces (Figure 5.7(c)). Cells on post microtextures of PDMS2 expressed the highest levels of Col I mRNA on day 30.

**Extracellular matrix (ECM) expression**

Cells on all substrates stained positive for AP, which is used as an early marker of osteoblastic differentiation (Figure 5.7(a)). Cells on the post microtextures stained more intensely for AP compared to smooth substrates on day 10, and AP increased on all substrates by day 30, especially on the PDMS2 and PDMS3 post microtextures. IHC staining showed minimal OC intensity on the all substrates on day 10, but greatly increased by day 30 (Figure 5.7(b)). Furthermore, the intensity of the OC IHC stain on PDMS2 and PDMS3 post microtextures was much greater compared to the other substrates.

**5.4 DISCUSSION**

PDMS substrates with variable stiffness and specially designed surface topographies were developed to systematically investigate the combined effects on human bone marrow-derived CTP behavior. These results highlight that surface post microtextures and higher stiffness level of PDMS would allow for the acceleration of
CTP progeny growth and associated osteogenic indicators such as AP and Col I expression, as well as OC secretion.

The most widely used form of PDMS is Sylgard ® 184 (Dow Corning Corporation), which derives from a two part polymer: a pre-polymer and a cross-linker. Different PDMS proportions were previously formulated to investigate possible alterations in PDMS properties due to the deviation from the manufacturer’s recommended 10:1 weight ratio, which corresponds to our PDMS2 notation. The PDMS2 formulation was reported to be resistant to the majority of chemicals tested, and exhibited higher elastic modulus compared to other PDMS formulations. Nonetheless, the PDMS3 formulations in this study exhibited the highest elastic modulus (stiffness) and corresponding cell behavior, such as osteogenic differentiation. Surface stiffness can be effectively changed by varying geometry of micropost and material used as the substrate. More specifically, changing micropost geometry varies post stiffness without altering bulk mechanical properties or surface chemistry of the material used to fabricate the substrate. Thus, surface post microtopography, with same size and density, but different PDMS formulation ratio, could be used to vary the surface mechanical environment and directly modify the substrate stiffness, and consequently, cell behavior.

Results from this study appear consistent with findings from recent studies by Rowlands et al., who demonstrated that the rate of mesenchymal stem cell proliferation was proportional to the substrate stiffness. In another report, Wang et al. observed up to a 2-fold increase in proliferation when the stiffness of substrates was increased. Yim et al. also observed that both surface topography as well as the
mechanical properties of the substrate can have a significant effect on interactions between stem cells and their ECM, influencing focal adhesion formation, the organization of the cytoskeleton, and consequent cell growth.

In the present study, there was no significant difference in OC mRNA expression between smooth substrates and post microtextures on day 30 (Figure 5.7(b)), while AP and Col I mRNA expression were consistently greater on post microtextures than on smooth surfaces (Figure 5.7(a) and (c)). These results indicate that CTP proliferation and early osteogenic differentiation are more likely to be affected by surface microtextures, while late osteogenic differentiation is more likely to be proportional to substrate stiffness. It is known that substrate stiffness can direct mesenchymal stem cells to differentiate into specific lineages: a soft substrate induces a neurogenic phenotype, while increasingly stiffer substrates induce myogenic and osteogenic phenotypes accordingly, because it is easier for the cells to develop a higher cytoskeletal tension on a stiffer substrate.\textsuperscript{174} Taken together, the observations from microtopography-induced and stiffness-directed differentiation suggest that physical interactions between the cells and the extracellular environment, either in the form of topography or stiffness, or the combination thereof, can modulate cell function and stem cell growth behavior.

The mechanisms dealing with how cells sense and change their behavior in response to combinations of different types of structural factors, such as substrate stiffness and surface topography, remain unclear. One mechanism that was reported by Yim et al.\textsuperscript{174} and Schwartz et al.\textsuperscript{175} is attributed to integrin signaling through focal adhesion complexes. The phosphorylation of focal adhesion kinase can be high on
stiffer substrates, resulting in growth factor activation of extracellular signal-regulated kinase to promote proliferation and osteogenic differentiation. Even though the clarification of how cells respond to substrate stiffness via a mechanotransduction cascade is still a much-debated topic, the fact that CTP proliferation and osteogenic differentiation can be accelerated by certain levels of PDMS stiffness suggests that cells can respond to changes in substrate stiffness in a physiologically relevant manner.

5.5 CONCLUSIONS

It is important to keep in mind that material composition, structure, and processing, all affect the material properties; therefore, alterations in any of these factors could lead to changes in more than one cellular behavior. The incorporation of micro-scale surface topography and optimal stiffness levels at the cell–substrate interface might provide an attractive approach to enhancing specific cellular responses, such as cell proliferation and osteogenic differentiation, without destabilizing the delicate biochemical environment. For the first time, this work provides a comparison of the combined influences of variable substrate stiffness and precise topographical features on bone marrow derived human CTP behavior. Although these are preliminary results that must be interpreted with care, it has been shown that culturing CTPs under osteogenic conditions on stiffer post microtextures enhances proliferation and osteogenic differentiation when compared to cells on less stiff smooth surfaces.
CHAPTER VI

STUDY OF MICROTEXTURED BIODEGRADABLE MATERIALS FOR BONE TISSUE ENGINEERING APPLICATIONS

Substantial portions of this Chapter have been published in Journal of Biomedical Material Research A 2009;90A:1198-1205.

6.1 INTRODUCTION

In suboptimal bone fracture repair conditions where attachment, migration, proliferation, and differentiation of bone forming cells is hindered, bone grafts can play an important role in promoting a microenvironment in which bone formation is enhanced. A current state-of-the-art approach to enhance bone regeneration has been the transplantation of adult stem cells derived from human bone marrow onto an implantable scaffold. At the interface of cell and scaffold surface, properties such as chemical composition, surface energy, and structural topography profoundly affect the overall behavior of the engineered cell and tissue construct. Many studies have been
conducted to show that surface topographies influence morphology, proliferation, migration, and differentiation of cells.142,157,158,162-165

Numerous research groups have focused on polydimethylsiloxane (PDMS) as the scaffold material of choice for producing surfaces with microtopography.89,90,109,110 PDMS is a material that has been used extensively in medical implants and biomedical devices because of its biocompatibility, low toxicity, and thermal stability.90,109 The practicability of PDMS for fabrication with both rapid prototyping and mass production techniques as well as lower cost makes it particularly attractive for the development of biomedical microelectromechanical system (bioMEMS).90,109 However, PDMS is not biodegradable, and therefore, not a suitable material for bone tissue engineering applications.90,116 The desire for a biocompatible and biodegradable material as a substitute for PDMS motivates an examination of alternate scaffold biomaterials, such as cellulose acetate (CA), poly octanediol citrate (POC), poly lactic-co-glycolic acid (PLGA), and cyclic acetal monomer-5-ethyl-5-dimethyl-1,3-dioxane-2-ethanol diacrylate (EH-Network), for bone tissue engineering applications (Figure 6.1). These four biodegradable materials are not only biocompatible and biodegradable materials, but they also lend themselves to the molding of very fine micro-scale and intricate structures. They are also known to be nontoxic and inexpensive. Many reports117,121,122,125,127,131,133-137,178 have confirmed that these materials are well suited for optimization and control of cell adhesion and growth.

In this study, human bone marrow cells containing connective tissue progenitor cells (CTPs) were cultured on the selected biodegradable substrates, as an alternative to
PDMS, comprised of both post microtextures and smooth surfaces for up to 30 days to investigate and compare the cell behavior.

![Figure 6.1](image)

**Figure 6.1** Alternate biomaterials such as cellulose acetate (CA)\textsuperscript{117-126}, poly octanediol citrate (POC)\textsuperscript{132-135}, poly lactic-co-glycolic acid (PLGA)\textsuperscript{127-131}, and cyclic acetal monomer-5-ethyl-5-dimethyl-1,3-dioxane-2-ethanol diacrylate (EH-Network)\textsuperscript{136,137} are potential candidates for bone tissue engineering applications.

### 6.2 MATERIALS AND METHODS

#### 6.2.1 SUBSTRATE PREPARATION AND FABRICATION

The microfabricated CA, POC, PLGA, EH-Network, and PDMS scaffolds were manufactured by soft lithography techniques (Figure 6.2). Briefly, a 6 μm thick layer of SU-8 2010 photoresist was coated on top of a silicon (Si) wafer. By using ultraviolet (UV) photolithography, the 10 μm diameter texture pattern was transferred from a photomask onto the photoresist, which was then developed to create a SU-8 mold. For CA and POC microtextured substrates, the patterned SU-8 served as the master mold. For PLGA and EH-Network substrates, a corresponding PDMS mold was produced from the
patterned SU-8 as follows. First, the liquid PDMS base and curing agent (Sylgard 184; Dow Corning) components were mixed at a ratio of 10:1, then poured on the patterned SU-8 mold and cured at 85°C for 2 h. After release, the patterned PDMS substrate was used to mold another PDMS substrate (with patterns equivalent to the SU-8 mold), which served as the master mold.

CA was prepared by mixing 1g powder-type CA (Aldrich Chemical, Milwaukee, WI) and 13 mL of spectroscopic grade acetone (Aldrich Chemical) for 2 h to obtain a clear solution.\textsuperscript{117,122} 20 wt% PLGA was dissolved in chloroform and stirred for 3 h at 50–60°C.\textsuperscript{131} POC was obtained by mixing 1M octanediol (Aldrich Chemical) and 1M citric acid (Sigma Aldrich) for 1 h at 140°C.\textsuperscript{133,135} EH-Network compound (obtained from Dr. John Fisher’s group at University of Maryland)\textsuperscript{136,137} was dissolved in benzoyl peroxide (Sigma Aldrich) for 30 min. All mixtures were poured onto the patterned SU-8 or PDMS molds and dried slowly for 1 to 3 days at room temperature or 80°C oven. The cured materials were released from their molds, and representative samples were inspected by scanning electron microscopy (SEM; JSM-5310, JEOL, USA; Peabody, MA). An unpatterned SU-8 film was used to produce the corresponding smooth surfaces. In addition, PDMS microtextures and smooth surfaces were also produced as previously described in Chapter 3.
Figure 6.2 Development of surface microtopographies on biodegradable materials (CA, POC, PLGA, and EH-Network) using soft lithography.

Biodegradability test

Normal CA exhibits very slow degradation, and therefore, biodegradability of CA can be accelerated using cellulase.\textsuperscript{117} Cellulase refers to a class of enzymes that catalyze the cellulolysis (or hydrolysis of cellulose), thereby selectively breaking cellulose into smaller length chains down to its glucose repeat units. The general resistance of most cellulose-based materials to natural degradation in physiological conditions offers the possibility of controlling the degradation rate by applying a tight control of cellulase availability, since this enzyme is normally absent in mammals, yet cytocompatible.\textsuperscript{117} For CA degradation tests in this study, we used cellulase from \textit{Trichoderma reesei} (Sigma, St. Louis, MO), treating CA substrates with about 70 enzyme units for 24 hours at 37 °C, prior to 10 weeks of incubation.\textsuperscript{117}

In order to estimate and compare the biodegradability of selected materials (PDMS, CA, cellulase treated CA (CA(CE)), POC and PLGA), specimens (1 cm X 1 cm) of the various biodegradable materials were placed in a tube containing 15 ml of phosphate-buffered saline (PBS). Specimens of each material were incubated at 37 °C.
and tested weekly for degradation for up to 10 weeks. Samples were washed with distilled water and dried under vacuum for one week. Afterwards, mass changes were calculated by comparing the initial mass with the mass measured at a given weekly time point.\textsuperscript{133,135} The biodegradability test was repeated four times for each material.

### 6.2.2 CELL CULTURE AND ANALYSES

As described by Muschler et al.,\textsuperscript{21} bone marrow aspirates were harvested from the anterior iliac crest with informed consent from four patients immediately prior to elective orthopedic procedures. Briefly, 2 mL samples of bone marrow were aspirated from the anterior iliac crest into 1 mL of saline containing 1000 units of heparin (Vector, Burlingame, CA). The heparinized marrow sample was suspended into 20 mL of heparinized carrier media (\(\alpha\)-minimal essential medium (\(\alpha\)-MEM) + 2 units/mL of Na-heparin; Gibco, Grand Island, NY) and centrifuged at 1500 rpm (400 X) for 10 min. The buffy coat was collected, resuspended in 20 mL of 0.3% bovine serum albumin-MEM (Gibco), and the number of nucleated cells was counted. The CA (non-cellulase treated), PLGA and PDMS substrates were sterilized for 30 min with 70% ethanol. (Note: POC and EH-Network was not plated with cells due to reasons described later in Sections 6.3.2 and 6.3.4). Cells were then plated on day 0 at a seeding concentration of 1 X 10\textsuperscript{6} cells per well and were cultured for 10 and 30 days under conditions promoting osteoblastic differentiation.\textsuperscript{20} Cell characteristics on all substrates were investigated using SEM, PicoGreen DNA quantification, fluorescent stains, and real time reverse transcript - polymerase chain reaction (real time RT-PCR). In this study, the PicoGreen DNA quantification was repeated three times and the real time RT-PCR was also repeated three times.
Scanning Electron Microscopy (SEM)

After the cells were cultivated for 10 or 30 days, the media was removed and the plated substrates were placed in a solution containing 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA), 3% sucrose (Sigma-Aldrich Co., Irvine, UK) and 0.1 M of PBS at 4°C and pH 7.4. After 1 h, the substrates were rinsed twice with PBS for 30 min at 4°C and washed with distilled water for 5 min. Dehydration was achieved by placing the plated substrates in 50% ethanol for 15 min while increasing the concentration of ethanol to 60, 70, 80, 90 and finally 100%. Dehydrated samples were then mounted on aluminum stubs, sputter-coated with gold-palladium, and examined using SEM.

PicoGreen DNA Quantification

CTP-seeded CA, PLGA and PDMS substrates were resuspended with 50 µl of lysis buffer (1% sodium dodecyl sulfate, 10 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM Tris-HCl, pH 8.1) to lyse the membranes of adherent CTP progeny. After 60 min, the samples were centrifuged at 14,000 rpm for 5 min and the supernatant was removed for analysis. A 40 µL sample of aqueous supernatant containing DNA was added to 0.96 mL TE buffer (10 mM Tris adjusted to pH 7.0 with HCl, 1 mM EDTA). As per the manufacturer’s instructions (Molecular Probes, Eugene OR), stock PicoGreen reagent was diluted 1:200 in TE buffer and 1 ml of that was added to each DNA containing sample. The tubes were capped, vortexed, and incubated at room temperature in the dark room for 3 min. The fluorescence was measured with a SpectraMax Gemini
fluorescence microplate reader (Molecular Devices Co., Sunnyvale, CA) at excitation and emission wavelengths of 480 and 520 nm, respectively. All calibration samples were assayed four times and a fresh calibration curve was generated for each 96 well plate. Baseline fluorescence was determined with a TE blank, the average of which was subtracted from the averaged fluorescence of other samples. Using this analysis, we determined that ~4.5 μg of DNA in 1 X 10⁶ adherent CTPs. Thus, we assumed that one cell has ~4.5 pg of DNA, and estimated the number of cells for each sample. Because individual donors differed with respect to the initial prevalence of CTPs, the cell count on the substrates was normalized to the control surfaces for each donor within the particular experiment.

Alkaline Phosphatase (AP) Stain

Samples were again stained in situ for AP, using the Vector Red substrate working solution (5 ml of 100 mM Tris-HCl adding 2 drops of Reagent 1, 2 and 3) for 30 min at room temperature in the dark, and then, washed in distilled water. The positively stained cells with AP activity appeared red when viewed under a fluorescent microscope.

Real Time Reverse Transcript – Polymerase Chain Reaction (Real Time RT-PCR)

The expression of osteoblast specific genes, such as AP, OC and collagen type I (Col I), were detected by real time RT-PCR. Total cellular RNA was isolated using RNeasy kit (Qiagen Inc., Valentia, CA) and reverse transcribed by conventional protocols with a Sensiscript Reverse Transcription kit (Qiagen Inc). The expression of the AP, OC, Col I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using a
real time RT-PCR analysis with Power SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA). GAPDH is an enzyme utilized in cellular metabolism and is assumed to be expressed at the same level in most cells; therefore, gene expression of GAPDH was used as an internal control to normalize out any differences in the amount of total isolated RNA. Primer sequences are presented in Table 3.1. Real Time quantitative PCR was performed on a 7500 Real Time PCR system (Applied Biosystems). Data analysis was carried out using 7500 Sequence Detection software (Applied Biosystems).

**Statistical Analysis**

The mean and standard deviation values were calculated using the data of all groups. All data was subjected to analysis of variance (ANOVA) and Tukey testing where appropriate (SPSS Version 10.0, SPSS Inc., Chicago, IL). Significance levels were set at $p < 0.05$.

6.3 RESULTS

6.3.1 SURFACE CHARACTERIZATION OF BIODEGRADABLE MATERIALS

SEM examination revealed the post microtextures and smooth surfaces of biodegradable substrates, CA, PLGA, POC and EH-Network (Figure 6.3). Post microtextures that were 6 μm in height, 10 μm in diameter, and 10 μm inter-space between posts substrates were manufactured by soft lithography techniques. None of the post microtextures and smooth surfaces exhibited any significant geometrical variations with respect to the SU-8/PDMS master.
Figure 6.3  SEM images of post microtextures and smooth surfaces of biodegradable substrates, CA, PLGA, POC and EH-Network. Post microtextures that were 6 μm in height, 10 μm in diameter, and 10 μm inter-space between posts substrates were manufactured by soft lithography techniques. Also presented are SEM images of corresponding PDMS post microtextures and smooth surfaces, which served as a control group.

6.3.2 PRELIMINARY ASSESSMENT OF EH-NETWORK

A preliminary investigation into CTP growth characteristics revealed that cells on the EH-network showed compatible cell attachment (Figure 6.4). However, the lack of sufficient raw material from our collaborator (Dr. Fisher’s group at the University of Maryland,\textsuperscript{136,\textsuperscript{137}}) prevented subsequent cell behavior investigations.
Figure 6.4 The morphology of CTPs grown on the post microtextures and corresponding smooth surfaces of EH-Network materials on day 9. CTPs on post microtextures mostly tended to attach next to the posts and spread between them. Cells on smooth surfaces exhibited arbitrary flattened shapes.

6.3.3 BIODEGRADABILITY OF BIOMATERIALS

The selected biodegradable materials (CA, PLGA, and POC) and control (PDMS) were tested for degradation in PBS by measuring mass loss over 10 weeks (Figure 6.5). All biodegradable materials retained >95% of their original mass for 3 weeks (Figure 6.5(a)). The mass of PLGA polymer was reduced by ~18%, whereas the mass of cellulase treated CA (CA(CE)) and POC post microtextures were reduced by ~13% and the mass of CA(CE) and POC smooth surfaces were reduced ~10% at week 10 (Figure 6.5(b)). PDMS did not show any mass loss in 10 weeks.
Figure 6.5  (a) The selected biodegradable materials (CA, PLGA, and POC) and control (PDMS) were tested for degradation by measuring mass loss over 10 weeks. The mass of PLGA polymer was reduced by ~18%, whereas the mass loss of cellulase treated CA (CA(CE)) and POC post microtextures (T) were reduced by ~13% and the mass of CA and POC smooth surfaces (S) were reduced ~10% at week 10. PDMS did not exhibit any significant mass changes over the 10 week testing period.
Figure 6.5 (continued) (b) The cellulase treated CA (CA(CE)) and POC post microtextures (T) had reduced in mass by ~13% at week 10, while the mass of CA(CE) and POC smooth surfaces (S) had reduced by ~10%. The highest degradation was exhibited by the PLGA substrates, which reduced in mass by ~18%. 
6.3.4. CHEMICAL ANALYSIS OF POC

While the POC post microtextures were made successfully and exhibited desirable biodegradability characteristics, this material was not suitable for cell cultivation experiments. It was discovered that the pH of the culture media exposed to POC decreased drastically in 2 days, resulting in pH values of 3-4 (Figure 6.6(a)). Further investigations using hydrogen-nuclear magnetic resonance (1H-NMR, Figure 6.6(b) and (c)) and x-ray photoelectron spectroscopy (XPS, Figure 6.6(d)) revealed that the chemical components and structures of our POC and reference POC \textsuperscript{133-135} were identical. Nevertheless, CTP growth was not possible on the POC substrates, and subsequent cell culture investigations were not conducted with this biomaterial.

\textbf{Figure 6.6}  (a) Biocompatibility test of POC in cell culture media. In 2 days, POC released higher acid levels of acid (resulting in pH values 3-4) during degradation and cells could not survive in this environment.
The (b,c) 1H-NMR and (d) XPS test revealed that the chemical components and structures of our POC and reference POC are identical.
6.3.5 CELL MORPHOLOGY ON CA AND PLGA SUBSTRATES

Figure 6.7 The morphology of cells grown on the post microtextures and the corresponding smooth surfaces of biodegradable materials. CTPs on CA post microtextures on day 9 mostly tended to attach next to the posts and spread between them while directing their processes toward posts and other cells. By day 30, numerous cells had spread over the top of the CA post microtextures and covered most of the surface with ECM. However, it is difficult to distinguish cell morphology on PLGA microtextures on day 9 and 30 because of its fast degradation. Cells on all smooth surfaces exhibited arbitrary flattened shapes and migrated without any preferred orientation for up to 30 days.

The SEM images revealed that human CTPs attached to post microtextures and smooth surfaces with varying cell morphology (Figure 6.7 and Figure 6.8). Cells on CA and PLGA smooth surfaces exhibited arbitrary flattened shapes and proliferated without any preferred orientation for up to 30 days. However, the morphology of cells grown on the post microtextures was different from those on the corresponding smooth surfaces. CTPs on CA post microtextures on day 9 mostly tended to attach next to the posts and spread between them while directing their processes toward posts and other cells. By day 30, numerous cells had spread over the top of the CA post microtextures and covered most of the surface with ECM. However, it is difficult to distinguish cell morphology on PLGA microtextures on day 9 and 30 because of its fast degradation (Figure 6.7 and 6.9).
With degradation, the microposts became blunt and tiny pores were observed on both microtextures and smooth surfaces (Figure 6.9).

**Figure 6.8** Additional SEM images showing the morphology of cells grown on the post microtextures and the corresponding smooth surfaces of CA.
Figure 6.9  Additional SEM images showing the morphology of cells (white arrows) grown on the post microtextures and the corresponding smooth surfaces of PLGA. On day 30, cells on PLGA covered most of the surface with ECM. However, it is difficult to distinguish cell morphology on PLGA microtextures on day 9 and 30 because of its fast degradation. With degradation, the microposts became blunt and tiny pores were observed on both microtextures and smooth surfaces.

6.3.6 CELL PROLIFERATION ON CA AND PLGA SUBSTRATES

Cell numbers calculated using PicoGreen DNA quantification analysis revealed that post microtextures of CA, PLGA, and PDMS exhibited a greater number of CTPs compared to those of smooth surfaces for 30 days (Figure 6.10). From day 9 to day 30, the number of CTPs increased significantly ($p < 0.05$) on all substrates. On day 30, CA post microtextures exhibited the highest cell numbers compared to those of PLGA and PDMS ($p < 0.05$). However, there was no significant difference in cell proliferation between PLGA post microtextures and smooth surfaces on day 30.
Cell numbers on CA, PLGA, and PDMS post microtextures and smooth surfaces. DNA quantification analysis revealed that post microtextures of CA and PDMS exhibited a greater number of CTPs compared to those of smooth surfaces for 30 days. From day 9 to day 30, the number of CTPs increased significantly ($p < 0.05$) on all substrates. On day 30, CA post microtextures exhibited the highest cell proliferation compared to those of PLGA and PDMS ($p < 0.05$). However, there was no significant difference to cell proliferation between PLGA post microtextures and smooth surfaces on day 30. * denotes statistical significance compared to other substrates ($p < 0.05$) and # denotes statistical significance compared to control (PDMS) substrates ($p < 0.05$).

### 6.3.7 OSTEOSTIGENIC DIFFERENTIATION OF CTP ON CA AND PLGA SUBSTRATES

Cells on all CA substrates stained positive for AP, which is used as an early marker of osteoblastic differentiation (Figure 6.11). Cells on the CA post microtextures stained more intensely for AP compared to smooth substrates on day 9, and AP increased on all substrates by day 30, especially on the CA post microtextures compared to the PDMS substrates. Clear fluorescent images of cells on PLGA substrates could not be obtained. This is attributed to the auto-fluorescence and fast degradation of PLGA material (Figure 6.11).
Fluorescent microscopy images of CTPs on PDMS, CA and PLGA post microtextures, and smooth surfaces on: (a) day 9, and (b) day 30. Fluorescent images show cells stained with AP (red color). CTPs on the CA post microtextures stained more intensely for AP, a marker of osteoblastic phenotype, compared to PDMS post microtextures and all smooth surfaces on day 9, and AP increased on all scaffolds by day 30. Clear fluorescent images of cells on PLGA substrates could not be obtained. This is attributed to the fast degradation and auto-fluorescence of PLGA material (PLGA (no cells)).

The results of real time RT-PCR revealed that AP mRNA expression on PDMS post microtextures had decreased by day 30, while increasing on CA post microtextures (Figure 6.12(a)). In contrast, AP expression on PDMS and PLGA smooth surfaces had increased by day 30, except for the CA smooth surface. The mRNA expression of OC significantly increased from day 9 to day 30 ($p < 0.05$) on all substrates (Figure 6.12(b)).
The OC expression increased significantly on post microtextures relative to smooth surfaces by day 30 for PDMS and CA ($p < 0.05$). However, there was no significant difference between the OC mRNA expressions between PLGA post microtextures and smooth substrates on day 30. Col I expression increased over time on PLGA surfaces, and was consistently greater on post microtextures than on smooth surfaces excluding CA on day 9 (Figure 6.12(c)). Col I expression on both PDMS post microtextures and smooth surfaces, however, did not exhibit significant changes for 30 days.

Figure 6.12 mRNA expression of: (a) AP, (b) OC, and (c) Col I from CTPs after 9 and 30 days on post microtextures and smooth surfaces of PDMS, CA and PLGA. (a) AP mRNA expression on PLGA and PDMS post microtextures had decreased by day 30, while CA post microtextures increased with time. In contrast, AP expression on PDMS and PLGA smooth surfaces had increased by day 30, except for the CA smooth surface.
Figure 6.12 (continued) (b) The mRNA expression of OC significantly increased from day 9 to day 30 on all substrates ($p < 0.05$). The OC expression was increased on post microtextures relative to smooth surfaces by day 30. The highest level of OC expression was on CA and PDMS post microtextures on day 30. However, there was no significant difference to the OC mRNA expressions on PLGA post microtextures and smooth substrates on day 30. (c) Col I expression increased over time on PLGA surfaces ($p < 0.05$) but was consistently greater on post microtextures than on smooth surfaces excluding CA on day 9. Col I expression on both PDMS post microtextures and smooth surfaces, however, did not exhibit significant changes for 30 days. Numerical values denote mean and standard errors. * denotes statistical significance compared to same substrates on day 9 and # denotes statistical significance compared to smooth counterpart ($p < 0.05$).
6.4 DISCUSSION

The desire for a biocompatible and biodegradable material as a substitute for PDMS motivates an examination of alternate biomaterials, such as CA, POC, PLGA, and EH-Network. This study demonstrated the successful microfabrication of biodegradable materials, and subsequent cell culture on CA and PLGA. In particular, CA exhibited the higher cell proliferation and osteogenic differentiation compared to PLGA and PDMS.

Although POC post microtextures were fabricated successfully, the material resulted in high acidity levels (pH 3-4) during degradation (in 1~2 days, Figure 6.6(a)). In an effort to reduce acidity of POC that was synthesized using poly octanediol and citric acid, the amount of citric acid was reduced by up to 50% from the recommended protocol. However, this change did not sufficiently decrease acidity of the POC, and resulted in more compliant POC substrates.

For PLGA, many groups have reported that periodic changing of the buffer solution in vitro can prevent build up of an acidic environment. In this present study, the media buffer was replaced every 3 days, which was sufficient for successful cell culture experiments. Moreover, it is known that, in vivo, the final degradation products of PLGA, such as lactic or glycolic acids, are involved in the metabolic cycles of bio-organisms, and are gradually removed from the body by normal metabolic pathways in 6~12 months. Many studies have confirmed that PLGA exhibits acceptable biocompatibility, and this material is well suited for optimization of cell growth.

The PLGA microposts became shorter and more blunted with degradation (Figure 6.9). Tiny (nano-scale) pores were observed on microtextures as well as smooth surfaces,
probably owing to the dissolution of low molecular weight PLGA fragments.\textsuperscript{178} The fast degradation and blunting of PLGA post microtextures likely diminished the effects of surface microtopography on cells, thereby resulting in no significant difference in cell proliferation and osteogenic differentiation between PLGA post microtextures and smooth surfaces (Figure 6.10 and 6.12). Nevertheless, there was still more cell proliferation on PLGA compared to PDMS (Figure 6.10). Craighead et al.\textsuperscript{62} reported that nano-scale (less than 500nm) surface topographies, which are on the size order of cellular focal contacts may provide an effective stimulus for promoting differential cell attachment, growth, and differentiation. Thus, the effects of nano-scale surface topographies after degradation on biodegradable substrates on cell behavior should be studied in the future.

This study has shown that CA exhibited the highest cell proliferation and osteogenic differentiation compared to PLGA and PDMS. These results are consistent with previous reports, which demonstrate that substrate stiffness influences cell growth behavior. It is known that the elastic modulus of CA (\textasciitilde 1000 MPa)\textsuperscript{183} and PLGA (\textasciitilde 400 MPa)\textsuperscript{184} are higher compared to that of PDMS (\textasciitilde 2 MPa)\textsuperscript{112,113}. A previous study (Chapter V) revealed that a higher stiffness level of PDMS accelerates CTP progeny growth and associated osteogenic differentiation. Even though the mechanism of how cells respond to substrate stiffness via mechanotransduction cascade is still a much-debated topic, the fact that CTP proliferation and osteogenic differentiation can be accelerated by higher levels of substrate stiffness suggests that biodegradable materials with surface microtextures and higher stiffness could provide a valuable strategy for bone tissue engineering.
6.5 CONCLUSION

For the first time, this work provides a comparison of several biodegradable scaffolds with surface microtextures on bone marrow derived human CTP behavior. It has been shown that culturing primary bone marrow-derived human CTPs and their progeny under osteogenic conditions on CA and PLGA microtextured scaffolds enhances proliferation and osteogenic differentiation compared to those on PDMS surfaces. More specifically, CA exhibited the highest cell proliferation and osteogenic differentiation compared to PLGA and PDMS. The results suggest a significant role for biodegradable microtextured CA materials in modifying the behavior of osteogenic cells. An investigation into the proliferation and osteogenic differentiation of CTP progeny in primary culture on biodegradable and biocompatible materials with surface post microtextures should provide a valuable model in which to explore the relationship between adult stem cell behavior and critical topographical parameters in the engineering of scaffolds for the enhancement of bone fracture healing.
CHAPTER VII
CONCLUSIONS AND FUTURE WORK

7.1 CONCLUSIONS

Autogenous cancellous bone is the gold-standard graft to achieve optimum skeletal incorporation in spinal fusion, bone defects, and fracture repair. However, host morbidity and a number of related complications create the need for an alternative bone graft material. A current state-of-the-art approach to bone regeneration consists of combining CTPs derived from bone marrow with a 3D biodegradable scaffold. The success of this approach not only requires a sufficient number of CTPs, but also an appropriate osteoactive environment. The scaffold supplies this regenerative environment through stimuli such as growth factors and cytokines. In addition to these biochemical stimuli, the scaffold architecture and surface topography play a crucial role in the appropriate development of cells and subsequent tissue formation.

Newer technologies have enabled the fabrication of micro/nano scale features that can influence biological processes within the same size scale of basic biological
structures. In tissue engineering, microfabrication has provided researchers with tools that have helped answer important biological questions. In addition, microfabrication related techniques have been used to create topographies of defined size that can selectively stimulate specific cell behavior. Therefore, precisely defined, miniature features produced using microfabrication techniques might be used to selectively stimulate CTPs within scaffolds to enhance cell growth and subsequent bone formation. This dissertation investigated various microfabrication techniques and material properties required for the development of a scaffold prototype with precise surface microtextures to provide osteoconductive stimuli to CTPs.

The first part of the dissertation presented investigations into the effects of surface post microtextures on CTPs. Chapter III demonstrated that CTPs preferentially grew and differentiated on post microtextures that were 10 \( \mu \text{m} \) in diameter, 6 \( \mu \text{m} \) in height and 10 \( \mu \text{m} \) inter-space between posts compared with cells on smooth surfaces. Chapter IV presented the development of PDMS post microtextures with varying geometries (micropost heights and inter-spaces) and an assessment of their corresponding effects on human bone marrow-derived CTPs. Microfabrication and soft lithography allowed for the construction of precise and reproducible microtextures of various geometries. PDMS post microtextures with 10 \( \mu \text{m} \) diameter, 10 \( \mu \text{m} \) and 20 \( \mu \text{m} \) heights, and 10 \( \mu \text{m} \) inter-space between posts accelerated cell growth compared to cells on other PDMS post microtextures and smooth PDMS.

The physical stimulation provided by the mechanical characteristics or surface topographies of scaffold materials play a crucial role in stimulating cell growth and directing cells to produce bone tissue in a desirable manner. Therefore, in Chapter V, the
influence of mechanical stiffness and surface topographical properties of PDMS on CTP growth was investigated to obtain information pertinent to the design and fabrication of bone scaffolds. Like surface topographical properties, stiffness of biomaterials plays a crucial role in stimulating cell growth and osteogenic differentiation to produce bone tissue in a desirable manner.

Chapter VI presented the investigation and comparison of CTP growth on selected biodegradable materials (CA and PLGA) as an alternative to PDMS. Moreover, this chapter presented a transfer microfabrication protocol to create biodegradable material microtextures, which could be utilized for other BioMEMS and bone tissue engineering applications. Results from a preliminary investigation into cell growth on these biodegradable materials demonstrated increased CTP growth characteristics compared with PDMS. More specifically, CA post microtextures exhibited the greatest cell growth behavior compared to PLGA and PDMS.

The cell-material surface interactions described in this dissertation have been undertaken to determine which surface properties can elicit specific cell responses in order to optimize the scaffold for its particular application. These studies demonstrate a valuable in vitro model, based on the precise and reproducible patterning capabilities of microfabrication and related MEMS techniques, in which to explore the relationship between topographical features of bone tissue engineering scaffolds and the likely response of human adult stem cells and progenitor cells in the settings of bone repair in vivo.

This dissertation contributes the following innovations and discoveries:
Chapter III provides insight into the role of precisely defined post microtextures on primary human CTP behavior.

Chapter IV presents CTP behavior on surface microtextures with optimal sizes of post geometry and arrangement using microfabrication related techniques to create osteoconductive topographical surfaces.

Chapter V investigates CTP behavior on surfaces of varying stiffness, and combination of these properties with surface topography of PDMS that are relevant to BioMEMS applications.

Chapter VI describes the translation of microfabrication techniques from PDMS to biodegradable materials such as CA and PDMS, as well as their effects on CTP behavior.

7.2 INVESTIGATIONAL LIMITATIONS AND FUTURE WORK

This dissertation motivates the clinical application of biocompatible substrates incorporating surface microtextures for selective stimulation of CTPs to enhance bone regeneration. The ultimate objective of the dissertation is to develop biodegradable and biocompatible scaffolds with precise geometrical features (post microtextures) that can provide CTPs with an osteoconductive environment similar to that provided by autogenous bone grafts. However, the interpretation of the results in the dissertation must be considered within the context of various experimental limitations. Consequently, the knowledge presented in this dissertation must be augmented by completion of additional tasks that are outlined below.
First, numerous groups have examined the effect of surface topographies on cell behavior (Table 2.2 and Table 3.2). In general, the dissertation results are consistent with previous reports, which suggest that surface microtopography influences osteogenesis. In addition, it has been demonstrated that, in spite of the identical surface chemistry, cells cultured on post microtextures appear to exhibit a shortened lag phase relative to smooth surfaces. Thus, even when the initial numbers of cells attached to the substrates are identical, post microtextures ultimately result in increased cell number and appear to accelerate osteogenesis. There are many factors contributing to the increased cell numbers and expression of osteogenic markers on different surface topographies. The incorporation of micro- and nano-scale topographies at the cell–substrate interface might provide an attractive approach to enhancing specific cell behavior without destabilizing the delicate biochemical environment. However, the intra-cellular mechanisms by which cell behaviors changes in response to different geometrical and biochemical stimuli remain unclear. Further investigation is needed to elucidate all of the possible factors and establish definitive mechanistic links between cell-surface interactions and their intra-cellular “mechanotransduction”, the process by which cells convert mechanical stimuli into a chemical response.\textsuperscript{75,109,110,170}

Second, although PDMS has previously served as a model material to demonstrate the feasibility of 3D scaffold microfabrication technique, it would be beneficial to translate this 3D fabrication technique from PDMS to biodegradable materials compatible with bone graft applications. As a possible direction for future research, we speculate that the previously reported fabrication processes (Chapter II, Microarchitecture) would permit the development of 3D scaffolds using alternative
biodegradable materials such as cellulose acetate (CA) and poly(lactic-co-glycolic acid) (PLGA), which have been topographically patterned in 2D in this dissertation (Chapter VI).

Third, while current dissertation work has centered on biologically properties (osteogenesis), mechanical properties of bone graft materials cannot be ignored. Mechanical properties of biomaterials that were investigated in the current dissertation (~MPa) are lower than those properties of normal bone tissue or other metallic biomaterials (~GPa). Thus, there is a clinical need to combine these biodegradable materials with metallic materials to produce a biodegradable coating that promotes bone infiltration so that tissue integration occurs rapidly and robustly onto a metallic surface. Applied to bone grafts, the ability to enhance osteoblast proliferation on implant surfaces using microtopography may increase host/implant stability, and therefore, decrease the chances of implant failure. Thus, surface coatings of implants should be optimized through their microtopography and other relevant parameters to ensure that the engineered architecture is best suited to the formation of biologically and mechanically robust bone tissue at the bone grafts interface. For these reasons, future work could encompass the production and testing of microfabricated CA or PLGA coatings on other biomaterials, which have mechanical loading properties similar to natural bone tissue, as an initial step in the development of a biomaterial that supports bone growth and integration. Through the use of a microfabricated CA or PLGA coating on metallic materials, enhanced bone ingrowth may occur on metallic material surfaces while CA or PLGA degrades.
Finally, the feasibility demonstration of CTP-seeded scaffolds with surface topography for bone tissue formation will require experiments in an animal model for performance assessment \textit{in vivo}. These experiments could be initially performed \textit{in vitro} with the 3D biodegradable scaffold with various surface topographies to verify enhanced CTP growth characteristics. Subsequently, the performance of 3D scaffolds should be investigated in the animal model to provide insight into their behavior for ultimate human bone graft applications.
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