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Modulation of Marrow Stromal Cell Differentiation in Bone Tissue Engineering Constructs

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ABSTRACT

Modulation of Marrow Stromal Cell Differentiation in

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Better understanding of the factors that affect marrow stromal cell differentiation will allow researchers to optimize the design of bone tissue engineering constructs toward healing large bone defects in human patients. This research characterizes the effects of scaffold properties and culture supplements on the osteoblastic differentiation of marrow stromal cells (MSCs) seeded on solid, porous scaffolds and cultured in a flow perfusion bioreactor. This bioreactor creates a culture environment similar to that experienced by osteoblasts in vivo by minimizing diffusional constraints and providing mechanical stimulation to the cells through fluid shear. For these studies, MSCs were seeded on scaffolds, cultured under static or flow perfusion conditions, and assayed for DNA, alkaline phosphatase activity, osteopontin, and calcium to assess osteoblastic differentiation. Light and electron microscopy were used to visualize cell morphology and matrix deposition. The results show that brief exposure of MSCs to dexamethasone, a chemical stimulus typically required for osteoblast differentiation, was required prior to seeding on a titanium fiber mesh scaffold for ectopic bone formation to occur in a subcutaneous implantation site. However, in the absence of dexamethasone, either flow perfusion culture or decellularized bone-like extracellular matrix deposited on titanium fiber mesh induced osteoblastic differentiation in MSCs.
Altering the diameter of titanium fibers composing the mesh affected the osteoblastic differentiation of seeded MSCs in flow perfusion culture; wider fibers were conducive to early osteoblast differentiation while thinner fibers were conducive to later differentiation and matrix deposition. Alternatively, coating the titanium scaffold surface with the adhesion peptide RGD resulted in increased cell adhesion strength leading to delayed osteoblastic differentiation \textit{in vitro}, but had no effect on bone formation \textit{in vivo}. Flow perfusion culture of MSCs seeded on porous calcium phosphate ceramic scaffolds resulted in better cell distribution within the scaffold and enhanced osteoblastic differentiation compared to static culture. These results show that scaffold geometry influences cell behavior in a flow perfusion bioreactor, emphasizing the importance of scaffold design in bone tissue engineering. In addition, undifferentiated marrow stromal cells can be induced toward the osteoblastic phenotype by signals other than dexamethasone, including bone-like extracellular matrix and fluid flow mediated shear stress.
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CHAPTER 1: INTRODUCTION AND BACKGROUND

There is a significant need for therapies to enhance healing in large skeletal defects because there exist over 1 million cases each year of patients requiring bone graft procedures to correct such defects\(^1\). These defects can arise for a variety of reasons including trauma, congenital deformity, and tumor resection and thus exist in a wide range of shapes, sizes, and functional locations. The most successful of current treatments for large bone defects is autologous bone graft. This therapy is attractive because there is no risk of immune rejection to the transplanted tissue; however, there are two major drawbacks associated with this procedure. First, there is a limited supply of donor bone, which is harvested primarily from a section of femur or tibia, from the trabecular bone of the iliac crest, or from a whole rib or fibula. Thus, there may not be enough donor tissue for proper shape reconstruction of the defect that can also support the necessary mechanical load during healing.\(^2\) Second, autologous bone graft therapies are associated with a risk of morbidity at the donor site which was healthy to begin with. Due to these issues, there is a need for alternative strategies to bone healing that allow exact shape reconstruction, are mechanically strong, and are biocompatible in both the short and long term. To this end, bone tissue engineering has evolved as a practical method of regenerating large bony defects.

Tissue engineering strategies for bone regeneration must factor in the biological, mechanical and surgical issues involved with the specific application of each construct. Currently, these strategies utilize solid support scaffolds, bioactive molecules, and osteogenic cells either alone or in various combinations with one another. The solid support provides a space filling function to provide a surface for anchorage dependent
bone cells on the perimeter of the defect to infiltrate the space and begin the healing process within the defect. Bioactive molecules can act as chemoattractants to recruit bone forming cells to the site of the defect or to encourage the osteodifferentiation of bone precursor cells already at the defect site. Osteogenic cells are the prime ingredient for healing of large bone defects as they are the tool that will lay down the new bone tissue and eventually integrate the defect site with the surrounding healthy tissue. A strategy that incorporates all three of these components would likely have the best possibility of regenerating a defect in an appropriate amount of time. Therefore, this research aims to create tissue engineering constructs by seeding osteoprogenitor cells on solid porous scaffolds and culturing these scaffold/cell constructs in vitro to create an extracellular matrix rich in bioactive molecules. These scaffold/cell/extracellular matrix constructs can then be implanted to induce bone tissue formation in vivo.

In order to contrive the best possible tissue engineered scaffold for bone regeneration, it is important to understand the physiological process of natural bone formation such that the body’s natural healing mechanisms can be exploited to their utmost potential.

**Bone Structure**

All mature bones in the human skeleton are composed of a central marrow cavity surrounded by bone tissue which is covered by periosteum. The bone marrow contains extensive blood vessels which supply the bone tissue with nutrients as well as a store of cells capable of initiating bone repair.

Bone tissue exists in two forms, cancellous and cortical bone. Cancellous bone is highly porous (50-90%) and is capable of deformation in order to absorb high mechanical
loads and distribute them evenly over a wider area. Thus, much of the cancellous bone that exists within the skeleton is located within short and flat bones as well as at the end of long bones near synovial joints. Because of the highly porous nature of cancellous bone, it possesses a high surface area and a majority of cells in cancellous bone rest on this surface in close proximity to the marrow and its blood vessels. In contrast, cortical bone is only about 10% porous resulting in a much stiffer and much stronger tissue than cancellous bone. About 80% of the human skeleton is composed of cortical bone which is the main type of bone found within weight bearing regions of the skeleton, but also surrounds cancellous bone in all types of bones. Because of the low porosity of cortical bone, the majority of bone cells are completely embedded within the bone matrix resulting in limited access to the marrow and its blood vessels. Thus, cancellous bone tends to have a much higher metabolic rate than cortical bone and responds more rapidly to changes in mechanical loads. However, cortical bone is still a highly active tissue.

Cortical and cancellous bone may consist of two different arrangements, woven bone or lamellar bone. Woven bone is sometimes referred to as primary bone in that it is the first type of bone laid down during embryonic development as well as after fracture. In time, this woven bone is remodeled into lamellar bone resulting in a very small amount of woven bone present in the adult skeleton. These two types of bone exhibit differences in regard to their formation, composition, organization and mechanical properties. Woven bone forms quite rapidly due to a high cell number per unit volume and has a random arrangement of collagen fibrils, resulting in isotropic behavior of the bone. Because of the random collagen arrangement, mineralization of woven bone is quite irregular as well, thereby resulting in a tissue that is relatively flexible and weak. In contrast, lamellar bone,
also referred to as secondary bone, forms at a much lower rate and is much more ordered than woven bone. Instead of a random arrangement, the collagen fibrils of lamellar bone are of relatively uniform diameter and are aligned into parallel sheets, forming lamellae. Due to the regularity of collagen fiber orientation, mineralization proceeds in a much more uniform manner compared to woven bone. The highly order structure of cortical bone results in a tissue that is stiffer and stronger than woven bone and behaves anisotropically, allowing the bones to be stronger in the direction of higher mechanical loads.³

Because the majority of the adult skeleton is composed of lamellar bone, it is important to understand the structure and organization of lamellar bone. Lamellar bone is composed of four main types of lamellae; trabecular, circumferential, interstitial, and osteon. The highly porous bone composing both the central marrow cavity and cancellous bone is organized into trabecular lamellae. Alternatively, the highly dense bone composing cortical bone is organized into inner and outer circumferential lamellae, interstitial lamellae and lamellae of osteons. Each of these lamellae is composed of highly oriented collagen fibrils; however, they are oriented differently in each lamella. The inner circumferential lamella is adjacent to and surrounds the trabecular lamellae composing the central marrow cavity of the bone. In this lamella, the collagen fibrils are oriented perpendicular to the axis of the bone, forming a circle, or circumference, around the central marrow cavity. The outer circumferential lamella is organized in the same manner as the inner circumferential lamella but is located at the outer perimeter of the bone near the periosteum.³
**Figure 1: Organization of lamellar bone**

Between these two circumferential lamellae lie the osteons, which form the majority of cortical bone. Each osteon is arranged into a cylindrical bundle with the collagen fibrils oriented longitudinally to the bone axis. At the center of each osteon is a canal, referred to as a haversian canal, which contains blood vessels, lymphatic vessels, and in some cases nerves. Each haversian canal is surrounded by several lamellae, and within each lamella lie osteocytes responsible for the metabolic activity of bone. Very small canals called canaliculi house the cell processes of osteocytes and radiate outward from the central haversian canal. The canaliculi connect osteocytes from different lamellae to each other and to the central canal. Because nutrient diffusion through mineralized bone matrix is minimal, the canaliculi allow for nutrient transport to cells in all lamellae of the osteon as well as a means for rapid cell-cell communication. The outer lamella of each osteon is covered with a thin layer of organic matrix or cement line. This cement line separates each osteon from the next as canaliculi and collagen fibrils do not
cross cement lines. This separation prevents crack propagation across the bone, allowing the bone to repair cracks before fracture occurs.3

The interstitial lamella makes up the irregularly shaped spaces that exist between osteons of cortical bone. The collagen fibrils of the interstitial lamellae are oriented longitudinally allowing efficient space filling of bone tissue between the cylindrically shaped osteons. Throughout the length of cortical bone there are also intermittent vascular canals that run crosswise between the marrow space and osteons, osteon to osteon, and osteon to periosteum. These vascular canals, or Volkmann canals, allow nutrient transport to the osteons near the perimeter of the cortex as well as to the periosteum, thus connecting all of the lamellae into a single unified bone tissue.3

The outer surface of bone tissue is covered by periosteum. This thin membranous-like tissue comprises two layers, an outer fibrous layer and an inner vascular and cellular layer. While the function of the outer layer is mainly connectivity of one bone to the next, the inner layer has important functions in regard to regular bone metabolism as well as wound healing. This inner layer, or cambium layer, has a dense vascular bed that supplies nutrients to the surface of the bone and also contains a store of progenitor cells that are capable of differentiating into osteoblasts for normal bone growth and maintenance or on a larger scale for bone repair in response to injury.3

The highly vascular nature of the periosteum, the extensive vascular network within cortical bone lamellae, and the extremely vascularized bone marrow are indicative of the highly metabolic nature of bone tissue. The close proximity of cells to the marrow in cancellous bone and the extensive vascular canals lying within the lamellae of cortical bone allow that no bone cell is more than 300 µm from a blood vessel. This is especially
important as the dense mineralized extracellular matrix of bone severely hinders diffusion of nutrients from and waste products to the blood vessels.\(^3\)

**Bone Components**

Bone is a highly organized tissue that consists of several different components that interact with one another to create the complex tissue. These three main components are bone cells, organic extracellular matrix and inorganic extracellular matrix.

The bone cells are responsible for the formation, resorption, remodeling and repair of bone tissue and are composed of three main cell types; osteoblasts, osteocytes, and osteoclasts. Osteoblasts are the cells responsible for the formation of new bone. These cells are derived from undifferentiated mesenchymal cells that reside within the vast network of bone canals, the bone marrow and the periosteum. When needed, these cells are recruited to the necessary site where they proliferate and then differentiate into the osteoblasts required to start forming new bone. The primary role of osteoblasts is the synthesis and secretion of organic extracellular matrix molecules, although they also function to balance electrolyte levels in the extracellular fluid with the osseous fluid and aid the mineralization of the bone matrix. During bone synthesis, there are large numbers of active osteoblasts present that may follow one of three different fates when they are no longer actively forming bone. First, they may remain on the surface of the bone in a resting state until needed to once again initiate bone formation. Second, they may become embedded within the extracellular matrix and become osteocytes, performing tissue maintenance. Finally, they may disappear from the site. Because many more cells are present during active bone formation than become osteocytes or resting surface osteoblasts, the majority of cells are removed by some unknown mechanism.
Those osteoblasts that become osteocytes account for more than 90% of the bone cells found in the mature skeleton. These cells have many processes that extend outward through the canaliculi and connect with other cells within the bone, allowing for good cell-cell communication within the tissue. This is vitally important in that those cells that lie on the surface of the bone are very sensitive to stresses on the bone and require a speedy network to transfer these signals to osteocytes so they may adjust their activity. The interconnected cell network allows the cells to sense bone deformations and to coordinate bone resorption and formation as well as the flow of ions between the mineralized matrix and the extracellular fluid space in response to these stimuli. In this manner, bone tissue will increase when exposed to higher stresses and decrease when exposed to lower stresses over a long period of time.

The third type of cell found within bone tissue is the osteoclast, which is responsible for the resorption of old bone for repair and remodeling purposes. Unlike the osteoblasts and osteocytes, these cells do not originate from mesenchymal stem cells, but rather from hematopoietic stem cells as do other cells from the monocyte family. These precursor cells can be found in the marrow as well as in the circulating blood and when activated will proliferate and then fuse to form large multinucleated cells. These giant cells are very efficient at resorbing bone by attaching to the surface of bone and creating a sealed off space between the cell and the bone matrix. Endosomes containing large amounts of proton pumps move to the cell membrane bordering this space where the pumps work to lower the pH of the extracellular pocket from 7 to about 4. This acidic environment allows the mineral components of the bone matrix to dissolve and activates acid proteases released from the cell to digest the organic matrix. In this manner,
osteoclasts form characteristic resorption cavities in the bone matrix to allow for deposition of new bone. Once completing its resorptive function, an osteoclast may divide into mononuclear cells and remain in a resting state until reactivated to form new osteoclasts.

The cellular component of bone comprises only a fraction of the total volume of bone tissue. The largest portion of bone tissue is the extracellular matrix, constituting more than 90% of the tissue volume. It is composed of both an organic and inorganic component and is responsible for the mechanical properties of bone. The inorganic component consists primarily of calcium, carbonate, and acid phosphate ions arranged in a crystalline-like structure to give bone its ability to resist compression and to also serve as an ion reservoir. The organic component consists primarily of collagen fibrils that give bone its form and ability to resist tension. Other components of the organic matrix include non-collagenous proteins such as osteocalcin, osteonectin, and bone sialoproteins that effect matrix organization and matrix mineralization. In addition, bone matrix also contains a variety of growth factors including transforming growth factor-β, insulin-like growth factors 1 and 2, bone morphogenic proteins, platelet-derived growth factors, interleukins 1 and 6, and colony stimulating factors that primarily affect cell behavior. Such cell behaviors as recruitment of stem cells, differentiation of stem cells, bone formation, and bone resorption can be effected by these growth factors.

Like all tissues, bone tissue is highly hydrated and thus consists of an extracellular fluid to fill in the void spaces between the cells and the extracellular matrix molecules. Recall from the earlier discussion of bone structure that there exist vast networks of small canaliculi which allow cell processes to penetrate through the dense mineralized matrix to
communicate with other cells. These small canals are filled with fluid which flows in response to mechanical stimuli to the bone tissue, creating shear stresses and streaming potentials within the bone tissue that may affect cell behavior. In response to a mechanical load, these micropores will deform with some increasing in volume while others decrease, leading to a difference in pressure which results in fluid flow. This fluid flow leads to streaming ion potentials as well as cellular deformations. Thus, the osteocytes are able to sense mechanical stimuli and react in an appropriate manner.\(^4\)

**BONE FORMATION IN VIVO**

The normal course of bone formation in vivo, whether it stems from embryonic development, normal growth, remodeling or fracture healing, follows a specific pathway. It begins with aggregation of undifferentiated mesenchymal cells or preosteoblasts followed by a period of proliferation to provide sufficient cell numbers for tissue formation. After this period of initial cell proliferation, the cells start to synthesize and secrete a loose organic matrix and they begin to differentiate toward osteoblasts. As these cells continue to differentiate, they lay down organic bone matrix that then mineralizes. This mineralization takes place in an organized fashion, with crystals first forming in specific hole zone regions of collagen. These crystals then grow along the collagen fibrils, eventually connecting the hole zone regions together. Once it begins, the mineralization process proceeds quite rapidly, with 60% of the final mineral content being formed within hours of initiation. However, after this initial blast of mineralization, the rate decreases and mineral continues to deposit slowly over an extended period of time. During mineralization, the water and non-collagenous protein concentrations in the bone matrix may change, but the collagen amounts and organization does not change.
appreciably. Osteoblasts cover the surface of this newly formed bone matrix and continue
to rapidly lay down more bone matrix until many of these cells are engulfed within the
matrix, becoming osteocytes, and the bone volume has reached a sufficient level. This
primary, or woven, bone has a relatively random configuration and after initial deposition,
osteoclasts come to the site of formation and start the remodeling process in order to
produce secondary or mature lamellar bone.

During embryogenesis, the size and shape of newly formed bone is determined
primarily by gene expression; however, movement and mechanical forces play an
increasing role as growth continues. In fact, changes in mechanical loading in the adult
skeleton can result in remodeling of bone to accommodate the new mechanical
environment. In order to retain normal bone density, bone tissue requires repetitive
mechanical loading and changes in this loading pattern over extended periods of time
lead to alterations in bone mass. Decreased loading from such phenomena as extended
bed rest or long-term exposure to the microgravity of outer space leads to decreased bone
mass. Increased mechanical loading due to, for example, large weight gain leads to
increased bone mass. Thus, the body is able to equilibrate bone mass with the necessary
mechanical integrity to support daily functions through the coordinated efforts of bone
formation and bone resorption.

This process of bone formation is mediated by cells which generally originate in
the bone marrow. The marrow is composed of a variety of cell types including adherent
stromal cells, endothelial cells, and non-adherent haematopoietic cells. Of these three cell
types, the stromal cells are considered to be the osteogenic precursor cells while the
haematopoietic cells may play a helper role in osteodifferentiation. The process begins
with proliferation of marrow stromal cells, characterized by the up-regulation of proliferative genes such as histones and protooncogenes. After a period of logarithmic growth, proliferation slows and there is an up-regulation of genes associated with the osteoblast phenotype including alkaline phosphatase, type I collagen, osteopontin, bone sialoprotein, and osteocalcin. Alkaline phosphatase levels initially increase, then peak, and finally decrease when mineralization is underway.\(^6\) Osteopontin is up-regulated throughout the differentiation process with peaks during proliferation and again near the onset of matrix mineralization.\(^7\) Bone sialoprotein is first detectable during matrix deposition, while osteocalcin appears during bone mineralization\(^6\). This differentiation process which occurs \textit{in vivo} also follows the same process when marrow stromal cells are cultured \textit{in vitro}\(^5\) and the protein markers mentioned above can be used to determine the extent of osteodifferentiation during \textit{in vitro} cell culture experiments.

\textbf{Bone Formation in Vitro}

The natural process of bone formation can be recapitulated through \textit{in vitro} experiments utilizing osteoprogenitor cells from a typical animal model such as the rat. These osteoprogenitor cells typically consist of either fetal calvaria cells or marrow stromal cells. Fetal calvarial cells are isolated via collagenase digestion of minced fetal calvarial bones whereas marrow stromal cells are isolated by flushing the marrow cavities of femura and tibiae of young adult rats.\(^5\) The cells of several animals are pooled together and plated in tissue culture flasks for subsequent culture in 2-dimensions. To promote the osteodifferentiation of these precursor cells, several factors have been identified as crucial elements in the cell culture process. The batch of fetal bovine serum (FBS) used in the culture medium has a strong effect on the ability of the progenitor cells to
differentiate into osteoblasts due to the wide variability in different serum lots to support mineralization. The culture medium must also be supplemented with 3 factors in order to promote osteodifferentiation. These factors include L-ascorbic acid, which induces collagen synthesis and deposition; β-glycerophosphate, which provides a source of inorganic phosphate for mineralization; and dexamethasone, a synthetic glucocorticoid that promotes bone nodule formation.5,7 While the first two components are relatively inactive additives that serve as reservoirs for molecules necessary for osteodifferentiation, dexamethasone is a potent chemical that exerts a powerful influence on mineralization, and the formation of nodules in rat marrow stromal cell cultures is entirely dependent on the presence of this glucocorticoid.8

It is necessary to grow marrow stromal cells in 2-dimensions for a brief period following initial harvest for several reasons. First, it allows isolation of the adherent cell population from the heterogenous mixture of cells found within the bone marrow. Second, it allows removal of cell and bone particulates from the bone marrow aspirate. Third, the number of marrow stromal cells initially obtained can be expanded to a quantity sufficient for successful tissue formation. After this initial culture phase, the cells can be lifted from the 2-D culture surface and seeded onto a scaffold material for 3-D culture. The subsequent 3-D bone tissue formation will eventually serve as a tissue engineered bone replacement for large bone defects. During the initial cell isolation and expansion phase of culture, marrow stromal cells may or may not be exposed to the assortment of medium supplements previously described, as early exposure to dexamethasone can result in an up to 5-fold reduction in cell number.8 In either case, marrow stromal cells
that have undergone this initial primary culture have been utilized in a variety of ways in order to promote the growth of bone tissue in vitro.

**Scaffold Materials for Bone Tissue Engineering**

Three main classes of scaffold materials have been investigated for the creation of bone tissue engineering constructs. Each of these scaffold materials has inherent advantages and disadvantages toward the tissue engineering of new bone.

Polymers are attractive scaffold materials in that their chemical and mechanical properties can be varied through synthesis and processing procedures to match a specific defect need. In addition, many polymers offer the advantage of being biodegradable, thus eliminating the potential hazards of a permanent implant such as chronic inflammation, infection, or bone resorption due to stress shielding. A variety of polymer scaffolds have been investigated as solid support structures for marrow stromal cell growth and osteodifferentiation toward the development of tissue engineered bone grafts. Poly(propylene fumarate-co-ethylene glycol) hydrogels were shown to resist cell attachment, yet when modified with short adhesion peptides such as RGD allowed attachment and migration of marrow derived osteoblasts in a dose dependent manner.\(^9\) Furthermore, when these hydrogels were formed into macroporous 3-dimensional structures, seeded marrow stromal cells differentiated into osteoblasts over 28 days in static culture with osteogenic medium.\(^10\) Similarly, oligo[poly(ethylene glycol) fumarate] (OPF) hydrogels were able to support marrow stromal cell adhesion and migration in a dose dependent fashion when modified with an adhesion peptide.\(^11\) In addition, marrow stromal cells encapsulated within unmodified OPF hydrogels differentiated into osteoblasts over 28 days of static culture in osteogenic medium.\(^12\) Poly(propylene
fumarate) (PPF) was shown to be a suitable surface for 2-dimensional culture and osteodifferentiation of marrow stromal cells. Moreover, when the marrow stromal cells were encapsulated within gelatin microspheres to protect them from the harsh chemical environment, they attached, proliferated and differentiated into osteoblasts on PPF surfaces that were not yet fully crosslinked.\textsuperscript{13} Poly(D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether (Me.PEG-PLA) films were shown to support marrow stromal cell adhesion and osteodifferentiation over 20 days in osteogenic medium.\textsuperscript{14} Marrow stromal cells showed enhanced osteodifferentiation on Me.PEG-PLA surfaces over plain poly(lactic acid) or poly(lactic-co-glycolic acid) surfaces when cultured under the same conditions.\textsuperscript{14}

In addition to polymers, calcium phosphate ceramic materials that imitate the inorganic component of bone, particularly the mineralized matrix, have been used as scaffolding for tissue engineered bone constructs. Normal mineralized bone matrix is composed primarily of carbonated apatite and the majority of synthetic calcium phosphate ceramics used in bone regeneration applications consist of thermally processed hydroxyapatite or tricalcium phosphate.\textsuperscript{15,16} Although these materials are meant to imitate the mineral component of bone, they are more crystalline and are therefore resorbed more slowly than native bone mineral or not at all. Calcium phosphate ceramic materials are also quite brittle with poor tensile strength, making them unsuitable for filling load-bearing defects. However, these materials are very attractive as bone defect fillers because they do not elicit a foreign body reaction and are tolerated well by host tissues.\textsuperscript{17} A variety of calcium phosphate materials are currently used by surgeons as stand alone materials for filling bone voids and their potential as scaffold materials for tissue
engineered bone constructs has recently been investigated\textsuperscript{17-19}. These ceramic materials are osteoconductive, meaning they will allow bone ingrowth when placed adjacent to normal host bone. However, they are not intrinsically osteoinductive, meaning they will not actively induce bone formation\textsuperscript{20}. Therefore, much work has been aimed at making calcium phosphate ceramics more osteoinductive by adding growth factors\textsuperscript{21-23} and/or osteoprogenitor cells to the scaffolds\textsuperscript{20,24-26}.

A third class of scaffold materials for bone tissue engineering constructs includes metals. Metals differ from both polymers and ceramics in that they are nonresorbable and thus will remain at the defect site indefinitely. Despite this drawback, metals are attractive scaffold materials in that they are inert, do not provoke an immune response, and are mechanically strong, making them suitable for load bearing bone regeneration applications. Indeed, metals, particularly titanium, have been used for many years for total joint replacement in both the hip and knee due to their superior mechanical properties. Surface modifications to these implants have furthered their success by facilitating integration with host bone to stabilize the implants. Recently, porous titanium scaffolds in the form of a fiber mesh have been investigated as a suitable scaffold material for facilitating bone regeneration. Titanium fiber mesh has been shown to be osteoconductive but not osteoinductive as a stand alone material and much effort has been aimed at increasing osteoinductivity by addition of coatings, growth factors, and/or osteoprogenitor cells\textsuperscript{27,28}. \textit{In vitro}, titanium fiber mesh can support the osteodifferentiation of marrow-derived progenitor cells, induced by dexamethasone or bone morphogenetic protein-2 (BMP-2) in the culture medium\textsuperscript{29,30}. \textit{In vivo}, titanium fiber mesh can support bone in-growth from surrounding tissue in an orthotopic site, but can
induce bone formation in an ectopic site when modified with a calcium phosphate coating,\textsuperscript{27,31} BMP-2 loading,\textsuperscript{29,32} transforming growth factor β-1 (TGF-β1),\textsuperscript{28} or seeded with marrow stromal cells.\textsuperscript{30,33} Thus, a combination approach combining a scaffold, cells and signaling molecules should be more successful in bone regeneration.

**Cell Seeding onto Tissue Engineering Scaffolds**

Many of the scaffold materials mentioned above have been used in conjunction with osteoprogenitor cells to create more osteoinductive tissue engineering constructs. An effective method of cell seeding is critical for promoting uniform cell distribution throughout the scaffold. Good cell distribution promotes uniform extracellular matrix distribution and thus uniform tissue formation within the construct. A variety of cell seeding techniques have been investigated for their effectiveness in promoting uniform seeding, the simplest of which involves placing a droplet of cell suspension on the top of the scaffold and letting the liquid slowly drip through the porosity of the scaffold. However, if measures are not taken to force the liquid through the scaffold, the droplet may spread and run over the side of the scaffold resulting in very little cell attachment to the scaffold itself. Thus, better consistency in seeding may be achieved by placing the scaffolds in a concentrated cell suspension combined with gentle agitation.\textsuperscript{34} This method, while allowing better consistency of seeding, is not ideal in that cells are not distributed evenly throughout the thickness of the scaffolds, but rather are concentrated near the surface of the scaffold.

In an attempt to improve the cell seeding distribution, several types of bioreactors have been investigated including spinner flasks, rotating wall vessels, and perfusion reactors. A spinner flask is a simple bioreactor consisting primarily of a flask with two
side arms. Scaffolds are suspended within the medium by needle-like shafts anchored to the cap of the flask. Medium is mixed with either a magnetic stir bar or a shaft with an impeller rotating at low speed to provide the lowest possible shear. Cells are suspended in medium, mixing is begun and cells are allowed to attach to the scaffolds for a given period of time. Vunjak-Novakovic\textsuperscript{35} seeded chondrocytes onto porous polyglycolic acid scaffolds in a spinner flask and found that 90-100% of cells in suspension were seeded on the scaffolds within 24 hours and that cells were uniformly distributed throughout the thickness of the scaffolds. Kim et al.\textsuperscript{36} studied the cell attachment of smooth muscle cells to polyglycolic acid fiber mesh scaffolds by three different seeding methods including a spinner flask. They showed greater cell attachment to the scaffolds in a spinner flask compared to both droplet and gentle agitation methods, as well as better cell distribution throughout the scaffold. Mauney et al.\textsuperscript{37} studied attachment of human bone marrow stromal cells on partially demineralized bone matrix and also saw more uniform cell distribution and greater cell seeding efficiency in a spinner flask compared to a static droplet/cell suspension method. Thus, the spinner flask resulted in better uniformity of cell seeding for a variety of cell types in several different scaffolds. However, despite the enhancement of cell attachment and uniformity of seeding in the spinner flask, Carrier et al.\textsuperscript{38} showed that some damage to the cells occurred due to shear from mixing of the vessel and Wendt et al.\textsuperscript{39} showed low cell seeding efficiency. This method of seeding is also very time-consuming, typically requiring a 24 hour period of cell attachment.

These drawbacks inspired these and other researchers to develop other dynamic cell seeding methods. Carrier et al.\textsuperscript{38} found that cardiac myocytes had greater cell attachment and suffered less cell damage when seeded onto polyglycolic acid fiber
meshes in a rotating wall vessel compared to a spinner flask. Wendt et al.\textsuperscript{39} developed a novel oscillating perfusion system where the scaffolds were press-fitted into flow chambers and a cell suspension was forced through the porosity of the scaffold. Flow of medium was continually reversed such that the cell suspension traveled back and forth through the scaffolds to increase opportunity for cell attachment. They showed a small increase in overall cell seeding efficiency, but immense increases in cell viability and uniformity of seeding over both a spinner flask and static seeding. Godbey et al.\textsuperscript{40} utilized the centrifugal force generated by a common laboratory centrifuge to seed bladder smooth muscle cells or human foreskin fibroblasts onto polyglycolic acid scaffolds from a dilute cell suspension. They showed better cell seeding efficiency and greater cell survival for a 10 min seeding time in the centrifuge compared to a 24 hour seeding time in a spinner flask.

**BIOREACTORS FOR BONE TISSUE ENGINEERING**

As discussed above, several types of bioreactors have been utilized for dynamic cell seeding of a variety of cell types onto a variety of scaffolds. These and other bioreactors have been more often used for the long term culture of cell-seeded scaffolds. The simplest method of 3-dimensional cell culture entails placing the scaffold/cell constructs into a well plate, covering with medium and simply placing in an incubator. However, the usefulness of this static culture method is severely limited in creating large tissue constructs *in vitro* as a result of poor mass transport of nutrients to and waste products from cells near the center of the scaffolds. Recall from the discussion of bone vascularity that no cell in bone tissue is more than 300 μm from a blood vessel and that the most active cells are located on the surface of the bone tissue where there is an
abundance of vessels for nutrient transport. Thus, in creating bone tissue engineering constructs in vitro, the actively proliferating and differentiating cells initially located at the center of a thick scaffold will suffer from nutrient deprivation and waste product buildup if the medium filling the porosity of the scaffold does not move in and out at an acceptable rate. In static culture, there is no bulk fluid movement and as such, cells must rely on the process of diffusion to carry nutrients from the medium surrounding the scaffold, through the porous network to the cell surface. As the nutrients diffuse inward, cells along the perimeter of the scaffold will take what they need first, leaving less and less available for cells in the interior creating a concentration gradient that will ultimately limit cell survival to a small region near the scaffold surface. The need for alternate methods of culturing these scaffold/cell constructs that reduce or eliminate the mass transport limitations inherent in static culture has spurred the development of a variety of bioreactors.

The spinner flask was the first type of bioreactor used for the long term culture of scaffold/cell tissue engineering constructs. In this simple design, a stirring mechanism is used to mix the medium exterior to the scaffold, creating a flow field to the surface of the scaffold for nutrient transport. Although nutrient transport within the scaffold is still governed by diffusion, the fluid flow around the scaffold exterior effectively moves the concentration gradient inward as the nutrient concentration at the scaffold surface is now the same as that in the bulk of the medium, allowing cell survival at a greater distance from the scaffold interior. This small increase in nutrient transport has resulted in great increases in cell survival and differentiation. Sikavitsas et al. 41 cultured marrow stromal cells on poly(D,L-lactic-co-glycolic acid) (PLGA) scaffolds in both static and spinner
flask culture in osteogenic medium for 21 days. They found increased cellularity over the first 2 weeks of culture in the spinner flask indicating a greater capacity to support cell growth as well as greater alkaline phosphatase activity and mineralization at 2 and 3 weeks of culture, indicative of osteoblastic differentiation. However, the mineralization front was still limited to the perimeter of the scaffold in spinner flask culture, although the layer of calcified matrix was thicker than in static culture. Others have found similar results when culturing MSCs on porous scaffolds in spinner flasks.42,43

A second type of bioreactor for long term culture of musculoskeletal tissues in the rotating wall vessel; a bioreactor developed by NASA to create a simulated microgravity environment while providing good mixing to the medium within the vessel. The rotating wall vessel is a horizontally mounted hollow cylinder housing an oxygenator membrane along the central axis of rotation. The space between the membrane and the outer vessel wall is filled with medium and the whole vessel is rotated. The medium rotates at a speed equivalent to the rotational speed of the vessel wall and this angular momentum offsets the pull of gravity on particles within the vessel. These particles can be microcarriers, scaffolds, or tissue aggregates and the speed of rotation can be adjusted to balance the gravitational force on particles of differing sizes. As for the spinner flask, the rotating wall vessel provides good mixing of medium to the scaffold surface while relying on diffusion to transport nutrients within the scaffold. Sikavitsas et al.41 compared the osteogenic differentiation of marrow stromal cells cultured on PLGA scaffolds in a rotating wall vessel to spinner flask and static culture. They found decreased scaffold cellularity over the first week compared to static culture and similar cellularity at 2 and 3 weeks of culture indicating an initial suppression of proliferation in the rotary vessel.
There was also much less alkaline phosphatase activity and calcification in the rotary vessel compared to static culture suggesting that there was a detrimental effect of this bioreactor on the osteoblastic differentiation of marrow stromal cells. Similar results were also observed by Goldstein et al.\textsuperscript{42} These results are not entirely surprising as bone cells \textit{in vivo} require mechanical stimulation to form and remodel bone. Astronauts who are in space for long periods of time lose substantial bone mass due to the absence of the gravitational force which normally provides the mechanical loading which signals bone growth and remodeling. Since the rotating wall vessel was designed to minimize the effects of gravity, creating a nearly weightless environment, these progenitor cells lack even the minimal mechanical signals provided by static culture. These results emphasize that while good mass transport is essential for developing tissue engineered constructs, the mechanical environment experienced by the cells plays an equally important role in osteoblast differentiation.

The third type bone tissue engineering bioreactor is a perfusion bioreactor which, by forcing medium through the porosity of the scaffold, completely mitigates the mass transport limitations inherent in static, spinner flask, and rotary vessel culture and introduces a mechanical stimulation to the cells in the form of fluid shear. Marrow stromal cells seeded on porous PLGA foams and cultured in a flow perfusion bioreactor resulted in increased alkaline phosphatase activity and more uniform cell distribution throughout the thickness of the scaffold compared to static culture.\textsuperscript{42,43} Using a novel flow perfusion bioreactor design developed in this laboratory\textsuperscript{44}, marrow stromal cells were cultured on titanium fiber mesh scaffolds and cultured at a medium flow rate of 0.5 ml/min for up to 16 days. There was enhanced alkaline phosphatase activity at day 8,
increased calcium deposition at day 16, and improved cell and extracellular matrix
distribution under flow perfusion compared to static controls.\(^{45}\) Further studies with a
marrow stromal cell/titanium fiber mesh studies in this flow perfusion bioreactor revealed
that increasing the flow rate of medium resulted in increasing calcified matrix deposition
in a dose dependent fashion.\(^{46}\) Increased fluid flow rate results in both better mass
transport through the scaffold as well as increased mechanical forces in the form of fluid
shear. In a subsequent study where fluid shear rate was increased without changing the
fluid flow rate, increasing fluid shear resulted in increasing calcified matrix deposition
and increased spatial distribution of cells and matrix within the titanium fiber mesh.\(^{47}\)
These studies highlight the importance of mechanical environment on the differentiation
of marrow stromal cells. Additionally, in this bioreactor system, fluid flow rate and fluid
shear can be easily modulated to accommodate specific mechanical requirements.
Furthermore, when marrow stromal cells were seeded onto starch-based polymer fiber
mesh scaffolds\(^{48}\) or resorbable poly(lactic acid) fiber mesh scaffolds\(^{49}\) there were
similar increases in calcium deposition and cellular spatial distribution at a low flow rate
of 0.3 ml/min, showing that the perfusion bioreactor system can accommodate a variety
of scaffold materials.

**Thesis Outline**

This thesis extends previous work on the flow perfusion bioreactor by examining
the effects of culture supplements and fiber diameter on the osteoblastic differentiation of
marrow stromal cells seeded on titanium fiber mesh scaffolds and cultured in a flow
perfusion bioreactor. In addition, this work also examines the effects of certain surface
modifications of the titanium fiber mesh scaffolds on the differentiation of marrow
stromal cells in static culture. These modifications include coating with the adhesion peptide arginine-glycine-aspartic acid (RGD) or coating with decellularized bone-like extracellular matrix. Finally, this research confirms the feasibility of culturing more brittle porous calcium phosphate ceramic scaffolds seeded with marrow stromal cells in a flow perfusion bioreactor. All of these studies reveal key factors related to the osteogenic differentiation of marrow stromal cells toward optimizing an in vitro culture environment for 3-dimensional bone tissue engineered constructs.

The following chapters will present five studies related to the culture of marrow stromal cells (MSCs) on either titanium or calcium phosphate porous scaffolds for the creation of bone tissue engineering constructs. Chapter 2 will layout the specific research aims addressed by this research. Chapter 3 reveals the effect of titanium fiber mesh pore size on the osteoblastic differentiation of seeded MSCs when cultured in a flow perfusion bioreactor. Chapter 4 reveals the effect of flow perfusion culture on the osteoblastic differentiation of MSCs seeded on titanium fiber mesh scaffolds in the absence of dexamethasone. Chapter 5 reveals the effect of decellularized extracellular matrix on the osteoblastic differentiation of MSCs seeded on titanium fiber mesh scaffolds and cultured in static culture. Chapter 6 reveals the effect of coating the surface of titanium fiber mesh scaffolds with the adhesion peptide RGD on the osteoblastic differentiation of seeded MSCs both in vitro and in vivo. This chapter also evaluates the dependence of initial cell differentiation state on eventual in vivo bone formation in an ectopic site. Chapter 7 demonstrates the advantage of flow perfusion culture over static culture of MSCs seeded on porous sintered calcium phosphate ceramic scaffolds. Finally, Chapter 8 will summarize the key points of each study and present directions for future work.
CHAPTER 2: SPECIFIC RESEARCH AIMS

The overall goal of this research was to modulate the osteoblastic differentiation of marrow stromal cells when seeded onto three-dimensional porous scaffolds to gain a better understanding of the key elements affecting bone formation in tissue engineering scaffolds. In vivo, marrow stromal cells are arranged in three-dimensions and receive differentiation signals from extracellular matrix molecules to which the cells are attached, soluble factors in the extracellular fluid, and mechanical forces exerted upon the cells. Cell differentiation in vitro within three-dimensional tissue engineering scaffolds is likely to be dependent on similar parameters. Therefore, the central hypothesis of this research was that marrow stromal cell differentiation in vitro is influenced by scaffold properties, culture supplements, and culture environment.

To test this hypothesis, the following specific aims were addressed:

1. Identify parameters other than dexamethasone that can induce the osteoblastic differentiation of marrow stromal cells.

2. Determine the effect of scaffold pore structure on marrow stromal cell differentiation in a flow perfusion bioreactor.

3. Determine the feasibility of culturing marrow stromal cells on porous calcium phosphate ceramics in a flow perfusion bioreactor.
CHAPTER 3: PORE SIZE OF FIBER MESH SCAFFOLDS AFFECTS THE
OSTEOBLASTIC DIFFERENTIATION OF SEENED MARROW STROMAL CELLS
CULTURED IN A FLOW PERFUSION BIOREACTOR

ABSTRACT

In this study, we cultured marrow stromal cells on titanium fiber meshes in a flow perfusion bioreactor and examined the effect of altering mesh pore size on cell behavior in an effort to develop a bone tissue construct composed of a scaffold, osteogenic cells and extracellular matrix. By altering the diameter of the mesh fibers (20 or 40 μm), scaffolds of 80% porosity with mean pore sizes of 65 or 119 μm were created. Cell/scaffold constructs were grown in static culture or under flow for up to 16 days and assayed for cellularity, alkaline phosphatase activity and Ca$^{+2}$ deposition while media samples were assayed for osteopontin. Cellularity was higher at early time points and Ca$^{+2}$ deposition was higher at later time points for flow constructs over static controls. The 65 μm pore size meshes had reduced cellularity in static culture. Under flow conditions, mass transport limitations are mitigated allowing uniform cell growth throughout the scaffold. The 65 μm pore size meshes had slightly greater cellularity under flow. There was greater alkaline phosphatase activity, osteopontin levels and calcium under flow at 8 days for the 40 μm mesh compared to the 20 μm mesh. However, by day 16, the trend was reversed indicating that under flow, the larger pore size enhanced marrow stromal cell differentiation initially while the smaller pore size

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enhanced differentiation later on. These results suggest that the time course of differentiation was dependent on scaffold pore size under flow conditions; however, the dependence was not linear with respect to time. Larger pore size was conducive to early osteoblast differentiation while smaller pore size was conducive to later differentiation and matrix deposition.

**INTRODUCTION**

Bone tissue engineering provides an alternative treatment toward healing large bone defects arising from maladies such as trauma, tumor resection or birth defect. Promising tissue engineering strategies have involved both singular and integrated approaches to bone regeneration utilizing various combinations of scaffolds, osteogenic cells, and signaling molecules. The scaffold provides a substratum for cell migration into the defect site or functions as a carrier for transplanted cells. Signaling molecules serve to recruit and differentiate osteogenic cells residing in the blood supply or neighboring healthy tissue. Osteogenic cells are the tools which synthesize and deposit new bone tissue within the defect site. Therefore, we are investigating a bone tissue engineering strategy which combines a titanium fiber mesh scaffold and marrow stromal cells along with in vitro culture to create a more osteoinductive construct.

Previous studies in our laboratory have shown that the osteogenic differentiation of bone marrow stromal cells seeded on titanium fiber meshes and cultured in a flow perfusion bioreactor is dependent on both the flow rate of medium through the constructs and the shear rate experienced by the seeded cells. Both increased flow rate and increased shear rate resulted in greater mineralized matrix formation after 16 days of culture, demonstrating that marrow stromal cells are sensitive to fluid flow mediated
environmental changes. Similar changes in cell culture environment can be created through alterations in the scaffold geometry which ultimately alter the fluid flow pathway through the scaffolds. A better understanding of how certain scaffold properties affect cell behavior will allow optimization of scaffold based tissue engineering constructs toward bone regeneration.

The aim of this study was to investigate the effect of scaffold mesh size on the differentiation of seeded marrow stromal cells cultured in a flow perfusion bioreactor. Titanium fiber mesh was chosen as the material for this study because it supports the attachment and osteogenic differentiation of marrow stromal cells and because it is non-biodegradable. While degradable materials may be ideal for tissue regeneration in vivo, a material that maintains its shape, size, and composition was necessary for this study to ensure consistent scaffold geometry throughout the culture period. The two titanium fiber mesh scaffolds used in these studies were of equal size and porosity, but were composed of fibers with differing diameters, resulting in scaffolds with differing mesh size. We seeded primary rat marrow stromal cells on the titanium fiber meshes and monitored proliferation, alkaline phosphatase activity, osteopontin secretion, and calcium deposition over 16 days in both a flow perfusion bioreactor and static culture. We hypothesize that differences in scaffold pore size will result in altered fluid flow through the scaffolds and ultimately result in altered shear stress experienced by cells seeded on the scaffold that will affect the osteoblastic differentiation of marrow stromal cells.
**Materials and Methods**

**Materials**

Scaffold material consisted of a non-woven sintered titanium fiber mesh (Bekaert, Zwevegem, Belgium), fabricated from thin titanium fibers. Two different fiber meshes were used for this study; the first was composed of titanium fibers of 20 μm diameter while the second was composed of titanium fibers of 40 μm diameter. Discs of 8 mm diameter were die-punched from the appropriate 0.8 mm thick sheet of fiber mesh to create the cell culture scaffolds. All chemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

**Mercury Porosimetry**

Scaffold volumetric porosity and pore size were determined via mercury porosimetry (Autoscan 500, Quantachrome Instruments, Boynton Beach, FL). A sample of the fiber mesh was placed into the sample chamber, which was then loaded into the porosimeter. A vacuum of 50 millitorr was applied to the sample chamber and the pressure was slowly increased (0.004 psi/sec) until mercury filled the void space around the sample (P ≈ 0.6 psi). At this point, data acquisition began and mercury was pushed into the sample chamber by slowly increasing the pressure at a rate of about 0.01 psi/sec. Once the volume of intruded mercury seemed to plateau, the pressure was increased at a faster rate of about 0.1 psi/sec until a total pressure of 50 psi was reached. The volume vs. pressure data were converted into porosity and pore size measurements using software supplied with the porosimeter (Quantachrome Autoscan, ver. 3.00). Three samples were analyzed for each type of mesh.
Cell culture

Rat bone marrow stromal cells (MSCs) were isolated and cultured using the method described by Maniotopoulos et al. Briefly, the tibiae and femora of 6-8 week old male Wistar rats (Harlan, Indianapolis, Indiana) were excised, the epiphyses were cut off and the diaphyses flushed with 5 ml of complete medium (α-minimum essential medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 50 μg/ml gentamicin, 100 μg/ml ampicillin, 0.5 μg/ml fungizone, 50 μg/ml L-ascorbic acid, 0.01 M β-glycerophosphate, and 10 nM dexamethasone). The resulting marrow pellet was broken up by trituration, the cells were plated in tissue culture flasks and cultured for 6 days in complete medium to expand the cell number. Titanium fiber meshes were press fitted into bioreactor cassettes in 6-well plates and seeded with 500,000 cells in 300 μl medium. After 2 hours, 10 ml medium was added to each well and cells were allowed to attach for 24 hours. Scaffold/cell constructs were then placed into either 6-well plates (static culture) or into the flow perfusion bioreactor (flow culture) for 4, 8 or 16 days. Three separate cell harvests were performed to generate the data, one for each time point. Marrow stromal cells from four different rats were pooled for each individual cell harvest to help reduce cell behavior variation from batch to batch.

Flow perfusion bioreactor

The flow perfusion bioreactor is described in detail elsewhere. Briefly, it consists of 6 flow chambers within a block of poly(methyl methacrylate) (PMMA). Each flow chamber holds one cassette press fit with a scaffold. The cassette is sandwiched by two neoprene o-rings to ensure no media leakage around the edge of the cassette. Each bioreactor contains 6 flow chambers on independent pumping circuits drawing from a
common media reservoir. Media flow within the bioreactor is driven by the action of a peristaltic pump and flows from the supply medium reservoir, through the pump, to the top of the flow chamber, through the scaffold, out the bottom of the chamber, and into the collection reservoir. Hydrostatic pressure drives medium from the collection reservoir back to the supply reservoir. Components of the flow circuit are connected with platinum-cured silicon tubing, which has a high permeability to both oxygen and carbon dioxide. Prior to use, all PMMA components (flow chamber, cassettes and screw tops) were sterilized with ethylene oxide gas while all other components (tubing, reservoirs, connectors and scaffolds) were sterilized by autoclaving.

The flow system was assembled using sterile technique in a laminar flow hood. The system was preconditioned by flowing medium containing a strong dose of antibiotic/antimycotic (400 unit/ml penicillin, 400 µg/ml streptomycin, 0.1 µg/ml fungizone) (Invitrogen, Grand Island, NY) through the bioreactor for 10 minutes before being replaced with 200 ml of the desired culture medium. Cassettes containing cell seeded scaffolds were placed into each flow chamber, the chambers were sealed, and the entire flow system was placed in a 37°C incubator with 95% air and 5% CO₂. The flow rate through the system was set to a low value (~0.3 ml/min) for 1 day to ensure good cell attachment before being increased to 1 ml/min for the duration of culture. Media were changed every 2 to 3 days and samples were removed after 4, 8 or 16 days and rinsed with phosphate buffered saline (PBS). Six scaffold/cell constructs were cultured for each time point: one sample was set aside for scanning electron microscopy (SEM) analysis and the others were stored in ddH₂O at -20°C until assays were performed.
Static Culture

Seeded scaffolds were removed from the cassettes after 1 day of attachment and cultured in standard 6-well plates with 4 ml of complete medium. Media were changed according to the same schedule as the flow system and samples removed after 4, 8 or 16 days and rinsed with PBS. Four scaffold/cell constructs were cultured for each time point: one sample was set aside for SEM analysis and the others were stored in ddH$_2$O at -20°C until assays were performed.

DNA Content

Scaffold cellularity was determined using the PicoGreen assay kit (Molecular Probes, Eugene, Oregon) to measure dsDNA content. The PicoGreen dye binds to dsDNA and the resulting fluorescence corresponds to the concentration of dsDNA in solution. Briefly, the frozen scaffold/cell constructs were thawed at room temperature, sonicated for 10 min and vortexed for 5-10 sec to allow the DNA into solution. Standards of calf thymus DNA in ddH$_2$O in concentrations ranging from 0 – 6 μg/ml were prepared and 50 μl of standard or sample was placed into individual wells of a 96-well plate. Tris-EDTA buffer and PicoGreen dye solution were prepared according to the manufacturer's instructions using reagents provided in the kit and added at 100 and 150 μl/well respectively. After a 10 min incubation in the dark at room temperature, the fluorescence was measured on a plate reader (FL x800, Bio-Tek Instruments Inc., Winooski, Vermont) using an emission wavelength of 490 nm and an absorbance wavelength of 520 nm. The cellularity of each scaffold was determined by correlating DNA with a known amount of MSCs.
Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity of MSCs was determined using a colorimetric endpoint assay to determine early osteoblast differentiation. The assay measures the conversion of the colorless substrate p-nitrophenol phosphate by the enzyme Alkaline Phosphatase to the yellow product p-nitrophenol, where the rate of color change corresponds to the amount of enzyme present in solution. ALP activity was determined by assaying aliquots of the same aqueous solutions that had been frozen, thawed, sonicated and vortexed for DNA quantification. As necessary, samples were diluted in ddH₂O up to 100x in order to stay within the detection range of the assay. Standards of p-nitrophenol in concentrations ranging from 0 – 250 μM were prepared from dilutions of a 1000 μM stock solution, and 80 μl of standard or sample was placed into individual wells of a 96-well plate. Alkaline buffer solution consisting of 1.5 M 2-amino-2-methyl-1-propanol at pH 10.3 was then added at 20 μl/well. Substrate solution was prepared by dissolving 40 mg 4-nitrophenyl phosphate disodium salt hexahydrate into 10 ml ddH₂O and added at 100 μl/well. The microplate was incubated for 1 hour at 37°C and the reaction was stopped by addition of 0.3 M NaOH at 100 μl/well. The absorbance of each well at 405 nm was then measured on a plate reader (PowerWave x340, Bio-Tek Instruments Inc., Winooski, Vermont). All samples were run in triplicate and compared against p-nitrophenol standards. Activity is expressed per cell as determined by the PicoGreen assay.

Calcium Content

The calcium content of each scaffold was assayed in order to quantify the amount of mineralized matrix present and was measured using a kit available from Sigma (St.
Louis, Missouri). This colorimetric endpoint assay measures the amount of purple-colored calcium-cresolphthalein complexone complex formed when cresolphthalein complexone binds to free calcium in an alkaline solution\(^{57}\) After the ALP assay, 1.4 ml 1N acetic acid was added to each sample and scaffolds were incubated in the resulting 0.5 M acetic acid overnight on a shaker table to allow the calcium into solution. Samples were diluted up to 20x in order to stay within the detection range of the assay. Ca\(^{2+}\) standards in concentrations ranging from 0 – 100 \(\mu\)g/ml were prepared from dilutions of a 1 mg/ml stock solution of CaCl\(_2\) and 20 \(\mu\)l of standard or sample was added to individual wells of a 96-well plate. Assay Working Solution was prepared by mixing equal parts Calcium Binding Reagent and Calcium Buffer Reagent provided in the kit and added at 300 \(\mu\)l/well. The microplate was incubated for 10 min at room temperature and the absorbance at 575 nm was measured on a plate reader. Samples were run in triplicate and compared against CaCl\(_2\) standards.

**Osteopontin Secretion**

The amount of osteopontin released into the media during culture was measured using an ELISA kit available from Assay Designs, Inc. (Ann Arbor, Michigan) to determine mid to late stage osteodifferentiation of MSCs on Ti scaffolds. Osteopontin is an extracellular protein that is produced by matrix-producing osteoblasts as well as bone resorbing osteoclasts.\(^{58}\) The assay was performed per instructions provided by the kit manufacturer. Briefly, media samples were collected over the culture period and frozen at -20°C until analysis could be performed. Samples were thawed, briefly vortexed and diluted up to 1000x in order to stay within the detection range of the assay. Standards in concentrations ranging from 0 – 4750 pg/ml were prepared from dilutions of a stock
solution of recombinant rat osteopontin and 100 µl of standard or media sample was added to individual wells of the microplate provided with the kit. The plate was covered and placed in 37°C incubator for 1 hr. Wells were aspirated and washed 7 times with 400 µl of wash buffer provided with the kit. Labeled Antibody Solution was prepared according to the manufacturer’s instructions and added at 100 µl/well and the plate was covered and incubated at 4°C for 30 min. Substrate Solution was prepared according to the manufacturer’s instructions and added at 100 µl/well and the plate was covered and incubated at room temperature for 30 min in the dark. Stop Solution consisting of 1N sulfuric acid was added at 100 µl/well and the absorbance at 450 nm with correction between 570 and 590 nm was measured on a plate reader. Samples were run in duplicate and compared against standards of recombinant rat osteopontin.

**SEM Analysis**

Samples were rinsed in calcium-free PBS and fixed in 2.5% gluteraldehyde at 4°C overnight. Scaffold/cell constructs were then rinsed 3 times in PBS and incubated at 4°C overnight in PBS. Samples were then dehydrated in a gradient series of ethanol, dried with tetramethylsilane, sputter coated with gold and examined on an XL-30 environmental scanning electron microscope (FEI Company, Hillsboro, Oregon).

**Statistical Analysis**

Results are expressed as means ± standard deviation. Significance was determined using Student’s t-test for porosimetry data, with a 95% confidence interval (p < 0.05). For cellularity, alkaline phosphatase and calcium data, significance was determined using Tukey-Kramer’s multiple comparison test with a 95% confidence interval (p< 0.05).
Osteopontin results are expressed as cumulative release with each point representing a pooled media sample of 4 (static culture) or 6 (flow culture) scaffolds. In order to conserve material so that all experimental conditions could be achieved, it was necessary to culture fewer constructs under static conditions as compared to flow perfusion conditions and this difference in sample size was accounted for during statistical analysis.

RESULTS

Porosimetry results for the two different mesh types are shown in Figure 2. There was no difference in scaffold porosity; however there was a significant difference in average pore size between the two mesh types. The average pore diameter in the 40 μm mesh was 119 ± 12 μm, almost double that of the 20 μm mesh (65 ± 3 μm).

Figure 2: Titanium fiber mesh scaffold porosity and pore size as determined by mercury porosimetry. Titanium scaffolds were composed of fibers with diameter of 40 μm or 20 μm. Results are presented as average ± standard deviation (n = 3). * represents significant difference between groups, p < 0.05.
At 1 day, there was no difference in DNA content of any of the meshes, indicating that cell seeding efficiency was consistent between the two mesh types (Figure 3). For static constructs there was no increase in cell number initially, followed by decreased cellularity at later time points. At 8 and 16 days, there were significantly less cells in the 20 μm mesh than the 40 μm mesh under static culture. In contrast, there was a significant increase in cell number at day 4 for both meshes under flow conditions, followed by a decline in cellularity at later time points; however, there was no difference between the two mesh types under flow perfusion.

![Graph showing cell numbers over time](Image)

**Figure 3:** Cellularity of titanium fiber mesh scaffold/marrow stromal cell constructs as determined by quantification of double stranded DNA using the PicoGreen assay and correlation to a known amount of MSCs. Titanium scaffolds were composed of fibers with diameter of 40 μm or 20 μm and scaffold/cell constructs were cultured statically in 6-well plates (n = 3) or in the flow perfusion bioreactor (n = 5) for 4, 8, or 16 days. Results are expressed on a per scaffold basis and are presented as average ± standard deviation. * represents significant difference between groups, p < 0.05.

There was a low level of alkaline phosphatase (ALP) activity at 4 days under all
culture conditions followed by a rise at day 8 and a decline at day 16 (Figure 4). There was a significant difference in ALP activity between meshes under flow conditions at all time points. There was also a trend to greater ALP activity in flow culture compared to static culture.

![Graph showing Alkaline Phosphatase Activity](image)

**Figure 4:** Alkaline phosphatase activity of MSCs seeded on titanium fiber mesh scaffolds. Titanium scaffolds were composed of fibers with diameter of 40 μm or 20 μm and scaffold/cell constructs were cultured statically in 6-well plates (n = 3) or in the flow perfusion bioreactor (n = 5) for 4, 8, or 16 days. Results are expressed on a per cell basis and are presented as average ± standard deviation. * represents significant difference between groups, p < 0.05.

Calcium deposition was negligible for all samples at 4 days (Figure 5). There was greater calcium deposition for flow constructs over static constructs at 8 and 16 days. There was no difference in calcium deposition between mesh types under static culture, however there was a difference between mesh types under flow culture at both 8 and 16 days. At 8 days, there was a greater amount of calcium in the 40 μm fiber mesh, but there was more calcium in the 20 μm mesh after 16 days of culture.
Figure 5: Mineralization of extracellular matrix produced by MSCs seeded on titanium fiber mesh scaffolds as determined by quantification of calcium content. Titanium scaffolds were composed of fibers with diameter of 40 μm or 20 μm and scaffold/cell constructs were cultured statically in 6-well plates (n = 3) or in the flow perfusion bioreactor (n=5) for 4, 8, or 16 days. Results are expressed on a per scaffold basis and are presented as average ± standard deviation. * represents significant difference between groups, p < 0.05.

The amount of secreted osteopontin was determined using media samples collected throughout the culture period and each point in Figure 6 represents an average value per scaffold resulting from pooled media from 6 constructs. Because each flow loop in the flow perfusion bioreactor draws from a common media reservoir, individual scaffold media samples were impossible to collect. There was initially, relatively high production of osteopontin by MSCs in the flow perfusion bioreactor followed by a decrease in production after about day 10. Osteopontin levels were much lower in static culture than in flow culture at all times during culture. There was not a large difference in protein production between the two mesh types.
Figure 6: Total osteopontin secretion from MSCs on titanium fiber mesh scaffolds as determined by ELISA for rat osteopontin. Titanium scaffolds were composed of fibers with diameter of 40 μm or 20 μm and scaffold/cell constructs were cultured statically in 6-well plates or in the flow perfusion bioreactor for (a) 8 days or (b) 16 days. Results are expressed on a per scaffold basis and reflect the value of a pooled media sample from 4 (static culture) or 6 (flow culture) constructs.
Samples were visualized using scanning electron microscopy to evaluate cell morphology and deposition of mineralized matrix (Figure 7). Figure 7A is a representative image of 4 days of flow culture, the cells are adherent to the fibers and well spread; some cells are beginning to span neighboring fibers. Figure 7B is representative of 8 days of flow culture showing many more cells covering both the scaffold fibers and the space between the fibers; small spaces are still available for fluid flow directly adjacent to the cells. Figure 7C is representative of 16 days of flow culture showing the entire surface of the scaffold to be covered in a dense layer of extracellular matrix; individual cells are no longer discernable within the matrix.

Figure 7: Scanning electron micrographs of Titanium/MSC constructs showing representative images of after 4 days (A), 8 days (B), or 16 days (C) of flow perfusion culture. A & B are magnification 250x, scale bars represent 100 μm, C is magnification 35x, scale bar represents 1 mm.
**Discussion**

Differentiation of marrow cells toward osteoblasts begins with a rapid cell growth phase. As proliferation slows, there is an up-regulation in alkaline phosphatase production that tapers off as mineralization gets underway.\(^6\) Osteopontin production is up-regulated throughout the differentiation process, peaking both during the cell-proliferation stage and again at the onset of bone deposition.\(^7\) After a period of organic matrix deposition, the extracellular matrix begins to calcify and there is a continuous increase in calcified matrix deposition until the tissue becomes fully mineralized.\(^3\) Thus we are able to monitor the osteodifferentiation of marrow stromal cells by assaying our cultures for these markers at various time points of culture.

In designing tissue engineering scaffolds, several key factors including material properties, surface properties, and pore architecture affect tissue formation.\(^59\) Scaffold pore architecture is described by factors such as overall porosity, pore morphology, and pore size. There have been several reports on the effect of scaffold pore size on the attachment, proliferation, and differentiation of a variety of cell types on a host of surfaces.\(^60-64\) These studies have illustrated that the effect of scaffold pore size on cell behavior varies widely with cell phenotype, stressing the importance of optimizing pore size for the particular desired tissue engineering application. These studies were carried out under static culture conditions, but the goal of this study was to determine if the pore size of a titanium fiber mesh affects differentiation of marrow stromal cells toward osteoblasts when cultured in a flow perfusion bioreactor. Experiments were carried out for up to 16 days since previous studies in this flow perfusion system have shown that to be sufficient time for significant mineralization to occur.\(^45-49\)
In terms of scaffold cellularity, an initial rise in scaffold cellularity was expected with a subsequent decrease as mineralization increased. This decline in cellularity at later time points can be attributed to both the trapping of DNA within the thick extracellular matrix, leading to an under representation of the cell number from the PicoGreen assay, as well as programmed cell death, resulting from the limited lifespan of terminally differentiated cells.\textsuperscript{65} We were interested in differences in scaffold cell number between the two different scaffolds. There was only a difference in scaffold cell number under static conditions at 8 and 16 days, with greater cellularity in the 40 \( \mu \)m mesh than the 20 \( \mu \)m mesh. This is not unexpected because porosimetry revealed the 40 \( \mu \)m mesh to have a pore size almost double that of the 20 \( \mu \)m mesh, making nutrient diffusion into the scaffold much easier in the 40 \( \mu \)m mesh and thereby allowing greater cell survival in the scaffold interior. Under flow conditions, because medium is flowing through the thickness of the scaffold, these mass transport limitations do not exist and we did not see any difference in cellularity.

We expected to see an increase in alkaline phosphatase (ALP) activity over the first week of culture followed by a decrease by day 16 when mineralization should be well underway. We observed this peak for all culture conditions, indicating that there was osteodifferentiation under all conditions. However, the ALP activity of the cells varied between groups for similar culture times. Specifically, there was a significant difference in ALP activity between the two scaffold types under flow conditions at all time points. There was initially greater ALP activity for cells in the 40 \( \mu \)m mesh, but by day 16, there was greater ALP activity in cells on the 20 \( \mu \)m mesh. These results indicate that marrow stromal cells within the 40 \( \mu \)m mesh were possibly differentiating at a faster rate, as the
peak in ALP activity was observed earlier. However, we need to take these data in
concert with osteopontin and calcium data as there is always the possibility that the actual
peak in alkaline phosphatase activity was missed, because we were only able to assay at 4,
8, and 16 days.

The amount of calcium deposited in the extracellular matrix correlates to the level
of tissue mineralization, with greater calcium levels associated with further
differentiation. The calcium data show much greater mineralization for flow cultures over
static cultures at all time points, consistent with scanning electron microscopy and
observations in previous studies that flow perfusion enhances osteodifferentiation over
static culture.\textsuperscript{45-49} There was no difference in calcium content between the two mesh
types in static culture, however there were significant differences in calcium levels
between the two mesh types under flow conditions. At 8 days, there was a greater amount
of calcium in the 40 \( \mu \text{m} \) mesh, but by day 16 there was more calcium in the 20 \( \mu \text{m} \) mesh.
These data support the alkaline phosphatase data in suggesting that osteodifferentiation
occurred faster initially in the 40 \( \mu \text{m} \) mesh under flow conditions. This observation can
be explained in terms of the mesh surface area. The larger fibers that compose the 40 \( \mu \text{m} \)
mesh result in a scaffold with reduced surface area for cell attachment compared to the 20
\( \mu \text{m} \) mesh. Because there was no difference in cellularity between the two scaffold types
under identical culture conditions, there was initially a greater number of cells per area
and thus closer cell proximity in the 40 \( \mu \text{m} \) mesh. Increased cell-cell communications in
early culture of marrow derived stromal cells has been shown to play an important role in
osteoblast differentiation.\textsuperscript{34,66,67}
The calcium data also suggest that by day 16, the cells on the 20 μm mesh were more differentiated than those on the 40 μm mesh under flow perfusion. We have previously shown that fluid shear force affects osteodifferentiation of marrow stromal cells, with a trend of greater cell differentiation with increasing fluid shear. To demonstrate how pore architecture can affect fluid shear, a fluid flow model based on the assumption of cylindrical pore geometry is presented. Although this assumption is not an accurate reflection of the pore structure of the titanium fiber mesh scaffolds, the following flow analysis provides an approximation of fluid flow through the fiber mesh. If we assume a cylindrical pore shape, the mean linear velocity of fluid through the porous network \( \bar{v} \) is proportional to the total flow rate \( Q \) and inversely proportional to scaffold porosity \( \varepsilon \) and scaffold diameter \( D \).

\[
\bar{v} \propto \frac{Q}{\varepsilon \pi D^2}
\] (1)

Shear stress at the pore wall \( \tau_w \) is proportional to the mean linear fluid velocity and inversely proportional to the pore diameter \( d_p \).

\[
\tau_w \propto \frac{\mu \bar{v}}{d_p}
\] (2)

By combining equations 1 and 2, it is evident that shear stress at the pore wall is inversely proportional to both scaffold porosity and pore diameter.

\[
\tau_w \propto \frac{\mu Q}{d_p \varepsilon D^2}
\] (3)

From equation 3, it is evident that decreasing the pore diameter leads to an increase in shear stress. As matrix is deposited onto the mesh, scaffold pore size is increasingly restricted, leading to increased shear force experienced by the cells along the perimeter of
the pores. For a given amount of extracellular matrix deposition, greater restriction to fluid flow occurs for smaller initial pore size, resulting in greater shear. This explains the enhanced osteodifferentiation in the 20 μm mesh after 16 days of culture. The nonlinearity of the dependence of scaffold pore size on differentiation suggests that mechanical stimulation in the form of shear stress is more important at later stages of osteoblast differentiation while cell-cell communications are more important early on.

Osteopontin protein is associated with the onset of mineralization in bone tissue and we expected to observe a correlation between osteopontin secretion and osteodifferentiation. Indeed, there was a direct correlation between cumulative osteopontin secretion, alkaline phosphatase activity and calcium deposition. In general, osteopontin levels were much higher for flow cultures compared to static controls. At 8 days, there was slightly greater total osteopontin secretion for marrow stromal cells on the 40 μm mesh over the 20 μm mesh in flow culture with the trend reversed at 16 days. These data directly support the alkaline phosphatase and calcium data indicating that indeed there was enhanced osteodifferentiation in the 40 μm mesh initially and the 20 μm mesh later on in the flow perfusion bioreactor. Under static culture, the osteopontin data were in agreement with the DNA data in that there were slightly elevated osteopontin levels for the 40 μm mesh over the 20 μm mesh in static culture. Because there were fewer cells in the 20 μm mesh it follows there should be lower osteopontin levels when expressed on a per scaffold basis.

The flow perfusion bioreactor currently allows for 4 flow perfusion culture conditions to be performed simultaneously. Because primary marrow stromal cells were used to seed the scaffolds, several cell harvests were performed to complete all the data
runs and the cells of 4 rats were combined in order to minimize animal to animal variation in cell performance. Since the comparisons of primary interest were between the two scaffold types at similar time-points, all scaffolds cultured for a specific time-point were seeded with cells from the same harvest. But, as a result of the separate cell harvests, the absolute amount of osteopontin in the culture medium for the 8 day culture period was three times higher at day 8 than the corresponding time-point for the 16 day culture period. This difference indicates that while trends remain the same between cell batches, the absolute values of certain parameters can vary based on the cell population obtained from the cell harvest. Further evidence of this phenomenon is provided by the large amounts of calcium observed for the 8 day flow perfusion run when previous studies showed very little calcium under similar conditions. These results emphasize the importance of cell population variation and illustrate a need for developing techniques to facilitate uniform cell selection from the heterogenous cell population of bone marrow aspirate.

**CONCLUSION**

The goal of this study was to investigate the effect of scaffold pore size on the osteodifferentiation of marrow stromal cells in a flow perfusion bioreactor. We observed that the time course of differentiation was dependent on scaffold pore size under flow conditions; however the dependence was not linear with respect to time. Larger pore size was conducive to early osteoblast differentiation while smaller pore size was conducive to later differentiation and matrix deposition. This dependence of MSC osteodifferentiation on the pore size of fiber mesh scaffolds resulted from differences in fluid shear experienced by the seeded cells stemming from differing pore architectures, as
well as differences in initial cell-cell interactions stemming from alterations in total scaffold surface area.
CHAPTER 4: FLOW PERFUSION CULTURE INDUCES THE OSTEOLASTIC Differentiation of Marrow Stromal Cell-Scaffold Constructs IN THE ABSENCE OF DEXAMETHASONE

ABSTRACT

Flow perfusion culture of scaffold/cell constructs has been shown to enhance the osteoblastic differentiation of rat bone marrow stromal cells over static culture in the presence of osteogenic supplements including dexamethasone. While dexamethasone is known to be a powerful induction agent of osteoblast differentiation in marrow stromal cells, we hypothesize that the mechanical shear force due to fluid flow in a flow perfusion bioreactor will be sufficient to induce osteoblast differentiation in the absence of dexamethasone. In this study, we examined the ability of marrow stromal cells seeded on titanium fiber mesh scaffolds to differentiate into osteoblasts in a flow perfusion bioreactor in both the presence and absence of dexamethasone. Scaffold/cell constructs were cultured for 8 or 16 days and osteoblastic differentiation was determined by analyzing the constructs for cellularity, alkaline phosphatase activity, and calcium content as well as media samples for osteopontin. For scaffold/cell constructs cultured under flow perfusion, there was greater scaffold cellularity, alkaline phosphatase activity, osteopontin secretion and calcium deposition compared to static controls, even in the absence of dexamethasone. When dexamethasone was present in the cell culture medium under flow perfusion conditions, there was further enhancement of osteogenic

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differentiation as evidenced by lower scaffold cellularity, greater osteopontin secretion and greater calcium deposition. These results suggest that flow perfusion culture alone induces osteogenic differentiation of rat marrow stromal cells and that there is a synergistic effect of enhanced osteogenic differentiation when both dexamethasone and flow perfusion culture are utilized.

**INTRODUCTION**

Bone tissue replacement can be necessary for treatment of large bone defects resulting from maladies such as birth defect, trauma, or tumor resection. A solid support scaffold is necessary to provide the proper framework for growth of new tissue in the defect site, and addition of cells and/or osteotropic biomolecules to the scaffold can enhance its bone regeneration potential. Previously, we have shown that marrow stromal cells cultured on titanium fiber mesh scaffolds in the presence of osteogenic supplements including dexamethasone produced large amounts of calcified matrix after 16 days in culture in a flow perfusion bioreactor whereas constructs grown in static culture produced minimal calcified matrix. This calcified matrix is a marker of late stage osteoblast differentiation of marrow stromal cells. We have demonstrated that the bioreactor has a two-fold contribution to cell differentiation in the form of mitigation of mass transport limitations to the scaffold interior as well as a mechanical stimulation of cells due to the fluid shear force within the scaffold.

In order to induce osteoblast differentiation in marrow stromal cells, the culture medium is usually supplemented with osteogenic agents including L-ascorbic acid, β-glycerophosphate, and dexamethasone. L-ascorbic acid enhances collagen synthesis and up-regulates ATPase and alkaline phosphatase activity and β-glycerophosphate
serves primarily as a source of inorganic phosphate ions.\textsuperscript{5,7} Dexamethasone is a synthetic glucocorticoid that exerts a powerful effect on the osteodifferentiation on marrow stromal cells. Continual exposure to dexamethasone, beginning shortly after cell harvest is required to drive and maintain the osteoblastic phenotype of marrow derived progenitor cells.\textsuperscript{68,70-72} While maintenance of the osteoblastic phenotype is desirable for long term cultures, dexamethasone has been known to drive marrow stromal cells down the osteogenic pathway so quickly that they do not proliferate well. This issue becomes especially problematic for autologous cell therapies where a large number of cells are required, because only a limited number of marrow derived progenitor cells can be harvested from the donor. As a result of this and other possible unknown negative effects of dexamethasone, it would be advantageous to culture marrow stromal cells \textit{in vitro} in the absence of dexamethasone while still encouraging osteodifferentiation.

Because previous studies of marrow stromal cell behavior in a flow perfusion bioreactor showed enhanced osteodifferentiation over static culture, we hypothesize that the mechanical stimulation due to fluid flow in the bioreactor will promote osteodifferentiation of marrow stromal cells even in the absence of dexamethasone. In this study, we harvested fresh rat marrow stromal cells, culture expanded them for 6 days in either the presence or absence of dexamethasone, seeded them onto titanium fiber meshes and cultured them either in static or flow perfusion culture in the presence or absence of dexamethasone. Osteodifferentiation was determined by measuring several markers for osteoblastic phenotype including alkaline phosphatase (an early marker), osteopontin (a mid-stage marker) and calcium deposition (a late stage marker).
MATERIALS AND METHODS

Materials

Scaffold material consisted of a non-woven sintered titanium fiber mesh (Bekaert, Zwevegem, Belgium), fabricated from 40 μm diameter titanium fibers. Discs of 8 mm diameter were die-punched from a 0.8 mm thick sheet of fiber mesh and the resulting scaffolds had a volumetric porosity of 80% with an average pore size of 120 μm. All chemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Cell Culture

Rat bone marrow stromal cells (MSCs) were isolated and cultured using the method described by Maniatopoulos et al. Briefly, 6-8 week old male Wistar rats (Harlan, Indianapolis, Indiana) were anesthetized with 4% isoflurane in oxygen and then euthanized by inhalation of CO₂. The leg bones were excised, and the soft tissue was removed from the femora and tibiae. The epiphyses were cut off and the diaphyses flushed with 5 ml of augmented medium (α-MEM supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 50 μg/ml gentamicin, 100 μg/ml ampicillin, 0.5 μg/ml fungizone, 50 μg/ml L-ascorbic acid, and 0.01 M β-glycerophosphate). The resulting marrow pellet was broken up by trituration, the cells were plated in 75 cm² tissue culture flasks and cultured for 6 days at 37 °C in a humidified atmosphere of 95% air, 5% CO₂ in augmented medium either with (+dex) or without (-dex) 10 nM dexamethasone to expand the cell number. Medium was changed at 1 and 3 days to remove the non-adherent cell population. At the end of this primary
culture period, cells were lifted with 2 ml concentrated trypsin solution (0.25% trypsin / 0.02% EDTA), centrifuged at 400 g for 5 min, and resuspended in a known amount of medium.

Titanium fiber meshes were press fitted into bioreactor cassettes in 6-well plates and seeded with 500,000 cells in 300 µl of media. After 2 hours, 10 ml medium was added to each well and cells were allowed to attach for 24 hours. Cell/scaffold constructs were then placed into either 6-well plates (static culture) or into the flow perfusion bioreactor (flow culture) for 8 or 16 days. During extended cell culture, cell/scaffold constructs were grown in either +dex or -dex medium, resulting in 8 experimental groups (Figure 8).

![Diagram](image)

**Figure 8:** Experimental design depicting the cell culture conditions for each of the 8 groups studied at both culture times. Primary rat marrow stromal cells were obtained in four separate cell harvests, with each batch containing a pool of cells from 4 donors.

Four separate cell harvests were performed to generate the data; one cell harvest was performed per primary culture condition (+dex or -dex) per time point (8 or 16 days).
Marrow stromal cells from four different rats were pooled for each individual cell harvest to help reduce cell behavior variation from batch to batch.

**Flow Perfusion Culture System**

The flow perfusion bioreactor is described in detail elsewhere.\textsuperscript{44} Briefly, it consists of 6 flow chambers within a block of poly(methyl methacrylate) (PMMA). Each flow chamber holds one cassette press fit with a scaffold. The cassette is sandwiched by two neoprene o-rings to ensure no media leakage around the edge of the cassette. Each bioreactor contains 6 flow chambers on independent pumping circuits drawing from a common media reservoir. Media flow within the bioreactor is driven by the action of a peristaltic pump and flows from the supply medium reservoir, through the pump, to the top of the flow chamber, through the scaffold, out the bottom of the chamber, and into the collection reservoir. Hydrostatic pressure drives medium from the collection reservoir back to the supply reservoir. Components of the flow circuit are connected with platinum-cured silicon tubing, which has a high permeability to both oxygen and carbon dioxide. Prior to use, all PMMA components (flow chamber, cassettes and screw tops) were sterilized with ethylene oxide gas while all other components (tubing, reservoirs, connectors and scaffolds) were sterilized by autoclaving.

The flow system was assembled using sterile technique in a laminar flow hood. The system was preconditioned by flowing medium containing a strong dose of antibiotic/antimycotic (400 unit/ml penicillin, 400 \(\mu\)g/ml streptomycin, 0.1 \(\mu\)g/ml fungizone) (Invitrogen, Grand Island, NY) through the bioreactor for 10 minutes before being replaced with the desired culture medium. Cassettes containing cell seeded scaffolds were placed into each flow chamber, the chambers were sealed, and the entire
flow system was placed in a 37°C incubator with 95% air and 5% CO₂. The flow rate through the system was set to a low value (~0.3 ml/min) for 1 day to ensure good cell attachment before being increased to 1 ml/min for the duration of culture. Media were changed every 2 to 3 days and samples were removed after 8 or 16 days and rinsed with PBS. Six scaffold/cell constructs were cultured for each time point: one sample was set aside for SEM analysis and the others were stored in 1.4 ml ddH₂O at -20°C.

Static Culture

Seeded scaffolds were removed from the cassettes after 1 day of attachment and cultured in standard 6-well plates. Media were changed according to the same schedule as the flow system and samples were removed after 8 or 16 days and rinsed with PBS. Four scaffold/cell constructs were cultured for each time point: one sample was set aside for SEM analysis and the others were stored in 1.4 ml ddH₂O at -20°C.

Scaffold Cellularity

Scaffold cellularity was determined using the PicoGreen assay kit (Molecular Probes, Eugene, Oregon) to measure dsDNA content. The PicoGreen dye binds to dsDNA and the resulting fluorescence corresponds to the concentration of dsDNA in solution.⁵⁵ Briefly, frozen scaffolds were thawed at room temperature, sonicated for 10 min, and vortexed for 5-10 sec to allow the DNA into solution. Standards of calf thymus DNA in ddH₂O in concentrations ranging from 0 – 6 µg/ml were prepared and 50 µl of standard or sample was placed into individual wells of a 96-well plate. Tris-EDTA buffer and PicoGreen dye solution were prepared according to the manufacturer’s instructions using reagents provided in the kit and added at 100 and 150 µl/well respectively. After a
10 min incubation in the dark at room temperature, the fluorescence was measured on a plate reader (FL x800, Bio-Tek Instruments Inc., Winooski, Vermont) using an emission wavelength of 490 nm and an absorbance wavelength of 520 nm. The cellularity of each scaffold was determined by correlating DNA with a known amount of MSCs.

Alkaline Phosphatase Activity

The alkaline phosphatase (ALP) activity of each scaffold was measured using a colorimetric endpoint assay to determine early osteoblastic differentiation of MSCs on Ti scaffolds. The assay measures the conversion of the colorless substrate p-nitrophenol phosphate by the enzyme Alkaline Phosphatase to the yellow product p-nitrophenol, where the rate of color change corresponds to the amount of enzyme present in solution. ALP activity was determined by assaying aliquots of the same aqueous solutions that had been frozen, thawed, sonicated and vortexed for DNA quantification. As necessary, samples were diluted in ddH₂O up to 100x in order to stay within the detection range of the assay. Standards of p-nitrophenol in concentrations ranging from 0 – 250 μM were prepared from dilutions of a 1000 μM stock solution, and 80 μl of standard or sample was placed into individual wells of a 96-well plate. Alkaline buffer solution consisting of 1.5 M 2-amino-2-methyl-1-propanol at pH 10.3 was then added at 20 μl/well. Substrate solution was prepared by dissolving 40 mg 4-nitrophenyl phosphate disodium salt hexahydrate (into 10 ml ddH₂O and added at 100 μl/well. The microplate was incubated for 1 hour at 37°C and the reaction stopped by the addition of 0.3 M NaOH at 100 μl/well. The absorbance of each well at 405 nm was then measured on a plate reader (PowerWave x340, Bio-Tek Instruments Inc., Winooski, Vermont). All samples were run in triplicate and activity is expressed per cell as determined by PicoGreen assay.
Osteopontin Content

The amount of osteopontin released into the media during culture was measured using an ELISA kit available from Assay Designs, Inc. (Ann Arbor, Michigan) to determine mid to late stage osteodifferentiation of MSCs on Ti scaffolds. Osteopontin is an extracellular protein that is produced by matrix-producing osteoblasts as well as bone resorbing osteoclasts. Media samples were collected over the culture period and frozen at -20°C until analysis could be performed. Samples were thawed, briefly vortexed and diluted up to 1000x in order to stay within the detection range of the assay. The assay was performed per instructions provided by the kit manufacturer in a 96-well plate and the absorbance of each well at 450 nm with correction between 570 and 590 nm was measured on a plate reader. Samples were run in duplicate.

Calcium Content

The calcium content of each scaffold was measured using a reagent available from Diagnostic Chemicals Ltd. (Oxford, CT) to quantify the amount of mineralized matrix present, a late stage marker of osteodifferentiation. This colorimetric endpoint assay measures the amount of blue-purple colored calcium-Arsenazo++ complex formed when Arsenazo III binds to free calcium in an acid solution. After the ALP assay, 1.4 ml 1N acetic acid was added to each sample and scaffolds were incubated in the resulting 0.5 M acetic acid overnight on a shaker table to allow the calcium into solution. Samples were diluted up to 20x in order to stay within the detection range of the assay. Ca^{2+} standards in concentrations ranging from 0 – 100 μg/ml were prepared from dilutions of a 1 mg/ml stock solution of CaCl_2 and 20 μl of standard or sample was added to individual wells of a 96-well plate. Arsenazo III reagent was used as provided and added at 300
μl/well. The microplate was incubated for 10 min at room temperature and the absorbance at 650 nm was measured on a plate reader. Samples were run in triplicate.

**Scanning Electron Microscopy**

Samples were rinsed in calcium-free PBS and fixed in 2.5% gluteraldehyde at 4°C overnight. Scaffold/cell constructs were then rinsed 3 times in PBS and incubated at 4°C overnight in PBS. Samples were then dehydrated in a gradient series of ethanol, dried with tetramethylsilane, sputter coated with gold and examined on an XL-30 environmental scanning electron microscope (FEI Company, Hillsboro, Oregon).

**Statistical Analysis**

Results are expressed as means ± standard deviations. Significance was determined using Dunnett’s T3 multiple comparison test for unequal sample sizes and unequal variances with a 95% confidence interval. Osteopontin results are expressed as cumulative release with each point representing a pooled media sample of 4 (static culture) or 6 (flow culture) scaffolds. In order to conserve material so that all experimental conditions could be achieved, it was necessary to culture fewer constructs under static conditions as compared to flow perfusion conditions and this difference in sample size was accounted for during statistical analysis.

**RESULTS**

Scaffold cellularity is shown in Figure 9. The same general trend was observed for all experimental groups where there was an increase in cell number at day 8 followed by a decline in cell number by day 16. There was significantly higher scaffold cellularity for flow constructs cultured in −dex medium compared to flow constructs in +dex
medium during extended culture. Additionally, for constructs cultured in –dex medium during extended culture, there was greater cellularity in flow perfusion culture compared to static culture, regardless of whether dex was present in the medium during primary culture.

Figure 9: Cellularity of scaffold/cell constructs in millions of cells per construct. (A) Primary culture in medium without dexamethasone (B) Primary culture in medium with dexamethasone * indicates significant difference between +dex and –dex medium for same culture condition ** indicates significant difference between static and flow culture for same medium condition, p < 0.05. n = 3 for static culture, n = 5 for flow culture.

Figure 10: Intracellular alkaline phosphatase activity of scaffold/cell constructs on per cell basis. (A) Primary culture in medium without dexamethasone (B) Primary culture in medium with dexamethasone * indicates significant difference between +dex and –dex medium for same culture condition ** indicates significant difference between static and flow culture for same medium condition, p < 0.05. n = 3 for static culture, n = 5 for flow culture.

Alkaline phosphatase (ALP) expression for cell/scaffold constructs is represented in Figure 10 on a per cell basis. Generally, there was an increase in cellular ALP activity
from day 8 to day 16 except for flow constructs cultured in the presence of
dexamethasone in both primary and extended culture. This group exhibited high ALP
activity at day 8 followed by a decline at day 16. For constructs cultured without
dexamethasone during extended culture, there was significantly higher ALP activity
under flow perfusion compared to static culture all time points for both preculture
conditions.

Mineralization of cell/scaffold constructs was determined by dissolving the
calcium within the extracellular matrix in 0.5N acetic acid and measuring the
concentration of calcium ions in solution. Figure 11 shows calcium content on a per
scaffold basis. There was significantly more calcium under flow perfusion compared to
static culture at day 16 for all groups. At this time point, there was also greater calcium
content in +dex constructs compared to –dex constructs, for both static and flow culture
for both preculture conditions.

Figure 11: Calcium content of scaffold/cell constructs in total calcium per construct. (A) Primary
culture in medium without dexamethasone (B) Primary culture in medium with dexamethasone *
indicates significant difference between +dex and –dex medium for same culture condition **
indicates significant difference between static and flow culture for same medium condition, p < 0.05. 
n = 3 for static culture, n = 5 for flow culture.
Osteopontin is a secreted protein and its levels were measured in samples of cell culture medium by a capture ELISA. Because each loop in the flow perfusion bioreactor draws medium from a common reservoir, one medium sample contained all the protein secreted from 6 different samples. Therefore, each point in Figure 12 represents a pooled mean from these 6 samples and statistical analysis was not possible. However, there was a trend in all cases to greater osteopontin production in flow perfusion culture compared to static culture and for +dex medium compared to −dex medium, especially in flow perfusion culture.

Figure 12: Secreted osteopontin protein from scaffold/constructs in cumulative release over 16 days of culture. Individual data points represent a pooled mean of secreted osteopontin protein from 4 (static) or 6 (flow) scaffold/cell constructs. (A) primary culture -dex (B) primary culture +dex.

Figure 13: Scanning electron microscopy images of scaffold/cell constructs cultured for 16 days at 250x magnification. (A) Flow perfusion culture, +dex (B) Static culture, -dex
Scanning electron microscopy was used to visualize the scaffold/cell constructs in order to assess cell morphology. Figure 13 shows microscopy images of scaffold/cell constructs after 16 days of culture under flow in +dex medium (Figure 13A) or in static culture in –dex medium (Figure 13B) at 250x magnification. Cells are visible both on the titanium fibers and within the extracellular matrix between fibers. For flow perfusion culture +dex, the cells were larger, more spread, and appeared to be surrounded by a thicker extracellular matrix. Also, under flow perfusion there was the presence of pore-like structures that were not present in constructs cultured under static conditions.

**DISCUSSION**

The goal of this study was to determine if flow perfusion culture could induce the osteoblast phenotype in rat marrow stromal cells seeded on titanium fiber mesh scaffolds. To this end, these scaffold/cell constructs were cultured under flow or static conditions in either the presence or absence of dexamethasone. The presence of dexamethasone allowed us to compare these results to previous studies conducted in our laboratory,\textsuperscript{45-49,51} while the absence of dexamethasone allowed us to elucidate the effect of flow perfusion alone on osteodifferentiation. By combining flow and dexamethasone we were also able to determine if there was a synergistic effect of the two variables on osteodifferentiation. Reports in the literature suggest that the cells retain a greater osteogenic capacity when dex is present in the medium immediately after harvest, and as such we typically culture expand marrow stromal cells in +dex medium.\textsuperscript{70,74} However, in order to elucidate the effect of flow perfusion culture in the absence of dexamethasone, it was necessary to also preculture the marrow stromal cells in the absence of dexamethasone.
Dexamethasone is a widely used glucocorticoid to induce the osteoblast phenotype in marrow derived progenitor cells when cultured *in vitro*. Under normal physiological bone formation process, these progenitor cells proliferate rapidly and as proliferation tapers off, the cells start down the differentiation pathway. Because dexamethasone is such a potent induction agent, when freshly harvested marrow stromal cells are exposed to dexamethasone immediately after harvest, they may start down the differentiation pathway before they are done proliferating. Thus, when marrow stromal cells are cultured in the absence of dexamethasone, greater cell numbers and a slower differentiation pathway have been observed. Our results follow this trend under flow perfusion conditions; there were more cells on each scaffold for −dex culture compared to +dex culture. We did not observe the same effect under static culture, where there was no significant difference in cellularity between scaffold/cell constructs cultured with or without dexamethasone.

In static culture, there are mass transport limitations to the interior of the scaffolds such that only cells around the perimeter of the scaffold have access to nutrients in the medium. Thus, there is a limited amount of space available to support cell growth and by day 8 of culture, cell number was maximized for the available space under both +dex and −dex conditions. Under flow perfusion, these transport limitations are removed and the scaffolds were able to support greater cell growth as evidenced by higher cell numbers for −dex culture under flow perfusion compared to static culture and higher cell numbers for −dex culture compared to +dex culture under flow perfusion, however this latter trend was not statistically significant at 8 days for −dex primary culture. In addition, we observed a slight decrease in scaffold cellularity from day 8 to day 16 which is consistent
with our previous work\textsuperscript{45-49,51} and can be attributed to both an inability to extract all of the DNA from the cells at day 16 due to entrapment in thick extracellular matrix and programmed cell death resulting from the limited lifespan of terminally differentiated cells.\textsuperscript{65} The former seems to be a greater factor as the most pronounced decreases in cellularity from day 8 to day 16 correspond to those conditions where there was the greatest mineralization at day 16.

Alkaline phosphatase (ALP) is known to be an early marker for the osteoblast phenotype, being up-regulated at the onset of differentiation and subsequently declining as differentiation progresses. Previously, for scaffold/cell constructs cultured in the presence of dexamethasone, we observed a peak in ALP activity around day 8 of culture.\textsuperscript{46-48} For this study, we observed this typical peak under flow perfusion for scaffolds cultured in dexamethasone during both primary and extended culture. For all other conditions, there was a trend to higher ALP activity with increased culture time, which is probably due to a different rate of osteodifferentiation than previously observed, resulting in a peak in alkaline phosphatase activity either after or between the time points chosen for this study. However, the data do suggest that there was increased osteodifferentiation under flow; for –dex medium, there was always greater ALP activity for flow over static culture. While a similar trend was observed for +dex constructs, the difference was not statistically significant in most cases.

Both scaffold cellularity and cell ALP production reflect the early stages of osteodifferentiation of marrow stromal cells and our data suggest that there was a positive effect of flow perfusion culture on osteodifferentiation. But, because these markers can be non-specific to osteoblast differentiation, later stage markers for the osteoblast
phenotype offer more convincing evidence as to the actual cell phenotype. Osteopontin is a protein that is secreted by osteoblasts throughout the differentiation process, but is up-regulated at the onset of matrix mineralization. For all static cultures, there was a low level of osteopontin in the culture medium over the entire culture period, however under flow perfusion there was an increase in the rate of production between days 6 and 12.

Matrix mineralization is an end stage marker for the osteoblastic phenotype and was analyzed by measuring the amount of calcium deposited onto the scaffolds over the culture period. There was very little calcium deposition by day 8; however, there was a significant amount of calcium at day 16 for all groups except that cultured without dexamethasone under static conditions. This time course of increasing calcium content is consistent with the osteopontin data, which suggest that mineralization began somewhere around day 6 for flow perfusion culture and was very pronounced by day 16. At this last time point, there was greater calcification for flow constructs over the corresponding static constructs and for flow constructs, there was greater calcification for +dex constructs over –dex constructs. These data suggest that there was an enhancement of osteodifferentiation for scaffold/cell constructs by flow perfusion and by dexamethasone and these two factors together provided a synergistic effect resulting in very high mineral content.

The calcium data correspond nicely to a study conducted by Aubin in which MSCs were cultured on tissue culture plates in primary culture (7 days) and secondary culture (21 days) in supplemented medium either with or without dexamethasone to determine the osteogenic capacity of the heterogeneous marrow-derived cell population. The number of bone nodules formed after 21 days of secondary culture was dependent on
the presence of dex in the medium in the following order from most to least number of nodules: +dex +dex, −dex +dex, +dex −dex, −dex −dex. There were still bone nodules in those cultures never exposed to dex, indicating that there were two cell populations present in the bone marrow, those that required dexamethasone and those that did not to differentiate into osteoblasts. She concluded that most of the adherent stromal cell population are more primitive osteoprogenitor cells, requiring dexamethasone to differentiate.

Upon initial inspection, it appears that calcium content decreased from day 8 to day 16 for those cells that were not exposed to dexamethasone in either primary or extended culture. However, this is an artifact of using different primary cell batches for each time point and demonstrates that even when pooling marrow cells from several donors, there is still batch to batch variation in the performance level of these cells. Certain cell harvests have greater innate osteogenic potential than others, and because a different cell harvest was performed for each primary culture method (+dex or −dex during initial 6 day culture period) for each time point (8 or 16 days), direct comparisons between different primary culture conditions at similar time points for similar extended culture conditions would be unreliable. However, based on the calcium results for scaffold/cell constructs cultured without dexamethasone under flow perfusion, it appears that initial exposure to dexamethasone may offer these cells a boost down the osteogenic pathway which flow perfusion culture helps to maintain.

**Conclusions**

We observed that titanium fiber mesh scaffold/marrow stromal cell constructs grown under flow perfusion were more mineralized than their static counterparts, even in
the absence of dexamethasone. However, there was a synergistic effect between flow perfusion and presence of dexamethasone in the culture medium, resulting in abundant mineralized matrix after 16 days of culture. There was also an indication that osteogenic differentiation might be enhanced under flow perfusion by exposure to dexamethasone only during the initial cell expansion phase. Finally, no osteogenic differentiation was observed for scaffold/cell constructs when exposed to neither dexamethasone nor flow perfusion.
CHAPTER 5: EFFECT OF BONE EXTRACELLULAR MATRIX SYNTHESIZED IN VITRO ON THE OSTEOSTATIC DIFFERENTIATION OF MARROW STROMAL CELLS

ABSTRACT

Alternative materials for bone grafts are gaining greater importance in dentistry and orthopaedics, as the limitations of conventional methods become more apparent. We are investigating the generation of osteoinductive matrix in vitro by culturing cell/scaffold constructs. The main strategy involved the use of a scaffold composed of titanium fibers seeded with progenitor cells. In this study, we investigated the effect of extracellular matrix (ECM) laid down by osteoblastic cells on the differentiation of marrow stromal cells (MSC) towards osteoblasts. Primary rat MSCs were harvested from bone marrow, cultured in dexamethasone, and seeded directly onto the scaffolds. Constructs were grown in static culture for 12 days and then decellularized by rapid freeze-thaw cycling. Decellularized scaffolds were re-seeded with pre-cultured MSCs at a density of 2.5x10^5 cells/construct and osteogenicity was determined according to DNA, alkaline phosphatase, calcium and osteopontin analysis. DNA content was higher for cells grown on decellularized scaffolds with a maximum content of about 1.3x10^6 cells/construct. Calcium was deposited at a greater rate by cells grown on decellularized scaffolds than the constructs with only one seeding on day 16. The Ti/MSC constructs showed negligible calcium content by day 16, compared with 213.2 ±13.6 μg/construct.

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for the Ti/ECM/MSC constructs cultured without any osteogenic supplements after 16 days. These results indicate that bonelike ECM synthesized *in vitro* can enhance the osteoblastic differentiation of MSCs.

**INTRODUCTION**

Culture of osteoblasts in 3-D is important in developing tissue engineered constructs for regeneration of large bone defects. To date, osteoblasts and osteoprogenitor cells have been cultured on a variety of scaffold materials and their ability to induce bone formation has been examined. While some scaffolds have shown an innate ability to support bone formation during *in vitro* culture, others have been surface modified with various molecules to aid their osteoinductive capacities. The most common of these enhancers include molecules present in native bone extracellular matrix including hydroxyapatite and collagen type I. Other approaches to enhancing bone formation by cell/scaffold constructs have involved the delivery of certain growth factors that are known to participate in normal osteoblast differentiation including bone morphogenic proteins, connective tissue growth factor, and transforming growth factors from the TGF-β family. However, an ideal cell/scaffold/signal construct has yet to be developed for use in bone tissue engineering applications.

One approach to development of more osteoinductive materials involves use of native components of bone extracellular matrix (ECM) to create the scaffold. There has been much focus on the use of bone-derived materials for the 3-D culture of osteoblast type cells, and these can generally be divided into two categories: demineralized bone matrix and ceramics. The first utilizes the organic components of bone ECM while the second utilized the inorganic components of bone ECM. While both
scaffold types have been found to have inherent bone inducing properties, there have been limited studies on the osteoinductive capacity of the combined organic and inorganic bone extracellular matrix molecules.\textsuperscript{76} We therefore sought to investigate the osteoinductive capacity of a decellularized bone ECM synthesized \textit{in vitro} on a titanium fiber mesh scaffold. Previously, it has been shown that when rat marrow stromal cells (MSCs) are seeded onto titanium fiber mesh and grown in the presence of osteogenic supplements, they will form bone-matrix \textit{in vitro}.\textsuperscript{96}

In this study, we investigated the osteoinductive properties of ECM produced by MSCs seeded onto titanium fiber meshes and grown in culture for 12 days. Bone formation on titanium meshes by MSCs has been shown to be successful in the presence of osteogenic supplements, including dexamethasone.\textsuperscript{76,77} We hypothesized that the ECM deposited on the titanium scaffold will induce osteoblastic differentiation of MSCs even in the absence of osteogenic supplements (where osteogenic supplements are defined as \(\beta\)-glycerophosphate and ascorbic acid in the presence of dexamethasone). The aim of this study was to investigate the effect of ECM deposited by osteoblast-like cells on the differentiation of freshly seeded MSCs in static culture. Differentiation of MSCs was determined by analysis of known indicators of the osteoblast phenotype including cell proliferation, production of alkaline phosphatase, secretion of osteopontin, and deposition of a calcified ECM.
METHODS AND MATERIALS

Scaffold Preparation

The constructs were prepared by die-punching discs from a sheet of titanium (Ti) fiber mesh prepared by Bekaert N.V. (Zwevegem, Belgium). Scaffolds were 5 mm in diameter and 0.8 mm thick, with fiber size at 20 μm and porosity of 86% according to manufacturer specifications. Scaffolds were sterilized by autoclaving prior to cell seeding.

Marrow Stromal Cell Isolation

Marrow Stromal Cells were harvested from the tibiae and femora of 6-8 week old male Wistar rats, weight 150-175g. Briefly, rats were euthanized using 4% isoflurane in CO₂, and the bones were aseptically excised from the hindlimbs.⁴⁶,⁵⁴ External soft tissue was discarded and the bones were placed in 50 mL DMEM supplemented with 200 μg/mL of a PSN cocktail for approximately 15 minutes. This concentration of antibiotics is 4 times the normal concentration used in cell culture and is used to avoid contamination during the harvesting process. The proximal end of the femur and the distal end of the tibiae were clipped. An 18-gauge needle was inserted into the hole in the knee joint at each end of the bone, and the marrow was flushed out from the shaft with α-MEM. The resulting marrow pellet was broken up by trituration, and the cell suspensions from all bones were combined in a centrifuge tube.

Generation of Ti/ECM Constructs

Cells were cultured in 75cm² flasks in α-MEM with osteogenic supplements (OS) (0.01M β-glycerophosphate, 50mg/L ascorbic acid, 10⁻⁸M dexamethasone) for 6 days. Medium was changed at 1 and 3 days to remove the nonadherent cell population. The
cells were trypsinized, centrifuged, resuspended in a known amount of medium and seeded on the meshes at 2.5 x 10⁵ cells in 100 µl of α-MEM. Cells were allowed to attach for 3 hours before adding 4mL complete medium to each well of a 6-well plate. After 24 hours of attachment, cell/scaffold constructs were transferred to 12-well plates and cultured for 12 days in complete medium. At the end of the culture period, constructs were rinsed in PBS and stored in sterile distilled and deionized water at -20°C.

The cellular component of the constructs was removed to produce the titanium fiber mesh scaffolds with deposited ECM (Ti/ECM). Scaffolds were thawed in a water bath at 37°C for 10 min, rinsed with phosphate buffered saline (PBS) to remove cellular debris, and frozen in liquid N₂ for 10 min. Constructs underwent 3 freeze/thaw cycles under sterile conditions to ensure complete removal of the cellular component. Samples were then placed in 6-well plates and allowed to air dry before seeding.

**Generation of Ti/ECM/MSC Constructs**

Fresh MSCs were harvested as discussed above and cultured for 6 days in augmented primary (α-MEM with β-glycerophosphate and ascorbic acid) or complete medium (α-MEM β-glycerophosphate, ascorbic acid and dexamethasone). The decellularized Ti/ECM constructs were then seeded with 2.5 x 10⁵ cells in 100 µl of primary medium. Cells were allowed to attach for 3 hours before adding 4mL primary or complete medium to each well of a 6-well plate. After 24 hours, Ti/ECM/MSC constructs were transferred to 12-well plates and cultured in primary or complete medium for 1, 4, 8, 12 or 16 days. Media were changed every 2 days over the culture period. Similarly, plain titanium fiber meshes were seeded with 2.5 x 10⁵ cells in 100 µl primary medium
(Ti/MSC constructs) and cultured in primary or complete medium for 1, 4, 8, 12, or 16 days. As a control, unseeded decellularized Ti/ECM scaffolds were cultured for the same time period in complete medium. Experimental groups were Ti/ECM/MSC constructs with OS, Ti/ECM/MSC constructs without OS, Ti/MSC constructs with OS and Ti/MSC constructs without OS and Ti/ECM with OS (see Table. 1). Each group consisted of n=6 samples. At the end of the culture period, all constructs were rinsed with PBS and stored in 1.5ml ddH₂O at -20°C until analysis was performed.

**Table 1: Summary of Experimental Groups and Culture Conditions Tested**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>ECM Deposited</th>
<th>MSCs Seeded</th>
<th>Osteogenic Supplements (OS)</th>
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<tbody>
<tr>
<td>Ti/MSC with OS</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ti/MSC</td>
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<tr>
<td>Ti/ECM/MSC</td>
<td>+</td>
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**Assays**

The cellularity of the constructs was determined using the PicoGreen assay kit from Molecular Probes (Eugene, OR) to measure dsDNA content. The PicoGreen dye binds to major grooves in dsDNA, and the resulting fluorescence activity corresponds to dsDNA concentration in solution. The cell/scaffold constructs were thawed at room temperature and sonicated for 10 min to allow cellular DNA into the solution. Working buffer provided with the kit was added to each well of a 96-well plate at 100µl/well. Standards and samples were then added to each well at 50µl/well in triplicate. PicoGreen dye solution was added to each well at 150µl/well and allowed to incubate for 10 min in
the dark at room temperature. Fluorescence at 545nm was read on a plate reader to
determine DNA concentrations per construct. Samples were run in triplicate and
compared against calf thymus DNA standards. A conversion factor of 3 pg DNA/cell
was used to calculate cell number based on MSC standards.

Alkaline phosphatase is an enzyme expressed by cells and is an early marker of
osteoblastic differentiation. Alkaline phosphatase enzyme activity of the constructs was
measured using an Alkaline Phosphatase Assay from Sigma (St. Louis, MO). The
enzyme modifies a substrate (p-nitrophenol phosphate) to produce a yellow product (p-
nitrophenol) which corresponds to the amount of enzyme in solution. Briefly, the
cell/scaffold constructs were thawed at room temperature, sonicated for 10 min, and
vortexed for 10 sec to allow the enzyme into solution. The samples were diluted 50 times
to ensure the results were within tolerance ranges of the assay. Standards and samples
were added at 80μl/well. Working alkaline buffer solution provided with the kit was
added at 20μl/well and the substrate solution was added at 100 μl/well. The plate was
covered and incubated for 1 hour at 37°C. After the incubation period, 100 μl of 0.3M
NaOH was added to each well to stop any further reactions. Absorbance was read at 405
nm on a plate reader to determine enzyme concentrations per construct. Samples were
run in triplicate and compared against p-nitrophenol standards.

Ca²⁺ presence is a late stage marker of osteoblast differentiation. Calcium content
of the constructs was determined using a calcium quantification assay provided by Sigma
(St. Louis, MO). A calcium binding agent changes color upon chelating dissolved
calciium, and this color change corresponds to calcium ion concentration. Samples were
thawed and sonicated for 10 minutes, then vortexed for 10 sec each. The cell/scaffold
constructs were placed in 0.5 N acetic acid and calcium was allowed to dissolve overnight on a shaker table, operating at 100 rpm. After vortexing for 10 sec, some of the later-day timepoints were diluted to ensure the results were within tolerance ranges of the assay. Samples and standards were added to a 96-well plate at 10 μl/well and the binding agent was added to the wells at 300 μl/well. Absorbance was read at 575 nm on a plate reader to determine calcium ion concentration per construct. Samples were run in triplicate and compared against CaCl₂ standards.

Osteopontin is a secreted protein that is a late stage marker of osteoblastic differentiation. Osteopontin content of the constructs was measured using an ELISA kit against rat osteopontin available from Assay Designs (Ann Arbor, MI). Media samples were collected every 2 days during the culture period and stored at -20°C. These samples were thawed and vortexed for 10 sec, and diluted up to 1000x in assay buffer. One hundred μl of sample or standard was allowed to incubate on the plate provided with the kit at room temperature for 1 hour. Each well was rinsed with wash buffer and 100 μl of secondary antibody solution was added to each well and allowed to incubate at 37°C for 30 min. Each well was rinsed thoroughly with wash buffer and 100 μl of labeled antibody was added to each well. The plate was incubated at 4°C in the dark for 30 min before 100μl of stop solution was added to each well. Absorbance was read at 450 nm with correction between 570-590 nm. Samples were run in duplicate and compared against rat osteopontin standards.
Statistical Analysis

All assays were performed in triplicate and results reported as means ± standard deviation for n = 6. Statistical analysis was performed on all results using analysis of variance. A two-tailed unpaired t-test between sample sets with a significance level of p < 0.05 was used for multiple comparison tests.

RESULTS

Figure 14 illustrates scaffold cellularity. The initial cell number for the Ti/ECM/MSC constructs was higher than expected, however the general trend observed is consistent with the other cultures. With the exception of the Ti/ECM OS group, all groups exhibited an increase in growth, a plateau, then a decrease from day 12- day 16. The Ti/ECM OS group exhibited a marked decrease in cell number over the entire time period.
Figure 14: Cellularity of Ti scaffolds seeded with MSCs over 16 days. The amount of dsDNA released from the constructs into the supernatant after sonication was measured. Experimental groups were titanium scaffolds with ECM and seeded with MSCs in osteogenic supplements (OS), titanium scaffolds with ECM and seeded with MSCs without OS, titanium scaffolds seeded with MSCs in OS, titanium scaffolds seeded with MSCs without OS and titanium scaffolds with ECM in OS. Data represent means ± standard deviation for n = 6.

Figure 15 shows the results of ALP activity of the constructs presented on a per cell basis. Enzyme activity was significantly higher for the Ti/ECM/MSC constructs in OS, without any observable peak. The Ti/MSC OS constructs exhibited a peak at the day 12 timepoint, while the Ti/ECM/MSC constructs showed comparatively greater enzyme activity with no observable peak. Alkaline phosphatase activity for the Ti/ECM constructs was not measured.
Figure 15: Alkaline Phosphatase activity of Ti scaffolds seeded with MSCs over 16 days. Enzyme activity was measured from release from constructs into supernatant after sonication and vortexing. Experimental groups were titanium scaffolds with ECM and seeded with MSCs in OS, titanium scaffolds with ECM and seeded with MSCs without OS, titanium scaffolds seeded with MSCs in OS, titanium scaffolds seeded with MSCs without OS and titanium scaffolds with ECM in OS. Data represent means ± standard deviation for n = 6.

Scaffold calcium content is shown in Figure 16. Constructs show an increase in calcium content over time. The Ti/ECM/MSC constructs, both with and without OS, show significantly higher matrix deposition when compared to their Ti/MSC counterparts, with the Ti/ECM/MSC OS constructs showing the greatest calcium deposition over time.
Figure 16: Calcium content of Ti meshes seeded with MSCs over 16 days. Samples were dissolved in 0.5 N acetic acid overnight and Ca²⁺ concentration in supernatant was measured. Experimental groups were titanium scaffolds with ECM and seeded with MSCs in OS, titanium scaffolds with ECM and seeded with MSCs without OS, titanium scaffolds seeded with MSCs in OS, titanium scaffolds seeded with MSCs without OS and titanium scaffolds with ECM in OS. Data represent means ± standard deviation for n = 6.

The osteopontin results, shown in Figure 17 show greatest detection of the protein for the Ti/MSC OS constructs, and least detection for Ti/MSC without OS constructs. The Ti/ECM/MSC OS constructs had the next highest, followed by the Ti/ECM/MSC constructs. Osteopontin levels were not measured for the Ti/ECM constructs since there were no cells present to generate the protein.
Figure 17: Osteopontin levels in media samples from Ti meshes seeded with MSCs over 16 days. Media samples every second day were measured for protein content. Experimental groups were titanium scaffolds with ECM and seeded with MSCs in OS, titanium scaffolds with ECM and seeded with MSCs without OS, titanium scaffolds seeded with MSCs in OS, titanium scaffolds seeded with MSCs without OS and titanium scaffolds with ECM in OS. Data represent means ± standard deviation for n=6.

**DISCUSSION**

The aim of this study was to investigate the effect of ECM deposited by osteoblast-like cells on the differentiation of freshly seeded MSCs in static culture. Investigation of the osteoinductive capacity of a decellularized bone extracellular matrix yielded results which show that late markers, such as calcium deposition and osteopontin activity, responded positively with increased expression.

Dexamethasone has been shown to direct osteoblastic differentiation of bone marrow stromal cells *in vitro* at both early and late stages toward terminal maturation. Thus, cultures treated with complete media (OS with
dexamethasone) were expected to show overall increased markers of osteoblastic differentiation compared to cultures treated only with primary media (no dexamethasone). This hypothesis was confirmed in all assay results, regardless whether ECM was present or not.

The cellularity of the scaffolds was quantified by analyzing DNA content using the PicoGreen assay, since dsDNA content has been shown to have a direct relationship with cell number.\textsuperscript{45,46,48} The general trend observed for the cellularity of Ti/MSC constructs was consistent with what is normally observed with this cell type, with a plateau phase occurring around day-4, then tapering off after day-8.\textsuperscript{45,46,48} This would indicate that there is no difference in the pattern of cell growth occurring between the experimental groups, however, the decellularized scaffolds were able to support more growth (p<0.05). It may be that these scaffolds had signal molecules remaining on the ECM surface that promoted cell attachment and growth. It is also possible that the decellularization process left behind some cell components, including cell membrane fragments. These fragments may have had growth promoting factors that encouraged the greater cell growth observed. These results are consistent with the calcium results, since increased matrix deposition would trap and inhibit DNA from being detected by the PicoGreen assay. Osteoblastic cells would be particularly difficult to detect since these cells are prone to surrounding themselves in matrix.

Alkaline phosphatase activity is a transient early marker of osteodifferentiation in marrow stromal cells and is generally a good indicator of differentiation.\textsuperscript{20} We expect to see an initial rise in activity followed by a decrease corresponding to further differentiation of the cells, when alkaline phosphatase production slows. We observed a
peak in activity occurring at the day-12 timepoint. Because time points were taken every 4 days, it is possible that the actual peak in ALP activity occurred between the measured time points. There was statistically no noticeable increase in alkaline phosphatase activity for Ti/MSC constructs cultured without osteogenic supplements indicating that these cells were not differentiating down the osteoblast pathway. In contrast, there was an increase in ALP activity for the same cells cultured on the decellularized Ti/ECM constructs, indicating that the ECM had a positive effect on osteoblast differentiation of marrow stromal cells (p<0.05). This observation is further corroborated by the calcium data which showed no measurable calcium in Ti/MSC constructs grown without OS while there was an increase in calcium content for Ti/ECM/MSC constructs grown without OS over the culture period. In addition, there was greater calcium deposition at 16 days on Ti/ECM/MSC constructs grown with OS than in the corresponding constructs without prior ECM deposition (p<0.05). Although, there was still much more calcified matrix in all samples grown with OS than those grown without OS. Clearly, the predeposited ECM had a beneficial effect on the differentiation of MSCs on titanium fiber mesh and this effect was enhanced in the presence of osteogenic supplements.

Calcium content of scaffolds is indicative of late stage differentiation of osteoblasts and we would expect to see a continual increase in calcium content over the culture period. All constructs that were seeded with MSCs showed a consistent increase in calcium content, with the highest value occurring at day-16. The day-1 calcium content for the decellularized scaffolds was expected to be relatively close to the day-12 Ti/MSC OS control group, since the decellularized scaffolds were precultured for 12 days in OS prior to decellularization. The results were consistent with such
expectations. The data indicate a beneficial effect of OS on calcium deposition, with both experimental and control groups exhibiting higher calcium deposition in OS than without (p<0.05). However, even with a lack of OS, the decellularized constructs (Ti/ECM/MSC) were able to generate more matrix than the control counterpart (Ti/MSC). This calcium deposition data for Ti/ECM OS (essentially plateaued calcium content) suggest that there were no cells present in the constructs following the decellularization process, consistent with expectations.

Osteopontin is expressed during the early stages of the differentiation of osteoblast progenitors in the bone marrow and is a direct indicator of osteoblastic proliferation in vitro.\textsuperscript{99} The relatively high ECM deposition exhibited by the Ti/ECM/MSC constructs in OS explains the relative osteopontin levels observed. Because osteopontin is a secreted protein, the high levels of ECM would easily sequester osteopontin within the scaffold, allowing less to be detected in the surrounding media. The Ti/MSC constructs showed ~24 μg/scaffold at day 16, compared with ~3 μg/scaffold for the Ti/ECM/MSC constructs, consistent with the calcium deposition observed.

**CONCLUSION**

MSCs grown on a decellularized scaffold with a pre-existing ECM exhibited greater cell numbers and calcium deposition, and relative osteopontin levels showed correlation between matrix deposition and detection of the protein. The data indicate that the presence of bone-like extracellular matrix deposited on titanium fiber mesh scaffolds can induce osteoblastic differentiation in bone progenitor cells in static culture. The osteoinductive capacity of a decellularized bone extracellular matrix was demonstrated by greater calcium deposition as well as increasing osteopontin levels exhibited by
cultured cells. Clinical applications of this methodology may reduce or eliminate the need for osteoblastic supplements to induce osteoblastic differentiation.
CHAPTER 6: ECTOPIC BONE FORMATION BY RAT MARROW STROMAL CELLS SEEDED ON TITANIUM FIBER MESH WITH A TETHERED RGD PePTIDE*

ABSTRACT

Titanium fiber mesh scaffolds have been shown to be a suitable material for culture of primary marrow stromal cells in an effort to create tissue engineered constructs for bone tissue replacement. In native bone tissue, these cells are known to attach to extracellular matrix molecules via integrin receptors for specific peptide sequences, and these attachments can be a source of cell signaling, affecting cell behaviors such as differentiation. In this study we examined the ability of primary rat marrow stromal cells at two different stages of osteoblastic differentiation to further differentiate into osteoblasts both in vitro and in vivo when seeded on titanium fiber mesh scaffolds either with or without RGD peptide tethered to the surface. In vitro, the tethered RGD peptide resulted in reduced initial cell proliferation and reduced calcium deposition after 16 days, indicative of delayed osteoblastic differentiation. In vivo, there was no effect of tethered RGD peptide on ectopic bone formation in a rat subcutaneous implant model. Scaffold/cell constructs exposed to dexamethasone for 4 days prior to implantation resulted in significant bone formation where as no bone formation was observed in –dex constructs. These results suggest that the osteoblastic differentiation of marrow stromal cells is delayed by surface tethered RGD peptide, and that the initial differentiation stage

* This chapter was submitted for publication in Biomaterials.
of implanted cells plays a bigger role than scaffold/cell attachment method in ectopic bone formation.

**INTRODUCTION**

Titanium is a biomaterial that has been used extensively in implants for hip and knee replacements due to its excellent biocompatibility, particularly with bone tissue. While titanium metal is not a degradable material, its supreme biocompatibility with bone tissue makes it an excellent model scaffold material for the tissue engineering of new bone. Our laboratory has been using titanium fiber mesh scaffolds to elucidate key elements in creating tissue engineered bone constructs combining cells, scaffolds, and signaling molecules. *In vitro*, these fiber mesh scaffolds support the adhesion and osteoblastic differentiation of primary marrow stromal cells in both static and flow perfusion culture. 34,45-48 *In vivo*, the material has been shown to be osteoconductive in an orthotopic site, 33 but not osteoinductive in an ectopic site 27 when used alone. However, addition of osteoprogenitor cells to these scaffolds resulted in enhanced bone formation within the fiber mesh scaffolds in both orthotopic 33 and ectopic 27 locations. Furthermore, bone formation on these scaffolds was enhanced by applying a calcium phosphate coating to the fiber mesh, 31,32 suggesting that the surface properties of these titanium fiber meshes play an important role in bone formation and other types of surface modifications may also benefit in vivo bone formation or in vitro osteoblastic differentiation of progenitor cells.

Osteoprogenitor cells are known to adhere to extracellular matrix proteins via cell surface integrins and these integrin binding events affect both cell attachment and differentiation. 100 These integrins are generally composed of dimers of alpha and beta
subunits, leading to a variety of combinations that can attach to specific binding domains on many different extracellular matrix proteins. Fibronectin is one such extracellular matrix protein that binds to osteoblastic cells via interactions between its own cell binding domains and three particular cell surface integrin dimers; α5β1, α3β1, and αvβ3. An RGD peptide sequence is located within fibronectin binding domains, and it is this particular sequence that interacts with both αvβ3 and α5β1 integrins. By introducing either whole protein or smaller peptide sequences to the surface of a tissue engineering scaffold, cell attachment to the scaffolds would be mediated in a manner more closely resembling native cell/matrix interactions, allowing for the cell signaling events that accompany such binding, such as those in the osteoblastic differentiation pathway. Van den Dolder et al. examined the effect of coating titanium fiber mesh scaffolds with fibronectin and/or collagen type I on the differentiation of marrow stromal cells seeded onto these scaffolds and showed that there was no effect of coating on osteoblastic differentiation over 16 days of in vitro culture. However, the conformation of proteins is known to change when adsorbed to solid surfaces and this alteration in structure may inactivate the binding domains of these proteins. Alternatively, a short peptide sequence such as RGD that is known to participate in integrin binding but does not require retention of a specific 3-dimensional structure could be tethered to the scaffold surface to affect cell attachment and/or differentiation.

Marrow stromal cells are primitive osteoprogenitor cells whose use in bone tissue engineering constructs has been well established. Because these cells are pluripotent, addition of osteogenic supplements such as L-ascorbic acid, β-glycerophosphate, and dexamethasone are typically needed to drive the osteoblastic
differentiation of these cells in vitro.\textsuperscript{54,68} The expression of cell surface integrins changes as osteoblast differentiation proceeds such that osteoblasts at various stages of differentiation may have different binding affinities for extracellular matrix molecules due to altered integrin expression.\textsuperscript{104}

We hypothesize that by tethering an adhesion peptide to the surface of titanium fiber mesh scaffolds, the osteoconductive behavior of the scaffold/cell constructs can be enhanced through one or two possible mechanisms: increased initial cell attachment to the scaffolds via the adhesion sequence or increased osteodifferentiation of progenitor cells due to integrin binding of cells to the scaffold surface. In this study, we examined the effect of tethering an RGD peptide to the surface of a titanium fiber mesh scaffold on the osteoblastic differentiation of marrow stromal cells both in vivo and in vitro. Two different cell populations were used at the time of seeding onto the scaffolds to determine if the initial stage of cell differentiation, and therefore pattern of integrin expression, had an effect on the eventual osteoblastic differentiation of these cells on peptide coated scaffolds. Dexamethasone is known to be a potent osteoblast inductive agent\textsuperscript{54,68,70,72} and has also been shown to affect integrin expression in differentiating osteoblasts with an up-regulation in $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins after 2 days of exposure.\textsuperscript{105} Therefore, two different cell populations were created by culturing either with or without dexamethasone for 3 days prior to cell seeding. Scaffold/cell constructs were cultured for 1 day after seeding in appropriate medium to allow for cell attachment before being implanted subcutaneously in the rat dorsum for 28 days or placed into well plates for 16 days of static culture. Osteoblastic differentiation was evaluated in vitro by quantifying scaffold
cellularity, cellular alkaline phosphatase activity, and construct calcium content while
differentiation was evaluated in vivo by histology.

MATERIALS AND METHODS

All chemical reagents were purchased from Sigma (St. Louis, MO) unless
otherwise specified.

Scaffolds

Plain titanium fiber mesh scaffolds were obtained by die-punching 6 mm diameter
discs from a 0.8 mm thick sheet of mesh composed of non-woven titanium fibers, 40 μm
in diameter (Bekaert, Zwevegem, Belgium). These discs were then sterilized by
autoclaving prior to use. Titanium fiber mesh scaffolds with tethered RGD peptide were
used as provided by Biomet Deutschland GmbH (Darmstadt, Germany). The peptide was
tethered to the titanium fiber mesh surface by the manufacturer as follows. Titanium fiber
mesh scaffolds were immersed in a solution of 100 μM cyclic RGD peptide in PBS (pH
7.4) over night. The specimens were washed three times with PBS, dried, packed and
then sterilized by gamma-irradiation with 25 kGy. To test for the quality of coating,
sterilized specimens were rehydrated in PBS and subsequently blocked with 5% bovine
serum albumin (BSA) in PBS for two hours. The specimens were then tested for cell
adhesion or submitted to a customized RGD specific enzyme linked immunosorbent
assay (ELISA) with colorimetric detection at 450 nm measured with a microplate reader
(SLT Rainbow). The resulting surface concentration of the peptide was not directly
measured, however the concentration of peptide in the coating solution has been shown to
result in increased cell plating efficiency on smooth titanium surfaces, indicating that there was sufficient surface modification to enhance cell attachment.

**Cell Culture**

Primary rat marrow stromal cells were harvested from the femora and tibiae of 6-8 week old male Fischer 344 rats (140-160g). Briefly, the leg bones were aseptically excised, the epiphyses cut off and the diaphyses were flushed with 5 ml primary medium (α-MEM with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 50 μg/ml gentamicin, 100 μg/ml ampicillin and 0.5 μg/ml fungizone). The resulting marrow pellet was broken up and the cells were plated into 75 cm² tissue culture flasks and cultured in incomplete medium (primary medium plus 50 μg/ml L-ascorbic acid and 0.1 M β-glycerophosphate). Medium was changed at 1 day and at 3 days was replaced with either incomplete medium or complete medium (incomplete medium plus 10 nM dexamethasone) and the cells were cultured for 3 more days. After this 6 day cell expansion phase, marrow stromal cells were seeded onto titanium fiber mesh scaffolds by adding a 100 μl droplet containing 300,000 cells to the top of each scaffold. Scaffolds were seeded in individual wells of a 6-well plate and cells were allowed to attach for 2 hours before 4 ml of the appropriate culture medium was added to each well. After 1 day, scaffold/cell constructs either were implanted subcutaneously for 28 days or remained in static culture for up to 16 days.

There were four experimental groups for each time point: 1) plain titanium scaffolds seeded with MSCs cultured without dexamethasone (Ti –dex) 2) plain titanium scaffolds seeded with MSCs cultured with dexamethasone (Ti +dex) 3) titanium scaffolds with tethered RGD peptide seeded with MSCs cultured without dexamethasone (Ti-RGD
-dex) 4) titanium scaffolds with tethered RGD peptide seeded with MSCs cultured with dexamethasone (Ti-RGD +dex). For each in vitro time point, 4 scaffold/cell constructs were cultured per group.

**Subcutaneous Implantation**

Titanium fiber mesh scaffolds with freshly seeded marrow stromal cells were implanted subcutaneously into the dorsa of 8 male Fischer 344 rats (330-360 g). One construct from each experimental group was implanted in each animal resulting in 4 implants per animal (32 implants total). Animals were anesthetized with a 4% isoflurane/oxygen mixture which was then reduced to 2% during surgical manipulation. The animals were placed in a ventral position, the backs clipped and disinfected and two small incisions were made along the vertebral column. A pocket was created on both sides of each incision using blunt dissection and 1 scaffold/cell construct was placed in each pocket. The incisions were closed with Vicryl 5-0 sutures. After 28 days, animals were sacrificed and the implants were retrieved. NIH guidelines for the care and use of laboratory animals were observed throughout the course of this study.

**Histology**

Implants were fixed in a solution of 4% formalin in PBS, dehydrated in a gradient series of ethanol, and embedded in methylmethacrylate. Thin sections (10 mm) were prepared using a modified sawing microtome technique and stained with methylene blue and basic fuchsin to visualize bone formation (bone appears red, cells appear blue). Three sections from each implant were imaged using a light microscope (Eclipse E600 Nikon,

**Histomorphometry**

Digital images of histological sections were obtained via a slide scanner (Epson Perfection 2400 photo scanner). These images were then analyzed using NIH Image software (version 1.62) to quantify the total implant area, the area occupied by the fiber mesh (black), and the area occupied by bone (red). The amount of bone formation within the scaffold was determined by dividing the bone area by the area available for tissue ingrowth (total area – area of fiber mesh). Sections were defined as positive for bone formation if the amount of bone was greater than 1%.

**Scaffold Cellularity**

Cellularity of scaffold/cell constructs cultured *in vitro* was determined using a PicoGreen assay kit from Molecular Probes (Eugene, OR) to quantify the amount of double stranded DNA. When PicoGreen dye binds to double stranded DNA in solution, the complex fluoresces and the amount of fluorescence corresponds to the concentration of DNA in solution.\(^{55}\) Briefly, scaffold/cell constructs were rinsed with PBS, placed in 1.4 ml of ddH\(_2\)O and stored at -20°C until analyzed. Samples were then thawed at room temperature for 1 hour and sonicated for 10 min to allow DNA into solution. 50 μl aliquots of sample were added to 100 μl of buffer and 150 μl of dye solution in individual wells of a 96-well plate and fluorescence at 520 nm was read on a plate reader (FL x800, Bio-Tek Instruments Inc., Winooski, Vermont). Samples were compared against calf thymus DNA standards and cellularity was calculated against MSC standards.
Alkaline Phosphatase

Alkaline phosphatase (ALP) activity was used as an early marker for osteoblastic differentiation of marrow stromal cells and was quantified by using a colorimetric endpoint assay using conversion of the colorless substrate p-nitrophenyl phosphate to yellow-colored p-nitrophenol. The same samples were used for ALP analysis as for DNA analysis. 80 µl aliquots of sample were added to 20 µl of alkaline buffer (1.5 M 2-amino-2-methyl-1-propanol at pH 10.3) and 100 µl of substrate solution (4 mg/ml) in individual wells of a 96-well plate. After the plate was incubated at 37°C for 1 hour, 100 µl of 0.3 N NaOH was added to stop the reaction and the absorbance at 405 nm was measured on a plate reader (PowerWave x340, Bio-Tek Instruments Inc., Winooski, Vermont). Samples were compared against p-nitrophenol standards.

Calcium Content

Calcium content was used as a late stage marker for osteoblastic differentiation and was quantified by a colorimetric endpoint assay that measures the the amount of blue-purple colored calcium-Arsenazo** complex formed when Arsenazo III binds to free calcium in an acid solution. After ALP analysis the same samples were used for calcium quantification. Samples were placed in 0.5 N acetic acid overnight on a shaker table to dissolve the deposited calcium. 20 µl aliquots of sample were added to 300 µl of Arsenazo III reagent (Diagnostic Chemicals Ltd., Oxford, CT) in individual wells of a 96-well plate and the absorbance at 650 nm was read on a plate reader. Samples were compared against CaCl₂ standards.
Statistics

Histology results are represented as proportion of implants that resulted in bone formation. Significance was determined using a Chi-square test of association at a 95% confidence level. All other data are represented as a mean ± standard deviation and significance was determined using Tukey's multiple comparison procedure at a 95% confidence level.

RESULTS AND DISCUSSION

The goal of this study was to determine if osteodifferentiation of marrow stromal cells seeded on titanium fiber mesh scaffolds could be enhanced by tethering the cell adhesion peptide RGD to the surface of the scaffold. We have shown titanium fiber mesh to be a suitable material for culturing osteoblasts in vitro and to be osteoconductive when placed in vivo. We've also shown that bone formation is further enhanced in these scaffolds by the addition of osteoprogenitor cells to the scaffolds prior to implantation in both an ectopic and orthotopic site. Cells are known to attach to extracellular matrix via integrin receptors for specific peptide sequences, and these adhesion peptides can also be a source of cell signaling to affect cell behaviors such as differentiation of progenitor cells. Therefore, we hypothesized that by tethering an adhesion peptide to the surface of our titanium fiber mesh scaffolds, the osteoconductive behavior of the scaffold/cell constructs could be enhanced through one or two possible mechanisms: increased initial cell attachment to the scaffolds via the adhesion sequence or increased osteodifferentiation of progenitor cells due to integrin binding of cells to the scaffold surface.
Primary rat marrow stromal cells were cultured on titanium fiber mesh scaffolds both *in vitro* and *in vivo* to assess the effect of RGD peptide on the osteodifferentiation of these cells. After harvesting, the marrow stromal cells were culture expanded for 6 days in order to provide sufficient cell numbers to complete these studies. Because progenitor cells express different cell surface markers, including those integrin receptors that bind to RGD peptide, at different stages of differentiation, we wanted to seed cells at two different stages of differentiation. In order to achieve this, we culture expanded all cells for the first 3 days in incomplete medium. For the next 3 days, half of the cells received medium containing dexamethasone (complete medium) and the other half continued receiving medium without dexamethasone. Since dexamethasone is known to induce osteoblastic differentiation in marrow stromal cells, we expected that those cells with 3 days of exposure to medium containing dexamethasone would be more osteoblastic than those cells that had not been exposed and we were able to seed cells at two different stages of differentiation onto the titanium fiber mesh scaffolds. During the subsequent extended *in vitro* culture period, scaffold/cell constructs continued to be cultured in either complete or incomplete medium.

Figure 18 shows the cellularity of scaffold/cell constructs cultured *in vitro* for up to 16 days. At day 1, there is no significant difference in the number of cells attached to scaffolds between the two types of fiber meshes, indicating that the tethered RGD peptide did not enhance cell attachment. Over the first 8 days of culture, scaffold cellularity increased for all groups, a trend that was expected as marrow stromal cells are known to undergo a period of rapid proliferation before proceeding down the differentiation pathway. By day 6, cell number plateaued for all groups indicating that the initial
proliferation phase was over. By day 8, all scaffolds had the same cellularity with the exception of the plain titanium scaffold/cell construct cultured in the presence of dexamethasone, which had a significantly higher cell number than all other groups.

![Graph showing cell number over days of culture for different scaffold conditions](image)

**Figure 18:** Cellularity of scaffold/cell constructs cultured in vitro for up to 16 days. Ti indicates a plain titanium fiber mesh; Ti-RGD indicates a titanium fiber mesh with RGD peptide tethered to the surface; -dex indicates cells were cultured in the absence of dexamethasone; +dex indicates cells were cultured in medium containing 10 nM dexamethasone. * indicates a statistically significant difference in means, p < 0.05.

Greater cellularity of the Ti +dex constructs over the Ti –dex constructs suggests that the presence of dexamethasone in the culture medium enhanced cell proliferation on the titanium fiber mesh scaffold. We would then expect to see increased cell proliferation in the Ti-RGD +dex constructs as well, however this trend was not observed. This is not surprising considering that the attachment of cells to scaffold via specific integrin receptors is likely a stronger bond than attachment of cells to the titanium surface. This
increased adhesion strength may hinder proliferation as cells are less able to migrate along the scaffold surface.\textsuperscript{107} By day 16, all constructs again had similar cellularity at a decreased value from day 8, a trend that we have observed in previous studies that is likely due to both the presence of a greater amount of extracellular matrix, which can inhibit nutrient transport to the center of the scaffolds and/or trap cellular DNA from escaping into solution for measurement, as well as programmed cell death in differentiating cells.

Alkaline phosphatase was used as an early marker of osteoblastic differentiation as this enzyme is up-regulated early in the differentiation pathway of osteoblasts, shortly after the initial cell proliferation phase.\textsuperscript{6} Alkaline phosphatase (ALP) activity per cell for scaffold/cell construct cultured \textit{in vitro} for up to 16 days is shown in Figure 19. ALP activity observed at day 1 showed a large standard deviation that was greatly reduced by day 2. Though there was no statistical difference in ALP activity between groups at day 1, because ALP activity is expressed on a per cell basis, the large standard deviation is likely due to the small number of cells initially attached to the scaffolds.
Figure 19: Alkaline phosphatase activity of marrow stromal cells cultured in vitro for up to 16 days on titanium fiber mesh scaffolds. Activity is expressed on a per cell basis. Ti indicates a plain titanium fiber mesh; Ti-RGD indicates a titanium fiber mesh with RGD peptide tethered to the surface; -dex indicates cells were cultured in the absence of dexamethasone; +dex indicates cells were cultured in medium containing 10 nM dexamethasone. * indicates a statistically significant difference in means, p < 0.05.

Overall, ALP activity was quite low over the first 6 days with no difference between any of the groups. This is in agreement with DNA data which showed this to be the cell proliferation phase. At day 8, there begins to be some separation in ALP activity with those constructs cultured with dexamethasone having greater ALP activity than their counterparts cultured in the absence of dexamethasone, although the difference was only significant for the Ti-RGD scaffolds. By day 16, there was much greater ALP activity for both the Ti and Ti-RGD constructs cultured in the presence of dexamethasone over their -dex counterparts, however there was still no difference between the different scaffold
types cultured under similar conditions. These data suggest that those cells cultured in the presence of dexamethasone were differentiating toward osteoblasts while those cultured in the absence of dexamethasone were not. In addition, for +dex constructs there was up-regulation in ALP activity and no difference in ALP activity between scaffold types at 16 days which suggests that although there was less initial cell proliferation in the Ti-RGD +dex constructs, these cells were still differentiating, lending support to the idea that the RGD peptide was contributing to a stronger cell/scaffold bond.

Calcium deposition was used as a late stage marker for osteoblast differentiation as a measure of extracellular matrix mineralization. Total calcium content of scaffold/cell constructs cultured in vitro for up to 16 days is shown in Figure 20. Because rapid increase in calcium content correlates to late stage osteoblast differentiation, it was not surprising that scaffold/cell constructs contained negligible calcium for all time points prior to day 16. At day 16, there was significant amount of calcium present in only the plain titanium scaffolds cultured in the presence of dexamethasone, in accordance with previous studies involving this scaffold. However, there was still no significant calcium deposition in Ti-RGD +dex constructs. ALP data suggest that these cells are differentiating, but the lack of calcium at day 16 suggests that cells seeded on these scaffolds were differentiating at a slower rate than their counterparts on plain titanium fiber mesh scaffolds. Recall that DNA data showed much greater cell numbers at day 8 on the Ti +dex scaffolds compared to Ti-RGD +dex constructs. The greater cell number would lead to greater cell-cell contacts which had been known to influence cell differentiation. Because the Ti-RGD +dex constructs never had this high cell density to enhance earlier osteoblastic differentiation, it is probable that these scaffold/cell
constructs were lagging behind their Ti +dex counterparts in the differentiation pathway.

![Graph showing calcium content over days of culture](image)

**Figure 20:** Calcium content of scaffold/cell constructs cultured in vitro for up to 16 days expressed as total calcium per construct. Ti indicates a plain titanium fiber mesh; Ti-RGD indicates a titanium fiber mesh with RGD peptide tethered to the surface; -dex indicates cells were cultured in the absence of dexamethasone; +dex indicates cells were cultured in medium containing 10 nM dexamethasone. * indicates a statistically significant difference in means, p < 0.05.

To summarize the *in vitro* data, there was osteodifferentiation of marrow stromal cells when cultured in the presence of dexamethasone, but none for those cells cultured in the absence of dexamethasone. For +dex constructs, there was slightly accelerated osteodifferentiation of marrow stromal cells when cultured on plain titanium scaffolds compared to titanium scaffolds with a tethered RGD peptide. Recall that for scaffold/cell constructs that were implanted subcutaneously for 28 days, -dex constructs represent marrow stromal cells that were cultured expanded for 6 days before seeding and 1 day after seeding in the absence of dexamethasone while +dex constructs represent marrow
stromal cells that were cultured expanded first for 3 days without dexamethasone and then for 3 days before seeding and 1 day after seeding in the presence of dexamethasone. Thus, we would expect that the cellular component of the +dex constructs were slightly more differentiated at the time of implantation than –dex constructs.

**Table 2: Ectopic Bone Formation in Subcutaneous Implants**

<table>
<thead>
<tr>
<th></th>
<th>% bone formation within implants</th>
<th>Implants with bone formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti –dex</td>
<td>0.2 ± 0.1</td>
<td>0/8</td>
</tr>
<tr>
<td>Ti +dex</td>
<td>6 ± 8</td>
<td>5/8</td>
</tr>
<tr>
<td>Ti-RGD –dex</td>
<td>1 ± 2</td>
<td>1/8</td>
</tr>
<tr>
<td>Ti-RGD +dex</td>
<td>4 ± 10</td>
<td>2/8</td>
</tr>
</tbody>
</table>

Table 2 shows a summary of the ectopic bone formation observed in the subcutaneous implants over the 28 day implantation period in terms of both the number of implants that exhibited some bone formation and the percentage of the implant that was filled with bone tissue. While statistical analysis of the bone percentage data did not yield any statistical difference between groups, the amount and location of bone formation observed within the plain titanium implant was similar to that observed in a study by Hartman *et al.*108 Chi-square analysis of the number of implants where bone formation was observed revealed that exposure of cells to dexamethasone prior to implantation resulted in greater bone formation whereas presence of RGD peptide on the scaffold surface did not have an effect. These results are in good agreement with *in vitro* data and lend further support to the idea that in the Ti-RGD +dex constructs, differentiation of marrow stromal cells was in fact occurring as indicated by alkaline phosphatase data, but the cells had not yet reached the stage of matrix mineralization by
day 16 of static culture. Figure 21 shows representative histological sections from each of the four implant groups. When bone formation did occur, it was present in the center of the implant rather than near the edges.

Figure 21: Representative histological sections of scaffold/cell constructs implanted subcutaneously in rat dorsa for 28 days. Sections were stained with methylene blue/basic fuchsin to visualize bone formation within the scaffolds, bone appears red while cells appear blue and the titanium fibers appear black. A) Ti–dex construct, typical section where bone formation did not occur B) Ti +dex construct, typical section where bone formation did occur C) Ti-RGD–dex construct, typical section where bone formation did not occur D) Ti-RGD +dex construct, typical section where bone formation did occur. Scale bars represent 1 mm.
Blum et al. observed similar ectopic bone formation in plain titanium fiber mesh seeded with rat marrow stromal cells that were cultured for 6 days in the presence of dexamethasone prior to implantation. They were evaluating the effect of genetic modification of marrow stromal cells with BMP-2 on in vivo bone formation and suspected that the reason they did not see great enhancement of ectopic bone formation by BMP-2 producing cells was that the cells were already starting to differentiate due to the dexamethasone exposure prior to implantation. Our observation that no bone formation was observed in scaffold/cell constructs when the cells had no exposure to dexamethasone would support these conclusions, and suggest that in order to create an osteoinductive construct, the cell population should not be undifferentiated cells, but rather preosteoblasts. These preosteoblastic cells express more strongly the receptor required for binding to RGD peptide, and we hypothesized that by tethering an RGD peptide to the surface of our titanium fiber mesh scaffolds, we could enhance cell attachment and subsequent bone formation, particularly for those cells that had already started down the differentiation pathway. However, this was not the case and indicates that the phenotype of the initial cell population has a greater influence on osteodifferentiation than the method of cell attachment to the scaffold.

**Conclusions**

In summary, there was osteodifferentiation of marrow stromal cells when cultured in the presence of dexamethasone, but not for those cells cultured in the absence of dexamethasone both in vitro and in vivo. There was no effect of tethering RGD peptide to the surface of titanium fiber mesh scaffolds on bone formation in vivo, although the tethered RGD peptide resulted in a slower rate of osteoblastic differentiation in vitro,
most likely due to increased binding strength of cells to scaffold via the RGD peptide.

These results clearly indicate the challenges involved in using bone marrow derived cells for bone tissue engineering applications as the initial phenotype of the marrow cells has a tremendous impact on eventual bone formation.
CHAPTER 7: FLOW PERFUSION CULTURE OF MARROW STROMAL CELLS
SEEDED ON POROUS BIPHASIC CALCIUM PHOSPHATE CERAMICS*

ABSTRACT

Calcium phosphate ceramics have been widely used for filling bone defects to aid in the regeneration of new bone tissue. Addition of osteogenic cells to porous ceramic scaffolds may accelerate the bone repair process. This study demonstrates the feasibility of culturing marrow stromal cells (MSCs) on porous biphasic calcium phosphate ceramic scaffolds in a flow perfusion bioreactor. The flow of medium through the scaffold porosity benefits cell differentiation by enhancing nutrient transport to the scaffold interior and by providing mechanical stimulation to cells in the form of fluid shear. Primary rat MSCs were seeded onto porous ceramic (60% hydroxyapatite, 40% β-tricalcium phosphate) scaffolds, cultured for up to 16 days in static or flow perfusion conditions, and assessed for osteoblastic differentiation. Cells were distributed throughout the entire scaffold by 16 days of flow perfusion culture whereas they were located only along the scaffold perimeter in static culture. At all culture times, flow perfused constructs demonstrated greater osteoblastic differentiation than statically cultured constructs as evidenced by alkaline phosphatase activity, osteopontin secretion into the culture medium, and histological evaluation. These results demonstrate the feasibility and benefit of culturing cell/ceramic constructs in a flow perfusion bioreactor for bone tissue engineering applications.

* This chapter was submitted for publication in Annals of Biomedical Engineering.
**INTRODUCTION**

Tissue engineering offers a promising approach toward healing of large bone defects and could help an estimated 200,000 patients each year.\(^{109}\) Popular approaches to tissue engineering utilize a scaffold, osteogenic cells, and bioactive factors either alone or in various combinations.\(^{110}\) We have been developing a tissue engineering strategy that combines osteoprogenitor cells with a titanium fiber mesh scaffold along with an *in vitro* culture period for regeneration of bone tissue. According this approach, previous work in our laboratory has demonstrated the benefit of flow perfusion culture on the osteoblastic differentiation of marrow stromal cells when seeded on titanium fiber mesh scaffolds.\(^{45-47,111}\) Titanium offers the advantage of good mechanical properties along with bone compatibility; however, titanium is inherently non-ideal as a scaffold material because it is not degradable. An alternative material that shows promise in this respect is porous calcium phosphate ceramic.\(^{112}\)

So-called bioactive ceramics came into use because they were reported to allow for the formation of a direct bond to living bone tissue. Particular interest has been focused almost exclusively on ceramics that closely resemble the mineral phase of bone, like hydroxyapatite \([\text{Ca}_{10} (\text{PO}_4)_6 \text{OH}_2]\) (HA) and tricalcium phosphate \([\text{Ca}_3(\text{PO}_4)_2]\) (TCP). HA is very stable under physiological conditions, this in contrast to TCP that is an ill-defined material and can be regarded as the dehydrated version of hydroxyapatite. TCP can be made by prolonged heating of pure hydroxyapatite at about 1400° C in the absence of water. Depending on the manufacturing conditions, two different TCP phases can exist, i.e. alpha-TCP and beta-TCP. The biological advantage of TCP above HA could be its degradability, resulting in a faster replacement by bone. However, the
degradation of TCP is not always as predictable as suggested and is controlled by many variables ranging from manufacturing to application site. Optimization of the degradability can be achieved by creating biphasic ceramic composed of a mixture of HA and beta-TCP. The material can be made macroporous in order to enhance bone ingrowth. Such macroporosity can be created by filling a polymer sponge, e.g. made of polyurethane, with a calciumphosphate slurry followed by a sintering process.\textsuperscript{113,114} During the sintering process, the polymer sponge is burned out leaving an interconnected porosity within the ceramic material. The resulting porous biphasic calcium phosphate ceramic scaffolds are biocompatible and very osteoconductive.\textsuperscript{115}

To further enhance the rate of bone formation into such porous bioceramics, much work has focused on making these materials more osteoinductive by addition of growth factors\textsuperscript{21-23} and/or osteoprogenitor cells to the materials.\textsuperscript{20,24-26} An integrated approach combining a porous ceramic scaffold, osteoprogenitor cells and an \textit{in vitro} culture period may create more optimal bone tissue engineering constructs. This \textit{in vitro} culture period allows deposition of extracellular matrix to act as the cell signaling element. The goal of this study was to determine the feasibility of using a flow perfusion bioreactor for the \textit{in vitro} culture of marrow stromal cells (MSCs) on porous calcium phosphate ceramic scaffolds. When compared to static culture, the flow of fluid through the scaffold porosity has been shown to enhance the osteoblastic differentiation of MSCs in fiber mesh scaffolds by both increasing the interior cells' access to nutrients and introducing a mechanical stimulation to the cells in the form of fluid shear.\textsuperscript{45-49} We hypothesize that these factors will also enhance cell distribution and differentiation of MSCs in porous
biphasic calcium phosphate ceramics despite large differences in pore architecture and material properties compared to the fiber mesh scaffolds used in previous studies.

In this study, we harvested fresh rat marrow stromal cells, culture expanded them for 6 days in osteogenic medium, seeded them onto porous calcium phosphate ceramic scaffolds and cultured them for 4, 8, or 16 days either in static or flow perfusion culture. Cell proliferation and osteogenic differentiation were determined by measuring scaffold cellularity and markers for osteoblastic phenotype including alkaline phosphatase and osteopontin. Constructs were also visualized by histology to determine cell distribution and matrix maturation.

**MATERIALS AND METHODS**

**Materials**

Porous calcium phosphate ceramic scaffolds composed of 60% hydroxyapatite/40% β-tricalcium phosphate were used as provided by CAM Implants BV (Leiden, the Netherlands). Scaffolds were cylinders, 8 mm diameter x 8 mm length and had a porosity of 90%. Prior to cell seeding, scaffolds were sterilized by exposure to ethylene oxide gas. The surface of the biphasic calcium phosphate ceramic scaffolds was visualized by scanning electron microscopy (XL-30 ESEM, FEI Company, Hillsboro, Oregon) to obtain information about the scaffold pore structure. All chemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

**Cell Culture**

Rat marrow stromal cells (MSCs) were harvested from the tibiae and femora of 6-8 week old male Wistar rats (Harlan, Indianapolis, IN) using the method described by
Maniotopoulos et al. Briefly, animals were anesthetized with 4% isoflurane in oxygen and then euthanized by inhalation of CO₂. The leg bones were excised, the soft tissue was removed, the epiphyses were cut off, and the diaphyses were flushed with 5 ml of osteogenic medium [α-MEM supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA), 50 μg/ml gentamicin, 100 μg/ml ampicillin, 2.5 μg/ml fungizone, 50 μg/ml L-ascorbic acid, 0.01 M β-glycerophosphate, and 10 nM dexamethasone]. The resulting marrow pellet was broken up by trituration, the cells were plated in 75 cm² tissue culture flasks and cultured for 6 days at 37 °C in a humidified atmosphere of 95% air, 5% CO₂ to expand the cell number. Media were changed at 1 and 3 days to remove the non-adherent cell population. At the end of this primary culture period, cells were lifted with 2 ml concentrated trypsin solution (0.25% trypsin / 0.02% EDTA), centrifuged at 400 g for 5 min, and resuspended in a known amount of medium.

Calcium phosphate ceramic scaffolds were encased in a flexible Teflon shell and then press fitted into bioreactor cassettes. Cassettes were placed into 6-well plates, covered with media, and placed in an incubator for 24 hours to prewet the scaffold surface. Media were aspirated and the scaffolds were seeded with a total of 1,500,000 cells in 300 μl of media. Three separate aliquots of 100 μl spaced 5 minutes apart were added dropwise to the top of surface of the scaffolds. After 2 hours, 10 ml medium was added to each well and cells were allowed to attach for 24 hours. Cell/scaffold constructs were then either removed from the bioreactor cassettes and placed into 6-well plates (static culture) or placed into the flow perfusion bioreactor (flow culture) for 4, 8 or 16 days in osteogenic medium. During the culture period, media samples were collected during medium changes every 2-3 days. At the end of the culture period, samples were
removed from culture (static culture) or removed from the bioreactor cassettes (flow culture) and rinsed with phosphate buffered saline. Four cell/scaffold constructs from each group were then placed into 2 ml of ddH2O and stored at -20 °C until biochemical analysis could be performed. The remaining two cell/scaffold constructs from each group were fixed in 10% neutral buffered formalin for histological preparation.

Flow Perfusion Culture System

The flow perfusion bioreactor is described in detail elsewhere. Briefly, it consists of 6 flow chambers within a block of poly(methyl methacrylate) (PMMA). Each flow chamber holds one cassette press fit with a scaffold. The cassette is sandwiched by two neoprene o-rings to ensure no media leakage around the edge of the cassette. Each bioreactor contains 6 flow chambers on independent pumping circuits drawing from a common media reservoir. Medium flow within the bioreactor is driven by the action of a peristaltic pump and flows from the supply medium reservoir, through the pump, to the top of the flow chamber, through the scaffold, out the bottom of the chamber, and into the collection reservoir. Hydrostatic pressure drives medium from the collection reservoir back to the supply reservoir. Components of the flow circuit are connected with platinum-cured silicon tubing, which has a high permeability to both oxygen and carbon dioxide. Prior to use, all PMMA components (flow chamber, cassettes and screw tops) were sterilized with ethylene oxide gas while all other components (tubing, reservoirs, connectors and scaffolds) were sterilized by autoclaving.

The flow system was assembled using sterile technique in a laminar flow hood. The system was preconditioned by flowing medium containing a strong dose of antibiotic/antimycotic (1000 unit/ml penicillin, 1000 μg/ml streptomycin, 0.25 μg/ml
fungizone) (Invitrogen, Grand Island, NY) through the bioreactor for 10 minutes before being replaced with osteogenic medium. Cassettes containing cell seeded scaffolds were placed into each flow chamber, the chambers were sealed, and the entire flow system was placed in a 37°C incubator with 95% air and 5% CO₂. The flow rate through the system was set to a low value (~0.3 ml/min) for 1 day to ensure good cell attachment before being increased to 1 ml/min for the duration of culture.

**Scaffold Cellularity**

Scaffold cellularity was determined by quantifying the amount of double stranded DNA and correlating to a known amount of marrow stromal cells. DNA was quantified using a PicoGreen assay kit (Molecular Probes, Eugene, OR). Briefly, frozen scaffolds were thawed at room temperature, sonicated for 10 min, and vortexed for 5-10 sec to allow the DNA into solution. Standards of calf thymus DNA in ddH₂O in concentrations ranging from 0 – 6 µg/ml were prepared and 50 µl of standard or sample was placed into individual wells of a 96-well plate. Tris-EDTA buffer and PicoGreen dye solution were prepared according to the manufacturer’s instructions using reagents provided in the kit and added at 100 and 150 µl/well respectively. After a 10 min incubation in the dark at room temperature, the fluorescence was measured on a plate reader (FL x800, Bio-Tek Instruments Inc., Winooski, Vermont) using an emission wavelength of 490 nm and an absorbance wavelength of 520 nm.

**Alkaline Phosphatase Activity**

The alkaline phosphatase (ALP) activity of each scaffold was measured using a colorimetric endpoint assay to determine early osteoblastic differentiation of MSCs.
ALP activity was determined by assaying aliquots of the same aqueous solutions that had been frozen, thawed, sonicated and vortexed for DNA quantification. As necessary, samples were diluted in ddH₂O up to 100x in order to stay within the detection range of the assay. Standards of p-nitrophenol in concentrations ranging from 0 – 250 µM were prepared from dilutions of a 1000 µM stock solution, and 80 µl of standard or sample was placed into individual wells of a 96-well plate. Alkaline buffer solution consisting of 1.5 M 2-amino-2-methyl-1-propanol at pH 10.3 was then added at 20 µl/well. Substrate solution was prepared by dissolving 40 mg 4-nitrophenyl phosphate disodium salt hexahydrate into 10 ml ddH₂O and added at 100 µl/well. The microplate was incubated for 1 hour at 37°C and the reaction stopped by the addition of 0.3 M NaOH at 100 µl/well. The absorbance of each well at 405 nm was then measured on a plate reader (PowerWave x340, Bio-Tek Instruments Inc., Winooski, Vermont). Activity is expressed per cell as determined by PicoGreen assay.

**Osteopontin**

The amount of osteopontin released into the media during culture was measured using an ELISA kit available from Assay Designs, Inc. (Ann Arbor, Michigan) to determine mid to late stage osteodifferentiation of MSCs. Media samples were collected every 2-3 days over the culture period and frozen at -20°C until analysis could be performed. Samples were thawed, briefly vortexed and diluted up to 1000x in order to stay within the detection range of the assay. The assay was performed per instructions provided by the kit manufacturer in a 96-well plate and the absorbance of each well at 450 nm with correction between 570 and 590 nm was measured on a plate reader. Samples were run in duplicate.
Histology

Two scaffold/cell constructs from each group were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, and embedded in glycol methacrylate (Technovit 7200 VLC, Exakt, Oklahoma City, OK). After polymerization, longitudinal sections (10 μm) were prepared by using the Exakt cutting and grinding system. Sections were stained with methylene blue/basic fuchsin and images were taken with a light microscope (Eclipse E600 Nikon, Melville, NY) equipped with a digital image capture system (3CCD Color Video Camera DXC-950P, Sony, Park Ridge, NJ). Methylene blue/basic fuchsin stains mineralized tissue red.

Statistical Analysis

DNA and ALP results are expressed as means ± standard deviations with n=4 for each group. Significance was determined using Tukey’s multiple comparison procedure at a 95% confidence interval. Osteopontin results are expressed as cumulative release with each point representing a pooled media sample of 6 scaffolds.

RESULTS

The scaffold surface was characterized by scanning electron microscopy to visualize the pore structure of the biphasic calcium phosphate ceramic scaffolds. Figure 22 shows the top surface of a blank ceramic scaffold showing the pores to be spherical in shape.
Figure 22: Scanning electron micrograph of the top surface of a cell-free scaffold at low magnification (26x). The bar represents 1 mm.

Scaffold cellularity was determined via quantification of double stranded DNA and comparison against a known amount of MSCs to determine cell number per scaffold (3.0 pg DNA/cell). These results are presented in Figure 23. Cells were seeded onto the ceramic scaffolds at 1,500,000 cells per scaffold and initial cell attachment was around 875,000 cells. There was an initial period of proliferation over the first 4 days, with much greater cell proliferation under flow perfusion culture compared to static culture. Under static culture conditions, scaffold cellularity remained constant over the entire culture period. Under flow perfusion conditions, there were significantly fewer cells at day 8 and day 16 compared to day 4.
Figure 23: Cellularity of calcium phosphate ceramic/marrow stromal cell constructs over 16 days of culture. Cells were cultured in osteogenic medium either in 6-well plates (static culture) or in a flow perfusion bioreactor (flow culture). Scaffold cellularity is presented as millions of cells per scaffold. Results are expressed as means ± standard deviation with n=4 for each bar. * indicates a significant difference from the corresponding value at day 4 (p<0.05). ** indicates a significant difference between flow and static culture at the same time point (p<0.05).

Alkaline phosphatase (ALP) is a transient marker of osteoblast differentiation that is up-regulated as proliferation slows and is subsequently down-regulated as the extracellular matrix begins to mineralize. Figure 24 shows the activity of ALP expressed per scaffold (24A) or per cell (24B). There was a low level of ALP activity which remained unchanged over the culture period under static conditions. The total ALP activity per scaffold (figure 24A) was much greater for flow perfusion conditions compared to static culture at all time points. However, when ALP activity was normalized to cell number (figure 24B), ALP activity was significantly greater under flow perfusion compared to static culture only at days 8 and 16. In addition, normalized ALP activity steadily
increased over the culture period in the flow perfusion bioreactor with activity at 4 days < 8 days < 16 days of culture.

![Graph A and B](image)

Figure 24: Activity of the alkaline phosphatase enzyme in cell/ceramic constructs over 16 days of culture. Cells were cultured in osteogenic medium either in 6-well plates (static culture) or in a flow perfusion bioreactor (flow culture). Enzyme activity is presented per scaffold (A) or per cell as determined by the DNA assay (B). Results are expressed as means ± standard deviation with n=4 for each bar. * indicates a significant difference from the corresponding value at day 4 (p<0.05), ** indicates a significant difference between flow and static culture at the same time point (p<0.05), # indicates a significant difference from the corresponding value at day 8 (p<0.05).

Osteopontin is a protein secreted by differentiating osteoblasts that is upregulated both during cell proliferation and again at the onset of matrix mineralization. \(^7\) Figure 25A shows the cumulative amount of osteopontin protein secreted into the culture medium expressed on a per scaffold basis. Each point in the figure was determined from a pooled media sample from 6 scaffolds. The protein was present in very low quantities over the culture period under static conditions. However, under flow perfusion conditions, a large amount of osteopontin was secreted into the culture medium over the entire course of the culture period. Figure 25B shows the rate of osteopontin secretion into the media. There was initially a high rate of protein production between day 2 and day 5, followed by a steady decrease in the rate of protein secretion which leveled out at day 9. There was then a slight elevation in protein production at day 14.
Figure 25:Amount of osteopontin protein secreted in the culture medium by cells cultured on porous ceramic constructs for up to 16 days. Cells were cultured in osteogenic medium either in 6-well plates (static culture) or in a flow perfusion bioreactor (flow culture). Medium samples containing the pooled osteopontin secretion from 6 scaffold/cell constructs were collected every 2-3 days during the culture period. Results are presented as cumulative protein release per scaffold (A) and rate of protein secretion into media (B).

At each time point, 2 scaffolds from each culture condition were fixed, embedded, sectioned and stained for histological evaluation. Representative histological sections stained with methylene blue/basic fuchsins are shown in figures 26 & 27. In these images, osteoblast nuclei stain blue while calcified bone matrix stains red. The ceramic appears black. In static culture, the cells were located near the perimeter of the scaffolds, they did not penetrate the porosity of the scaffold. There was no obvious difference between the various culture times. In contrast, under flow perfusion conditions there was a dramatic difference in tissue formation. After 4 days of culture, the majority of the cells were located near the top of the scaffold, but had penetrated about 1/3 of the total thickness of the scaffold (figures 26 A&B). The cells located on the scaffold surface as well as in the pore space (figure 26C). At this point, the cells were not staining as bone cells as evidenced by the lack of color in the immediate vicinity of the cells. By day 8, cells had penetrated the entire thickness of the scaffold, although there was still a small area in the center of the scaffold that appeared to be devoid of cells (figures 27 A&B).
Figure 26: Histological sections of ceramic/cell constructs after 4 days of flow perfusion culture in osteogenic media. Sections were stained with methylene blue/basic fuchs in to visualize the cells (blue) and any bone formation within the scaffolds (red). The calcium phosphate scaffold appears black in these images. A) Scaffold edge, B) Scaffold center, C) Higher magnification of scaffold edge showing cell morphology.

At this point, individual cells were beginning to stain dark and very small areas of red stained mineralized-deposits were visible in areas of high cell density. The deposits were mainly present at the inner surface of the pore walls. After 16 days of flow perfusion culture, cells were located throughout the entire scaffold and multiple pore walls were covered with circular regions of very intense red stained mineralized deposits. Mineralized tissue also appeared to bridge pore openings (figures 27 C&D). In general, the cell/scaffold constructs stained much darker at day 16 compared to both day 8 and
day 4. In addition, there were more areas of localized red stain, indicating deposition of greater amounts of bone-like extracellular matrix.

Figure 27: Histological sections of ceramic/cell constructs after 8 and 16 days of flow perfusion culture in osteogenic media. Sections were stained with methylene blue/basic fuchsin to visualize the cells (blue) and any bone formation within the scaffolds (red). The calcium phosphate scaffold appears black in these images. C) Scaffold edge at 8 days, D) Scaffold center at 8 days, E) Scaffold edge at 16 days, F) Scaffold center at 16 days. Scale bar is 0.5mm.

**DISCUSSION**

Numerous studies have reported on the *in vivo* formation of bone within porous calcium phosphate ceramics in orthotopic sites.\textsuperscript{116,117} The addition of marrow cells to these ceramics has also been reported to enhance bone formation in ectopic sites.\textsuperscript{25,26,118}
indicating the osteoinductive potential of ceramic/marrow cell constructs. However, limited information is available on the in vitro culture of osteoblastic cells on calcium phosphate ceramic materials. Toquet et al.\textsuperscript{20} reported on the culture of human bone marrow cells on porous biphasic calcium phosphate ceramic scaffolds in static culture. They observed increasing scaffold cellularity and alkaline phosphatase activity, indicating osteoblastic differentiation of these marrow cells over 21 days of culture on porous ceramic surface. In contrast, we did not see continued cell proliferation after day 4 for MSCs on ceramic surfaces in static culture, and saw only a slight elevation in alkaline phosphatase activity in static culture. However, different cell types were used for each study and although the scaffolds had the same general composition, it is likely there were differences in scaffold properties due to differing processing procedures that could affect cell behavior.

Flow perfusion culture allowed for increased cell proliferation compared to static culture. DNA analysis showed significant cell proliferation over the first 4 days of flow perfusion culture. Scaffold cellularity was determined by rupturing the cell membranes, allowing the DNA into solution and subsequently measuring the concentration of DNA in solution. Therefore, any mechanism by which the DNA is not allowed into solution would result in an under-representation of the actual cell number within the constructs. One such mechanism is the deposition of large amounts of extracellular matrix within the scaffold, creating a diffusional barrier for DNA to escape into the surrounding solution. Therefore, the DNA quantification data must be considered along with the histological data to give a more accurate depiction of scaffold cellularity. Histological sections of cell/scaffold constructs after 8 and 16 days of flow perfusion culture show that the
constructs were completely filled with both cells and mineralized matrix. This indicates that the quantitative DNA data for these time points showing a significant decrease in scaffold cellularity was likely due to entrapment of DNA by the extracellular matrix, which prevented the proper retrieval of DNA material.

ALP is a transient marker of osteoblast differentiation, with expression peaking near the onset of matrix mineralization. There was increasing ALP activity per cell over 16 days of perfusion culture and the order of magnitude of the values is consistent with previous studies involving the flow perfusion culture of marrow stromal cells on fiber mesh scaffolds. However, the differentiation profile in this study appears to be slightly delayed compared to these studies in that the peak value of ~35 pmol/hr/cell observed near day 8 in previous studies$^{46,47,111}$ was not achieved until day 16 in present study. The trend to increasing ALP activity over the 16 days of perfusion culture indicates that this peak in ALP expression is occurring sometime after day 8, most likely near the 16 day time point. However, it is difficult to determine exactly when expression peaked due to the limited number of time points. When ALP activity was expressed on a per scaffold basis, activity peaked at day 4, corresponding to rapid cell proliferation during this time period. The reduced ALP activity at days 8 and 16 is likely due to a similar entrapment mechanism as noted for the DNA measurements.

Osteopontin is a protein that is secreted throughout the osteoblast differentiation process, but it is up-regulated during proliferation and again at the onset of matrix mineralization. Because this is a secreted protein, it was possible to analyze more time points than in the case of ALP. There was a peak in the rate of osteopontin secretion at day 5 corresponding rapid cell proliferation during this time period. The slight increase
in the rate of osteopontin secretion at day 14 as shown in figure 4B suggests that matrix mineralization may be starting around this time point, in agreement with the alkaline phosphatase data.

Previous studies in our laboratory involving the culture of marrow stromal cells on fiber mesh scaffolds composed of poly(L-lactic acid),\(^{49}\) blends of starch with ethylene vinyl alcohol or poly(ε-caprolactone),\(^{48}\) or titanium metal\(^{111}\) have shown significant matrix mineralization after 16 days of flow perfusion culture. In these studies, mineralization was quantified by first dissolving the mineral component of the extracellular matrix in dilute organic acid and then measuring the concentration of calcium ions in solution. In the present study, this calcium quantification was not possible because the scaffold material was composed of calcium ions which also dissolved into the organic acid along. Likewise, von Kossa staining for mineral was also not possible. Therefore, histological sections of cell/scaffold constructs were obtained and stained with methylene blue and basic fuchsin to visualize the cells along with any mineralized tissue formation. The very dark circular spots observed in figure 6D resulted from sectioning through scaffold material interconnecting two pores, and the very intense red stain corresponds to mineralized matrix deposited on the pore interconnect. The increasing red staining area with flow perfusion culture time indicates that mineralized matrix was forming within the scaffold and was increasing with time, indicative of osteoblastic differentiation.

Overall, there was better cell distribution throughout the scaffold, greater scaffold cellularity, and more mineralized matrix production in ceramic/cell constructs cultured in the flow perfusion bioreactor compared to static culture. Therefore, flow perfusion
culture was beneficial to the osteoblastic differentiation of marrow stromal cells when seeded on porous calcium phosphate ceramic scaffolds. These ceramic scaffolds were more brittle, much thicker (~10 times taller), and had a very different pore structure compared with the fiber mesh scaffolds of previous flow perfusion studies. Despite these differences, flow perfusion culture still enhanced the osteoblastic differentiation of MSCs. Thus, this study shows that the flow perfusion bioreactor can accommodate a wide variety of scaffold types for the creation of bone tissue engineering constructs and that flow perfusion culture is beneficial to the osteoblastic differentiation of cells seeded on these scaffolds.

CONCLUSIONS

This study demonstrated the feasibility of culturing marrow stromal cells on porous biphasic calcium phosphate ceramic scaffolds in a flow perfusion bioreactor. Over 16 days of culture, flow perfusion enhanced cell proliferation, cell distribution, and osteoblastic differentiation of primary rat marrow stromal cells seeded onto porous calcium phosphate ceramics composed of 60% hydroxyapatite and 40% β-tricalcium phosphate. Alkaline phosphatase and osteopontin protein markers of osteoblastic differentiation indicated that extracellular matrix was beginning to mineralize around 16 days of flow perfusion culture while histological evaluation of scaffold/cell constructs revealed uniform distribution of cell and matrix throughout the thickness of the scaffold. Therefore, a flow perfusion bioreactor creates an improved culture environment for cell seeded calcium phosphate ceramics when compared to static culture.
CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS

The overall goal of this research was to modulate the osteoblastic differentiation of marrow stromal cells when seeded onto three-dimensional porous scaffolds to gain a better understanding of the key elements affecting bone formation in tissue engineering scaffolds. The central hypothesis of this research was that marrow stromal cell differentiation in vitro is influenced by scaffold properties, culture supplements, and culture environment. This hypothesis was confirmed by showing that scaffold pore size affects marrow stromal cell differentiation in a flow perfusion bioreactor; by identifying parameters other than dexamethasone that induced the osteoblastic differentiation of marrow stromal cells; and by demonstrating the feasibility of culturing marrow stromal cells on porous calcium phosphate ceramics in a flow perfusion bioreactor.

Chapter 3 demonstrated that titanium fiber mesh pore size affected the osteodifferentiation of seeded marrow stromal cells in a flow perfusion bioreactor. The time course of differentiation was dependent on scaffold pore size under flow conditions; however the dependence was not linear with respect to time. Larger pore size was conducive to early osteoblast differentiation while smaller pore size was conducive to later differentiation and matrix deposition. This dependence of MSC osteodifferentiation on the pore size of fiber mesh scaffolds resulted from differences in fluid shear experienced by the seeded cells stemming from differing pore architectures, as well as differences in initial cell-cell interactions stemming from alterations in total scaffold surface area.

Chapter 4 demonstrated that titanium fiber mesh scaffold/marrow stromal cell constructs grown under flow perfusion were more mineralized than their static
counterparts, even in the absence of dexamethasone. However, there was a synergistic effect between flow perfusion and presence of dexamethasone in the culture medium, resulting in abundant mineralized matrix after 16 days of culture. There was also an indication that osteogenic differentiation might be enhanced under flow perfusion by exposure to dexamethasone only during the initial cell expansion phase. Finally, no osteogenic differentiation was observed for scaffold/cell constructs when exposed to neither dexamethasone nor flow perfusion.

Chapter 5 showed that the presence of bone-like extracellular matrix deposited on titanium fiber mesh scaffolds can induce osteoblastic differentiation in bone progenitor cells in static culture. The osteoinductive capacity of this decellularized bone extracellular matrix was demonstrated by greater calcium deposition as well as increasing osteopontin levels exhibited by MSCs cultured on this surface. In addition, there was a correlation between matrix deposition and detection of osteopontin protein, indicating that this extracellular matrix may sequester soluble osteopontin.

Chapter 6 revealed that there was osteodifferentiation of marrow stromal cells seeded on titanium fiber mesh scaffolds when cultured in the presence of dexamethasone, but not for those cells cultured in the absence of dexamethasone both in vitro and in vivo. There was no effect of tethering RGD peptide to the surface of titanium fiber mesh scaffolds on bone formation in vivo, although the tethered RGD peptide resulted in a slower rate of osteoblastic differentiation in vitro, most likely due to increased binding strength of cells to scaffold via the RGD peptide. These results clearly indicate the challenges involved in using bone marrow derived cells for bone tissue engineering
applications as the initial phenotype of the marrow cells has a tremendous impact on eventual bone formation.

Finally, chapter 7 demonstrated the feasibility of culturing marrow stromal cells on porous biphasic calcium phosphate ceramic scaffolds in a flow perfusion bioreactor. Over 16 days of culture, flow perfusion enhanced cell proliferation, cell distribution, and osteoblastic differentiation of primary rat marrow stromal cells seeded onto porous calcium phosphate ceramics composed of 60% hydroxyapatite and 40% β-tricalcium phosphate. Alkaline phosphatase and osteopontin protein markers of osteoblastic differentiation indicated that extracellular matrix was beginning to mineralize around 16 days of flow perfusion culture while histological evaluation of scaffold/cell constructs revealed uniform distribution of cell and matrix throughout the thickness of the scaffold. Therefore, a flow perfusion bioreactor creates an improved culture environment for cell seeded calcium phosphate ceramics when compared to static culture.

In summary, this thesis has shown that many factors can influence the osteoblastic differentiation of marrow stromal cells when cultured on three-dimensional tissue engineering scaffolds. In creating ideal bone tissue engineering constructs consisting of a combination of a scaffold, cells, and bioactive factors; a flow perfusion bioreactor is a much more suitable culture environment than static culture in well plates. The bioreactor eliminates mass transport limitations to the scaffold interior and provides mechanical stimulation to the seeded cells through fluid shear. Scaffold properties such as pore size impact cell differentiation, especially in flow perfusion culture. In addition, the bone-like extracellular matrix created by the in vitro culture of marrow stromal cells on porous scaffolds creates an osteoinductive environment for the differentiation of other marrow
stromal cell populations. Therefore, bone tissue engineering constructs created by in vitro culture have excellent potential for bone regeneration applications in the clinic. However, more work is required to optimize this tissue engineering strategy. A biodegradable material with mechanical integrity similar to native bone and degradation properties similar to the rate of bone formation would be a more ideal scaffold material. It is also yet unclear what the optimum scaffold pore size and amount of in vitro generated extracellular matrix are to maximize bone formation. Finally, better characterization of the flow patterns within the flow perfusion bioreactor is needed to better understand the relationship between fluid shear and cell differentiation for creation of the ideal scaffold/culture combination.
CHAPTER 9: REFERENCES


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