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THE DEVELOPMENT OF A MESENCHYMAL STEM CELL BASED BONE GRAFT SYSTEM

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ABSTRACT

Greater than 5.5 million fractures are sustained by Americans each year, accounting for more than 500,000 bone graft surgeries. Bone grafts are the second most transplanted material, surpassed only by blood. The current "gold standard" for bone grafting involves harvesting bone material from the patient's iliac crest, due to its osteoinductive properties. Unfortunately, the surgery required to harvest material from the iliac crest causes additional pain for the patient, relies on a limited amount of available bone tissue, and results in, on average, a 30% rate of donor site morbidity. Both natural and synthetic substitutes have been developed to avoid complications and alleviate the pain associated with harvesting grafts from a patient's healthy bone; however, these substitutes are costly and lack the osteoinductive properties desired by surgeons for proper repair. Bone tissue engineering solutions combine scaffold materials with viable stem cells or bone cells in products that can be implanted into bony defects. Incorporating cells and growth factors within a bone graft prior to implantation improves upon its ability to induce bone formation and decreases the time needed for native tissue to proliferate and integrate with the graft. The long-term goal of this research is to develop a mesenchymal stem cell-based bone graft system with the potential of someday eliminating the use of autogenic tissue.

The first part of this research project was centered on the order of events of physiological remodeling: bone resorption by osteoclasts followed by the deposition of mineralized matrix by osteoblasts. These events indicate that the cells responsible for resorbing bone mineral may also possess the ability to recruit and/or induce osteoblast development and activation. A series of studies were developed to gain further insight into

the role that osteoclasts may play on the differentiation of mesenchymal stem cells (MSCs) when incorporated into a bone graft system. The results demonstrated that osteoclasts can positively influence expression of bone cell markers during the initial and final stages of osteoblast differentiation without having a significant effect on markers linked to the middle stage. The observed effects on osteoblasts could prove to be advantageous to bone tissue engineering applications.

A second set of studies were performed to examine the aspirate material obtained from the femoral shaft using the Reamer/Irrigator/Aspirator (RIA) device (Synthes, USA; Paoli, PA) as an untapped source of MSCs that could be incorporated into a bone graft system. The fat layer of aspirate, which has traditionally not been a topic of research or clinical interest, was the focus of this work. Viable, proliferating cells were successfully and consistently isolated, using the same techniques, from the fatty layer of RIA aspirate of multiple patient samples. Further characterization performed on the fat layer from multiple patient samples showed that the fatty layer of aspirate is mainly composed of four main fatty acids: oleic, palmitic, linoleic, and stearic. Finally, synthesis of the osteoblast cell markers alkaline phosphatase and calcium by cells isolated from the RIA fat layer was observed in both two-dimensional tissue culture polystyrene and three-dimensional ceramic bone graft granule cultures. The results of this work suggest that the fatty layer of RIA aspirate may be a new, untapped source for autologous progenitor cells with bone forming capabilities. Previously considered waste, the lipid rich fat layer of aspirate collected during reaming of the medullary canal may be a source of adult mesenchymal stem cells that could be used to stimulate new bone growth at the site of fracture repair.

DEDICATION

This work is dedicated, with great appreciation, to those that have provided me with the love and support needed to accomplish this goal: my parents, for being there to encourage me every step of the way and for your willingness to give of yourselves so that your children can succeed; my husband, for your unwavering desire to bring happiness, friendship, balance and laughter to my life; my sister and brother, for being wonderful friends and being examples of how to find my strengths and use them to do great things ; my grandparents, from whom I inherited my work ethic, ambition, and desire to help others; and finally, to the rest of an incredible support system composed of my family, Clemson family, and friends.

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PREFACE

Since the 1960s there have been two main approaches toward the repair of damaged or diseased tissue that lacks the capacity to properly heal by natural means: tissue transplantation and device implantation. These traditional approaches to bone repair are currently being integrated with innovative tissue engineering techniques, as researchers and clinicians shift their treatment focus towards regenerating functional tissue rather than just filling a defect to provide structural support. One major bone tissue engineering approach uses a patient's own cells that are harvested through needle aspiration or from the removal of bone tissue during surgical reaming. These cells are expanded and incorporated into implantable systems in hopes of enhancing the bone forming capabilities of traditional bone graft substitutes.

There are six chapters within this dissertation which describe research studies focused on the development of a bone graft system that incorporates mesenchymal stem cells with a synthetic bone graft substitute. The first chapter provides a review of the important cellular components and functions of bone tissue that must be considered when developing a successful bone grafting option for treating damaged tissue. Also included in this chapter are: an explanation of the important features of an ideal bone graft; a brief review of current natural and synthetic bone graft options; and an overview of the current tissue engineering approaches to bone graft development.

Chapters 2, 3, and 6 provide the methods, results, and conclusions of studies that examined how osteoclasts might be used to stimulate the differentiation of mesenchymal stem cells into bone forming cells. In Chapters 2 and 3, osteoclast conditioned medium was

applied to mesenchymal stem cells cultured in a two-dimensional system and fed osteogenic supplements. Results described in the second chapter suggest that the proliferation of stem cells can be positively influenced by osteoclast conditioned medium. The results from this research were presented at the Society for Biomaterials Annual meeting in Chicago, IL in April 2007. The work described in Chapter 3 was conducted to better characterize osteoclast differentiation within an *in vitro* test system, and three phases of osteoclasts differentiation were identified. The results of additional cellular studies, using conditioned medium collected for each of the phases, also suggested that osteoclast conditioned medium has a positive effect on the intracellular protein levels of stem cells; however, the overall differentiation of these cells into osteoblasts was inhibited.

The study described in Chapter 6 also examined the effects of osteoclasts on osteoblast differentiation; however, the two cell types were co-cultured on a resorbable, three-dimensional ceramic bone graft substitute. It was determined that the direct presence of osteoclasts also had an influence on the development of mesenchymal stem cells into osteoblasts. These results supported findings from the conditioned medium studies. Additionally, it was determined that the addition of RANKL to stem cell culture medium, which is needed to induce osteoclast maturation, may also effect the maturation of osteoblasts from pre-cursor cells.

The experiments described in Chapter 4 and 5 were the first to examine the fatty layer of aspirate material collected from reaming of the femoral shaft during fracture repair surgeries. This work was conducted with the intention of identifying a new source for autologous mesenchymal stem cells for use within a bone graft system. Cells with morphology comparable to that of cells from an established mesenchymal stem cell line were successfully

isolated from the lipid-rich layer and expanded in the laboratory. Additional studies performed using the isolated cells revealed that the cells acquired from the aspirate were capable of expressing osteoblast-specific markers when fed osteogenic supplemented medium during *in vitro* culture. These data suggest that the aspirate cells may possess osteogenic potential. These findings were presented at the Society for Biomaterials Annual meeting in Atlanta, GA in September 2008.

The cells isolated from the reamer aspirate were also tested for their ability to differentiate into osteoblasts when cultured on ceramic bone graft granules, as described in Chapter 5. Cells isolated from the aspirate fat layer grew successfully on three-dimensional ceramic substrates and results from this study served to support the theory that the fatty portion of RIA aspirate may be an untapped source for progenitor cells that could enhance bone formation if introduced to the site of fracture repair. The work in Chapter 5 was presented at the Society for Biomaterials Annual meeting in San Antonio, TX in April 2009.

The experiments presented in this dissertation serve to aid in the future development of a cell-based bone graft system that may be used in place of the traditional autograft or the less osteoinductive bone graft substitutes that are available today.

CHAPTER ONE

INTRODUCTION

1.1 Background and Significance of Problem

Over 2 million bone graft procedures are performed annually worldwide to repair defects caused by trauma or removal of tumors, or to fuse spinal vertebrae [1]. Bone grafts are the second most transplanted material into the body, surpassed only by blood, with sales that have reached over $1 billion and that continue to grow as the global population ages [7, 8]. An array of natural materials and synthetic bone substitutes are currently available to surgeons needing to perform a bone graft procedure; however, no clinical standard exists for determining which material or combination of materials may be the optimal choice for treating specific injuries.

As the life expectancy of humans continues to grow, people are staying active longer. Since the 1960s there have been two main approaches toward the repair of damaged or diseased tissue that is unable to heal by natural means: (1) tissue transplantation and (2) device implantation. Transplantation of tissue from a healthy area on the patient's own body to the area in need of repair has been the optimal and preferred method. Implantation uses materials harvested from donors, or synthetic materials that are designed to interact with host tissues following placement at the site of trauma [10, 11]. These traditional approaches to bone repair are currently being integrated with innovative tissue engineering techniques, as researchers and clinicians shift their treatment focus towards regenerating functional tissue rather than just filling a defect to provide structural support [12].

Both natural and synthetic materials placed into the body illicit local and systemic responses from the host. The biological host response differs depending on the material and can be the main factor in determining whether a treatment succeeds or fails [14]. Surface chemistry, surface topography, and degradation properties can all affect the biological response to an implant [15]. Metals are the current material of choice for load-bearing implants (total joint replacement, fracture fixation) due to their strength and resistance to fracture fatigue. Metal implants are not ideal, however, because they are not naturally absorbed by the body and can result in weakening of surrounding host tissue due to major differences in load distribution [14] or disruption of blood supply [16]. In cases where a significant amount of bone must be replaced and a metal implant stabilized, the current available non-metallic materials are: autologous or allogenic bone, polymers, ceramics, biologically derived proteins, demineralized bone matrix, and composites [17-19].

Autologous and allograft bone are the most widely used materials, with synthetic bone grafts accounting for only approximately 10% of grafting procedures worldwide [1]. An ideal bone graft material supports or stimulates [14, 20, 21]:

(i) Osteointegration - ability of graft material to chemically bond with the surface of host bone without an intervening layer of fibrous tissue. To be completely functional the graft must unite with the host bone (e.g. bioglass, hydroxyapatite).

(ii) Osteoconduction - ability of graft material to support bony and vascular ingrowth within its structure and onto its surface. The result of this process is an organized haversian system (e.g. bioglass, hydroxyapatite).

(iii) Osteoinduction - ability of graft material to induce differentiation of mesenchymal stem cells from surrounding tissue into the osteoblast phenotype (e.g. demineralized bone matrix, biologically derived proteins).

(iv) Osteogenesis - ability of graft material to induce formation of new bone by osteoblastic cells present within the graft (e.g. autograft).

While many scaffolding materials can readily facilitate osteoconduction, often growth factors must be applied to achieve osteoinduction, and progenitor cells must be incorporated to achieve osteogenesis [18]. Currently, autografts are the only clinically available option that may have all four characteristics [21]. New tissue engineering techniques, coupled with new biomaterials, are helping to improve graft options with the potential of some day replacing the need for the often scarce autogenic tissue.

Bone tissue engineering is a science that combines the knowledge of bone structure, function and mechanisms of failure with engineering methods to create improved treatment options for bone repair. Bone tissue is a complex material with a number of structural features and cellular functions that must be considered when developing a successful bone grafting option for treating damaged or diseased tissue.

1.2 Structure of Bone Tissue

Bone is a unique connective tissue whose functions in the body are: to support loads imposed on the body, provide a rigid frame that permits locomotion, protect internal organs against injury, act as a reservoir for mineral elements, and produce blood cells within the marrow [22, 23]. Approximately 206 intricately arranged bones are responsible for keeping the human body upright and for keeping the systems of organs in position despite constant

movement [24, 25]. The bones are also able to withstand tremendous forces in order to provide protection for the internal organs. The strength of healthy bone is reported to be four times stronger than concrete; one cubic inch can withstand loads up to 20,000 pounds [26]. The efficient arrangement of its elements on the molecular, cellular and tissue structures provides a relatively low weight material with a tensile strength comparable to cast iron [27].

Bone tissue is a composite material made up of an inorganic and an organic component. In general, the inorganic phase accounts for approximately 70% of the total material composition and the organic phase accounts for the remaining 30% (minus a negligible amount of water). The two components are tightly bound together and oriented along the longitudinal axis and the principal direction of stress [27, 28]. Fibrous protein and Type 1 collagen make up 90% of the organic phase; the remaining 10% is mainly noncollagenous protein filler. Although the exact composition remains unclear, the inorganic component is primarily made up of crystalline calcium phosphate [27, 29]. Calcium phosphate, chemically arranged as hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), is responsible for the bones' rigidity and resistance to crushing while collagen contributes to its strength and resilience [23]. The components of the inorganic phase are also responsible for regulating bone metabolism [27]. A number of impurities can be substituted into the phosphate groups that are located on the apatite lattice [30]. Imperfections in the crystals following these substitutions affect their solubility, making them more susceptible to the acidic environment created by the bone cells during the resorption process [27, 29], These substitutions can also affect the density, morphology, and hardness of the bone [30].

The major component of the organic phase, the structural protein Type 1 collagen, is also found in human skin, tendon and dentin. Collagen molecule orientation is said to be responsible for organizing the deposited apatite crystals parallel to the axis of collagen fibers [29]. Noncollagenous proteins that contribute to the function of bone are contained within the collagen matrix (Table 1.1).

Table 1.1: Major Bone Proteins

Protein	Stage of Expression	Function
Histone H4	Proliferation	Marker of proliferation; linked to DNA replication [2, 3]
Alkaline Phosphatase	Maturation	Hydrolysis of phosphate esthers and pyrophosphate to I increase phosphate supply [4]
Osteocalcin	Mineralization	Conversion of protein into calcium; mineral binding; attraction of osteoclasts; regulation of rate of mineralization or final shape of mineral crystals [5]
Bone Sialoprotein	Mineralization	Formation and remodeling of connective tissue matrix [6]
Osteopontin	Mineralization	Initiation of mineralization through calcium-binding properties [9]
Osteonectin	Mineralization	Help binding of calcium to collagen; binding of denatured collagen and HA; regulation of Ca+ concentration; organization of mineral within the matrix [9, 13]

1.3 Bone Cells

The major cell types that occupy bone tissue have different origins and respond to different stimuli [31]. Unlike man-made materials, bone is self renewing and can adapt to differing mechanical stresses and strains [27]. The bone cells are able to adapt by forming or resorbing bone tissue as needed. Mechanical loading is required in order to maintain a

healthy balance of mineralization and resorption within the tissue**.** Successful bone formation depends on

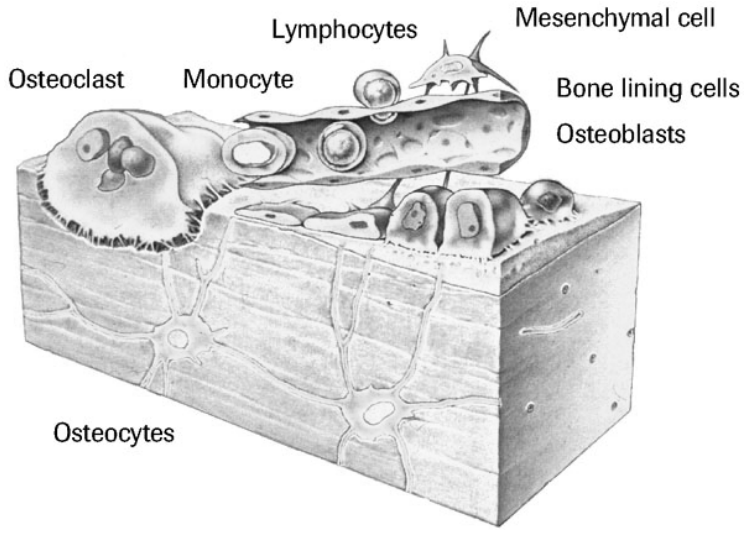

Figure 1.1: Physiological Arrangement of Bone Cells [32]

the expression of and exposure to a number of growth factors and proteins [33]. Three bone cell types (osteoblasts, osteoclasts and osteocytes) are constantly interacting as part of over one million bone remodeling units that are present in the human skeleton (Figure 1.1) [34].

1.3.1 Osteoblasts

Osteoblasts are cells derived from mesenchymal stem cells (MSCs) that differentiate along the osetoblastic lineage following expression of certain transcription factors. These MSCs are located in the bone marrow and within the periosteum. They are polarized with

nuclei located at the end of the cell, distal to the bone surface [27]. Osteoblasts line the surface of the bone and contain three major organelles; the nucleus, the Golgi apparatus, and the rough endoplasmic reticulum. Alkaline phosphatase is found on the surface of the cell membrane and, as this becomes mineralized, the osteoblasts mature into osteocytes [27]. Osteoblasts change shape as their function changes; growing larger and more rounded as the level of metabolic activity increases. After the cells are committed along the bone cell pathway, they secrete alkaline phosphatase and unmineralized bone matrix that forms the osteoid during maturation. Finally, this osteoid mineralizes to yield mature bone which expresses osteocalcin. As these new bone cells become embedded in the surrounding tissue matrix and generate processes, they form the osteocytes that comprise the majority of the bone cells [24, 27].

The differentiation of osteoblasts from MSCs is a complex process that is dependent on a number of transcription and growth factors [33]. Differentiation of these cells *in vitro* requires supplementing cell growth medium with L-ascorbic acid (Vitamin C) and β-glycerol phosphate [35, 36]. Ascorbic acid enhances the extracellular matrix deposition through collagen production and promotes alkaline phosphatase activity [37, 38]. The phosphate additive is connected to an increased presence of hydroxyapatite [37]. A third supplement, corticosteroid dexamethasone, has been shown to contribute to increasing the differentiation potential of MSCs but it may do so while inhibiting proliferation [33, 34, 39]. Expression of the transcription factor runt-related transcription factor 2 (Runx2), also known as core binding factor alph1 (Cbfa1), is also required for MSCs to commit to the osteoblast lineage [40-42]. Runx2 must bind to the promoter of several bone-specific genes, including osteopontin and osteocalcin, for proper skeletal growth. When expression of this

transcription factor is repressed in controlled experiments the result is bone development with symptoms of skeletal dysplasia [41-43].

The differentiation of MSCs into osteoblasts has been divided into three distinct phases that can be identified by examining the expression of specific genetic markers that are expressed during the differentiating process (Figure 1.2). The three main phases have been designated proliferation, maturation, and mineralization [44]. Although an exact map of gene expression is unknown, the three stages have been identified using the following markers: Runx2, Type 1 collagen, osteocalcin, alkaline phosphatase, and bone sialoprotein [45].

During the initial proliferation phase, Runx2, Dlx5 and Msx2 are required to steer the cells away from an adipocytic or chondrocytic phenotype and toward an osteoblastic phenotype [31]. Expression of the gene histone H4 (H4) is prominent during this phase of active cell replication and gradually tapers off as the stem cells progress into the maturation phase [46, 47]. Histones are proteins located within the chromatin of the nucleus whose synthesis is linked to DNA replication [47]. Type 1 collagen is also expressed in this phase but its levels peak as the cells start laying down matrix in the maturation stage. This second phase has been shown to correlate with high levels of alkaline phosphatase expression at approximately Day 14 after exposure to differentiation cocktail [34]. Type 1 collagen contains a RGD sequence that may help attract other osteoblasts, and alkaline phosphatase may contribute to supplying phosphate for the matrix through hydrolyzation of phosphate esters [34].

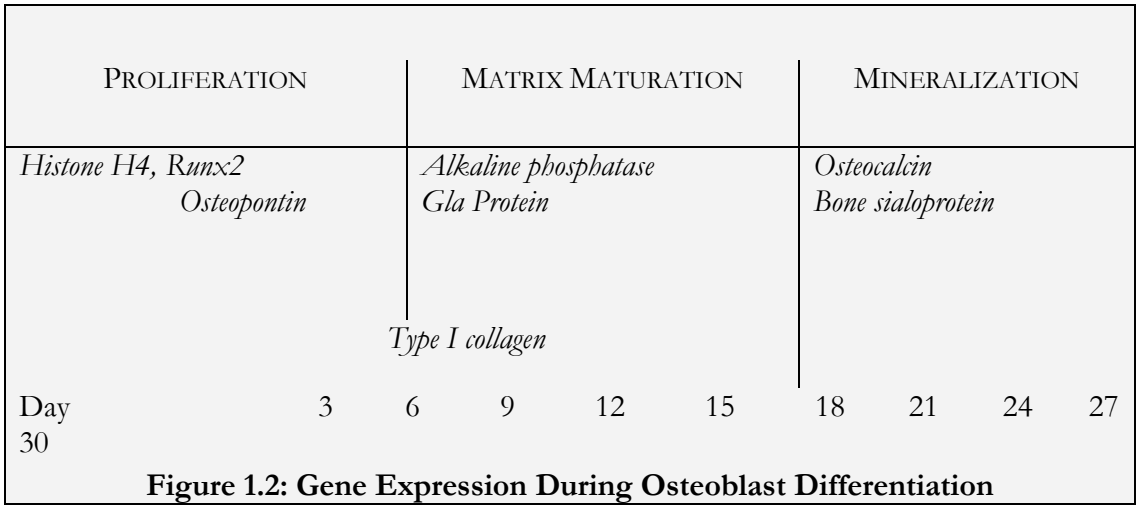

Figure 1.2: Gene Expression During Osteoblast Differentiation

Bone sialoprotein (BSP) is a primary marker for terminally differentiated osteoblasts in the mineralization phase [48]. Osteocalcin is an additional marker of the final mineralization phase. Studies of the BSP gene have shown that it helps control osteoblast differentiation via an autoregulation mechanism. This mechanism uses a positive feedback loop that contains the BSP promoter [48]. BSP along with osetopontin can contribute to mineralization via calcium binding [34].

Mature osteoblasts regulate bone formation and stimulate bone resorption through a signaling triad formed by the molecules receptor activator of nuclear factor kB (RANK), RANK-ligand (RANKL), and osteoprotegerin (OPG) (Figure 1.2). Of this triad, RANK-L and OPG are found on the surface of and expressed by osteoblasts, respectively. When osteoblasts and osteoclasts precursor cells are in physical contact with each other, this activation can occur [49].

1.3.2 Osteocytes

As the surface of the osteoblast is covered in mineralized bone matrix, the nucleus expands. When the terminally differentiated osteoblast is completely surrounded by the mineralized matrix it is considered an osteocyte [50]. Osteocytes are the most numerous of the bone cells, accounting for 90% of all the cells in this skeletal tissue [27]. Described as star-shaped, they are found between the lamellae, arranged around the central lumen [27]. They have an extensive network of processes that allow the cells to establish contact with surrounding osteocytes and the bone lining cells. It is believed that the cell-to-cell contact allows the osteocytes to communicate stress and strain signals in order to regulate the metabolic activity of bone [27].

It is believed that the osteocytes are responsible for sensing mechanical stress and strains experienced by the bones through integrins that attach these cells to the matrix. Mechanical forces cause a change in the fluid of the canaliculi which agitate the integrins. Osteocytes translate the changes experienced by the integrins as biochemical signals to the other bone cells via gap junctions. These signals act as controls for bone formation or resorption depending on the loads to which the cells are subjected. Osteocytes can undergo apoptosis during bone formation and resorption; evidence of these cells living for decades also exists [50].

1.3.3 Osteoclasts

Osteoclasts are highly specialized macrophage polykaryotic cells that are responsible for the resorption of old or damaged bone matrix [49]. Unlike osteoblasts, these cells are derived from the hematopoietic stem cells (HSCs) of the bone marrow that are also

responsible for the production of monocytes and macrophages. Monocytes migrate to the surface of the bone and differentiate into mature osteoclasts in response to normal microdamage that occurs within the bone micro-structure [49], [51]. Osteoclast formation is complete when the monocytes fuse to form multi-nucleated cells after exposure to stromal-derived stimulators.

The multinucleated osteoclasts, or "giant cells", can be identified histologically by the large size and ruffled borders that are unique to these cells' morphology. The ruffled, or brush, edges are formed by the cell membrane folding in order to increase the cell membrane surface area [27]. They can also be identified using assays or staining for the tartrate-resistant acid phosphatase (TRAP) enzyme which osteoclasts produce in elevated amounts relative to other cell types [52], [53].

A link between osteoclastogenesis and stromal cells was first observed in an *in vitro* co-culture of bone marrow HSCs with stromal cells [52]. Additional studies have shown that when osteoblast differentiation is absent or impaired in mice, osteoclast formation will not occur [51]. The discovery of a mechanism that links a receptor located on the osteoclasts [receptor-activator of NF-$\varkappa$B (RANK)] with a surface protein of osteoblasts [RANK-ligand (RANKL)] has helped explain the intricate coupling that exist between these two cell types and their distinct roles in the bone remodeling process (Figure 1.3).

Osteoblast release macrophage colony stimulating factor (M-CSF) stimulates the production of osteoclast genes in precursors. The presence of M-CSF may also help facilitate the binding of RANK on the surface of the precursor cells to RANKL which is produced by and expressed on the surface of osteoblasts [49],[54, 55]. After RANK/RANKL binding occurs the polarized osteoclast cell undergoes genetic and

structural changes in preparation of bone resorption. Studies have indicated that membrane-bound RANKL is more efficient than the soluble RANKL in inducing differentiation of osteoclasts [54]. This may explain the claim by some that cell-to-cell contact of stromal cells with pre-osteoclasts is necessary for the production of multinucleated cells [51].

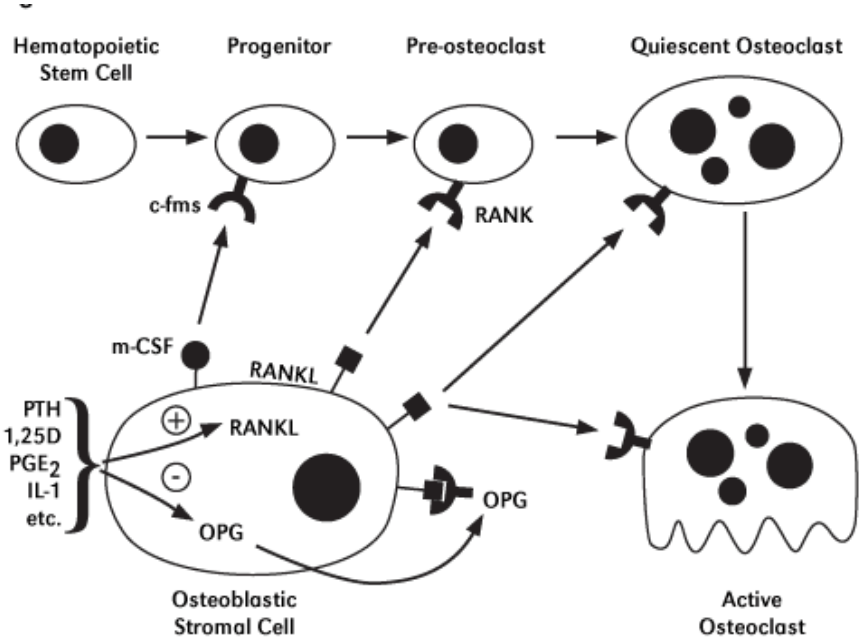

Figure 1.3: Interaction between Osteoblasts and Osteoclasts [56]

Resorption of bone occurs when the brush borders of the osteoclast create a small pocket with the bone surface and secrete hydrogen ions, creating an acidic environment that dissolves the bone matrix, exposing collagen bundles [27, 49]. The resorption pits that form when osteoclasts are active are called Howship's lacunae. Integrin proteins [vitronectin receptor ($\alpha v\beta 3$)] allow the osteoclasts to attach to the surface of damaged or aged bone and create an isolated area for ion secretion. Bone material is removed through acidic proteolytic

digestion [27]. Osteoclasts express the enzymes cathepsin B, D, L, K, and matrix metalloprotease-1 (MMP-1). Cathepsin K is produced in the largest amounts and is believed to be responsible for digestion of the bone matrix while MMP-1 is linked to cell migration [34].

The RANKL signaling pathway has a number of regulatory factors that can control the activation of osteoclasts when needed to keep bone at a healthy bone mass. A major regulatory glycoprotein, osteoprotegerin (OPG) is also produced by osteoblasts and is in the same biological family as RANK and RANKL. OPG acts as an inhibitor of bone resorption by acting as a decoy receptor for RANKL, blocking the binding of RANK [49], [54]. These inhibitory mechanisms are used by the body to maintain bone mass and bone density and are being explored for possible treatment of bone disorders [57]. Clinical use of electromagnetic stimulation has also been shown to interfere with the RANK-RANKL-OPG triad and could be applied clinically to help regulate bone resorption [58]. Additional studies have explored the role RANKL plays in T cell response to various infections; these findings suggest that RANKL is required for optimal response of these immune cells [59].

1.3.4 Bone Marrow Stem Cells

Stem cells are a unique set of cells that have the capacity for self-renewal and have not yet committed to a specific cell type [9, 60, 61]. These cells have been shown to be multi-potent, meaning that they possess the ability to differentiate into a number of different cell types. All cells in the body are derived from embryonic stem cells, found in the blastocyst, during the early stages of human development [9, 61, 62]. Stem cells are driven down specific paths of differentiation after stimulation by proteins and growth factors that are

specific to certain cell types. These precursor cells follow a closely regulated chain of proliferation, migration, and differentiation prior to maturation along most cell lineages [61]. In addition to the embryonic cells, stem cell lines have been identified in the fully developed adult body (Table 1.2). Locations of these mature stem cell lines include the brain, gut, epidermis, and the bone marrow [61]. These stem cell lines, often referred to as "adult stem cells" (ASC), produce specific cell types depending on their location of origin. A number of recent studies have challenged the idea that adult stem cells are restricted in their ability to produce a variety of cells types [62]. Marrow stromal cells may represent a source of post-natal stem cells that can be used for production of skeletal and other tissues [63].

Table 1.2: Classes of Stem Cells [61]

Type	Location	Cells Produced
Hematopoietic	Bone Marrow	T-cells, B-cells, white blood cells
Neural	Brain	Neurocytes, oligodendrocytes, blood
Epithelial	Bone Marrow, Gut, Epidermis	Vascular endothelial cells
Mesenchymal	Bone Marrow	Osteoblasts, chondrocytes, neurocytes, adipocytes
Embryonic	Blastocyst	All cell types

The stem cells located within the bone marrow can be divided into two major subsets: hematopoietic and mesenchymal. Bone marrow is widely used as a source for the mesenchymal stem cells (MSC) [61]. MSCs have been found to exhibit a high degree of plasticity with the ability to produce osteoblasts, chondrocytes, adipocytes, myotubes and

neurocytes [61, 64]. When isolated from marrow using plastic, adherent cell culture techniques, the initial MSCs display a homogeneous population of fibroblast-like cells that do not express antigens typical of hematopoietic cells (CD34, c-Kit, CD45) [61], [62].

Expanded MSCs subcultured from bone-marrow can contain an assortment of cells that are at different levels of commitment. Cells isolated from these cultures are not necessarily homogeneous but include differentiated bone cells and cells that have shown the ability to develop along multiple lineages (osteo/chondro, osteo/chondro/adipo). As the progenitors progress they also contain a subset of uncommitted cells and lose the ability of self-renewal [61].

Another potential source of stem cells in the body that may contribute to formation of bone cells are found in the adipose tissues [65]. These cells have been also shown to derive bone, cartilage and neurons, in addition to fat tissue [64]. Cells isolated from the fatty portion of the bone marrow have demonstrated the ability to mature along both the adipocyte and osteoblast cell lineages [61]. However, Gimble and coworkers have contested that although these cells share common functions, an inverse relationship exists between adipocytes and osteoblasts. They suggest that commitment along one pathway occurs at the expense of the other phenotype [65].

Bone marrow stem cells isolated from the marrow and the trabecular compartments have been shown to have the ability to form new bone when transplanted directly. These cells are also capable of contributing to bone formation when placed in a scaffold culture prior to implantation into the subcutaneous space in mice and rats [66]. Using these adult stem cells for the treatment of bone injuries is a major area of interest in orthopedic research [63]. A number of issues need to be addressed before these cells can be used in clinical

practice, including defining the optimal stage of commitment of the cells for transplantation and if they can be introduced to the patient via direct loading at the injury site or through systemic infusion.

Use of BMSCs for bone regeneration is popular practice because of their multi-lineage differentiation potential, their relative availability and ease of harvest, and their capacity to undergo multiple replications without losing multi-potent potential. These traits make them an attractive source for cell-based therapeutic approaches. Several experiments have demonstrated that BMSCs can be induced to transform into osteoblasts.

The rates of repair for skeletal defects and the number of MSCs in the body diminish with age. Human MSCs in bone marrow have been shown to decrease from 1 in 10,000 marrow cells at birth to 1 per 2,000,000 in geriatric patients [67]. These numbers indicate a correlation between the numbers of available MSCs in the body with the ability of the skeleton to self-repair. Although the number of available MSCs in the marrow decreases with age, there has been great success in expanding cultures of these cells in the laboratory to provide tissue engineering approaches for bone repair.

1.4 Bone Remodeling

The gross outward appearance of bone tissue suggests that it is a dense, homogeneous and static organ. In reality, bone is a dynamic tissue that undergoes constant resorption, renewal, and remodeling. Bone resorption and formation are coupled in healthy bone and occur in a very controlled manner, following a sequence of activation-resorption-formation [29, 31]. Coupling ensures that new bone will be formed wherever bone is removed [31]. If functioning properly, up to one-fifth of the skeleton is replaced per year. Briefly,

microcracks occur normally on the surface of our bones and osteoclasts are responsible for the resorption of this damaged material. Resorption creates a cavity similar in size to a haversion osteon at a rate of 40 to 60 microns per day [29]. Following resorption, the osteoblasts lay down new bone matrix which becomes mineralized and creates new osteons.

This remodeling mechanism keeps bone mineral new and healthy. When age, trauma, or a disease disrupts this process there is a resulting imbalance in the formation and resorption coupling, leaving bones either brittle or fragile. The remodeling process is activated when the osteoid layer of bone is disrupted and the underlying matrix is exposed. A few concepts have been developed to explain how this activation occurs: (1) growth factors stored in the bone matrix are released during resorption and activated by the low pH environment, allowing them to promote activity by the osteoblast. (2) change in bone topography within resorption pits is sensed by the bone cells via chemical communication [68].

The group of cells that are involved in activation, resorption, and formation are called a bone modeling unit (BMU). One proposed model of bone remodeling considers osteoblasts, osteoclasts and their precursors as part of the BMU [27, 69]. Bone lining cells that lift from the damaged surface seal off the area that is undergoing remodeling, creating a 'canopy' for the other active bone cells [70]. Different areas of the BMU can undergo resorption and formation simultaneously until the entire area is composed of new mineralized bone matrix [70].

1.5 Natural Bone Graft Options

1.5.1 Autografts

The use of a patient's own bone continues to be the standard for bone grafting techniques, despite the availability of numerous bone graft substitutes, due to the intrinsic osteoinductive capabilities of autograft [71]. Current options for autologous grafts are vascularized and nonvascularized cortical, cancellous, and bone marrow material. The current "gold standard" for autogenous bone graft material involves harvesting bone graft material from the patient's iliac crest.

Limited numbers of mature osteoblasts are transplanted within the bony material, but osteogenic precursor cells are present in enough numbers to give the implant the desired properties that continue to make it the preferred material for bone grafting [18, 21]. Initially, these grafts act strictly as scaffolds but gradually stimulate bone formation, despite a lack of vascularization. Ultimate success of the graft depends on the amount and rate of revascularization that occurs along with new bone growth [27]. Vascularization of cortical bone grafts ensues at a much slower rate than in cancellous bone grafts; the vascularization rate affects the chance of cell survival within the graft [72]. Osteoclasts actively resorb cortical bone grafts, resulting in an increase in porosity that makes them better suited for osteoconduction [27, 72].

Autogenous bone grafting has an excellent probability of success in spinal fusion, and cancellous grafts are excellent for nonunions with less than 6cm of bone loss. Cortical bone grafts have also been successful in larger defects (> 6cm) that require some structural support [18]. Intraoral autologous grafts are used extensively in periodontal and

maxillofacial procedures with a high level of success, often in combination with other bone graft materials [12].

Unfortunately, the surgery required to harvest iliac crest bone graft material causes additional pain for the patient, draws on a limited amount of tissue, and results in a 30% rate of donor site morbidity [7, 73]. An insufficient amount of bone available for harvest has been reported in 20% of surgical cases [8]. Autografts are naturally absorbed by the body, but this process may occur at a faster rate than new bone formation [74]. Comparing various implant materials for cranioplasty, autogenous bone grafts have a significantly higher rate of infection than synthetic titanium meshes [75, 76]. Implant slippage, graft fracture, and an inability to keep patients immobile for a requisite amount of time have all attributed to the failure of autografts used in orthopedics [77].

Both natural and synthetic substitutes have been developed to avoid the complications and pain associated with harvesting autograft material from a patient's healthy bone, but they are costly and lack the inductive properties desired by surgeons for proper repair, leaving autologous tissue as the standard by which all biologics are measured [71, 78].

1.5.2 Allografts

Allografts are products harvested from cadaver bones that have been preserved by flash freezing or freeze-drying, then vacuum-packed as whole or partial bone, morsels, or chips. Accounting for approximately one third of bone graft procedures in the United States, allografts are the preferred substitute for autografts with similar osteointegrative and osteoconductive characteristics [18]. Allografts contain no living cells, but some grafts are inductive when demineralized or broken into smaller particles [21, 76]. Allografts are

advantageous for large defects when inadequate autologous tissue is available for harvest but must be processed to avoid transmission of disease. The methods used to treat allograft material prior to use are expensive and increase the cost of this technology while diminishing the mechanical and biological properties [1].

Allografts have been used successfully for short-level fusions in the cervical spine but are not recommended for use in the thoracic and lumbar spine due to allograft weakness in stimulating bone growth [76]. Issues associated with allografts include a 17% rate of radiological non-union, bacterial infection, and negative immunological response from the host [21]. Between thirty and sixty percent of allografts experience some complication after ten years, and a higher level of variability is associated with their use clinically compared to autografts [14].

1.5.3 Demineralized Bone

Freeze-drying and flash freezing are two commonly used preservation methods that salvage the mineralized portion of the tissue, however, doing so may increase the chance for a negative response from the immune system [20]. Demineralized bone matrix (DBM) is a type of allograft produced by treating bone with hydrochloric acid to remove the mineral phase, leaving behind only an osteoconductive collagen matrix [79]. Various non-collagenous proteins and growth factors remain within the cortical bone-structured matrix, which may contribute to the osteoinductive properties of the demineralized material [1, 76]. Both human and bovine DBM are available commercially from a number of manufacturers and in a variety of forms [80, 81]. Companies claim that specific variations in processing and storage techniques provide advantages, such as increased concentrations of inductive factors,

over other competitor products. It is believed that removing the mineralized phase of the bone increases the exposure and availability of growth factors contained within the material [20].

The costs of DBM products differ little from synthetic bone grafts, averaging between $500-$1500 worth of material per surgery (approximately $100-$150 per cubic centimeter) depending on the amount of material and whether they have been designated as containing bone morphogenetic proteins (BMPs) [82]. BMPs are found in variable amounts, and the concentrations are normally so low they probably have very little effect on osteoinduction. In addition, there is high batch-to-batch variability within DBM products, which can lead to variable clinical outcomes [83].

DBM is best suited for non-load bearing applications and can be used in place of or as an autograft extender [81]. Common uses of DBM are for treating bone cysts and well-contained defects and other bony voids or gaps within the skeletal system [84]. DBM is often incorporated into a variety of carriers including: putty, paste, strips, and glycerol gels. The latter forms are intended to keep the DBM in place for ease of implantation. Carriers include calcium sulfate, collagen, and glycerol. The percentage of actual DBM material varies between products and is reported to be lower in products that use a carrier [80].

Clinical studies have shown that DBM mixed with autograft is as effective as autograft alone, in patients undergoing posterolateral spinal fusion [85], where at 24 months, the two groups had equivalent rates of diffusion. DBM has also been effective, in combination with internal fixation devices, in treating non-union fractures [86]. The degree of demineralization has been shown to play a role in the ability of DBM to support osteogenic differentiation of BMSCs *in vitro*. Studies have also shown an indirect correlation between

bone nodule formation and the amount of mineralized tissue that remains in the substrates [87]. These results illustrate why the variability in batches of DBM is a clinical problem.

1.5.4 Bone Growth Factors

A large number of growth factors have been shown to contribute to bone formation and repair. As technology improves, new growth factors continue to be identified; however, much attention is presently being given to the following: transforming growth factor-β1 (TGF-β1), transforming growth factor-α (TGF-α), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and bone morphogenetic proteins (BMP) [33]. Their functions vary widely but include promotion of vascular ingrowth (FGF, VEGF), induction of osteogenesis (TGF-β1, BMP), and stimulation of fracture healing (PDGF). The osteoinductivity of DBM bone grafts has been attributed to the presence of these important growth factors which they contain [88]. Each of these growth factors has been identified within autografts taken from the iliac crest. Schmidmaier and coworkers compared levels of osteogenic growth factor in two sources of autologous bone graft tissue: graft from the iliac crest and bone particles collected during reaming of the medullary canal [89]. The reaming debris was found to have higher levels of five growth factors: FGFa (fibroblast growth factor-acidic), PDGF, IGF-1, TGF-β1, and BMP-2. Significantly greater amounts of FGFb (fibroblast growth factor-basic) and VEGF were measured within the iliac crest material [89]. These results indicate that reaming debris may be a comparable source of autograft material for use in orthopedic surgery.

Attempts have also been made to combine osteogenic growth factors with synthetic bone graft substitutes in order to improve their osteoinductive capabilities. Clarke and colleagues studied IGF-1, FGF, and TGF-β1 absorbed onto ceramic bone void filler and compared to autograft. Of the three, FGF was found to be the most beneficial, but none had a significant effect on the formation of bone around a metal implant [90].

1.5.5 Bone Morphogenetic Proteins

The use of biologically derived materials as components in bone grafts is a growing area of interest as more is information is gathered about the functions of growth factors in bone development. Bone morphogenetic proteins are a subset of the TGF-β super family that have been isolated for their ability to induce bone formation *in vivo* [91]. These proteins are very powerful stimulants for bone formation that are found naturally at sites of bone formation. More than 16 human BMPs have been identified and numbered accordingly, with BMP-2 through BMP-9 (with the exception of 8) showing the most promising toward bone formation. BMP-2 and BMP-7 are the two that have shown to stimulate bone formation equally or even better than autogenous bone grafts [76, 92]. It is believed that BMPs induce the expression and function of a number of major proteins including: osteocalcin, bone sialoprotein, and alkaline phosphatase [91].

At present only a few products have been approved for clinical uses that contain BMPs but it is an area of active growth (Table 1.3). BMPs are available in small amounts naturally so scientist have used genetic engineering to produce the quantities that are needed for use in bone graft products. This technology ensures purity and reproducibility when isolating the growth factors. Initial work with BMPs for bone repair showed that the product was

quickly removed by body fluids when placed within the surgical site. As a result, the approved products use Type 1 collagen as a carrier for ease of delivery. BMPs must be delivered in the correct dose and over enough time for proper bone formation to occur. Further development is needed to improve carrier methods. Modifications could potentially reduce the amount of BMP needed initially, thereby allowing a reduction in the cost of the products [91]. Currently, for example, 3.5mg of OP-1 Putty from Stryker is sold at a cost of $4500.

Approval for these biological products was preceded by clinical studies that demonstrated their success in spinal fusion surgery. BMP-based products can now be used in tibial fractures as well [91]. Springer and colleagues explored the use of BMP-7 on cranial defects in growing animals and found that they induced higher quantities of bone growth over iliac bone grafts [93]. This protein has also successfully prevented non-union formation when applied immediately after fracture [94]. BMPs combined with HA and collagen gels have been examined for potential use in dental repair. These composites have the potential to induce bone formation that can stabilize HA material in mandibular augmentation [72].

A study on the use of BMPs in over 300,000 spinal fusion surgeries in the United States between 2002 and 2006 was conducted by the Department of Neurosurgery at Brigham and Women's Hospital in Boston, Massachusetts. The group found that hospital stays and inpatient costs were increased with the use of BMPs, regardless of the area of the spine where fusion was performed. Additionally, complication rates for fusions performed in the neck region were 50% higher in patients when a BMP product was used, compared to when one was not [95]. The Food and Drug Administration has issued warnings about the use of BMP products in areas of the neck, an area that is not approved for BMP use.

Table 1.3 : Clinically Available Bone Morphogenetic Protein Products				
Product	*Company*	*Contents*	*Uses*	*Form*
OP-1 Putty	Stryker	Genetically engineered human BMP-7 powder and bovine collagen	Spinal fusion Traumatic fracture	Putty
Infuse	Medtronic	Human BMP-2 solution with collagen sponge for delivery	Spinal fusion Traumatic fracture	Solution
MB52	DePuy Spine	BMP molecule (MP52) combined with calcium phosphate synthetic bone graft substitute (Healos)	In clinical development	In clinical development

1.6 Synthetic Bone Graft Options

Synthetic bone grafts have been developed to alleviate the complications associated with the use of human bone as a grafting material. Synthetic grafts are readily available and do not require the patient to undergo a tissue harvesting procedure, alleviating pain that may accompany an additional procedure [1]. Traditional synthetic bone grafts are osteointegrative and osteoconductive, with more recent attempts being made to increase osteoinductivity by incorporating bone morphogenetic proteins (BMPs) and/or cellular material within the graft [1]. The creation of a composite graft that combines osteoconductive matrices with a component containing osteogenic cells and osteoinductive growth factors could alleviate the use of autografts completely, potentially improving their functionality, speeding the healing process, and improving the rate of bone replacement.

Synthetic bone grafts should support new bone formation, support mechanical loads that are present at the defect site, elicit no foreign-body reaction, cause minimal fibrous tissue growth, and undergo remodeling by the body [21]. The mechanical properties of the graft need to be balanced to prevent stress shielding, a problem caused when the material is too stiff compared to natural bone. The graft must be strong enough to withstand cyclic loading and resist fatigue fracture [21].

When a synthetic implant is placed inside the body there is an initial acute inflammatory response that results in the mitotic expansion of mesenchymal cells that produce extracellular matrix around the implant site. If the implant is made of a metal or metal alloy, the extracellular matrix forms a fibrous membrane encapsulation around the implanted material. When a metal orthopedic implant is used within bone, a capsule of functional regenerated bone tissue rather than a fibrous membrane can form around the implant. The formation of regenerated bone tissue, with no fibrous interface, is desired to achieve integration of the implant with the native tissue. In cases where alumina or titanium devices are used, the interface between the regenerated bone capsule and implant is not strong enough to withstand high loading, especially in shear [67, 96].

It was first demonstrated by L.L. Hench that certain types of glass ceramics form a chemical bond between the surface of the implant and the regenerated bone tissue capsule; making the interface very strong, stable, and able to better withstand loading. Hench showed that glasses that contained specific portions of oxidized elements were able to form this chemical bond with the living bone tissue, now referred to as bioactive glasses [10]. After it was reported that these glass substrates had bioactive properties, hydroxyapatite ceramics and ceramic phosphates were also found to possess this important quality. Not all

ceramics are bioactive. Since this discovery, a number of ceramics have been used in bone reconstruction surgery with positive results [10, 11].

Some non-ceramic materials (i.e. aragonite, calcite) and organic polymers (i.e. polyethylene oxide (PEO) / polybutylene terephthalate (PBT) block polymer Polyactive ®) have also shown similar bone-bonding characteristics [67, 97].

1.6.1 Ceramic Bone Graft Properties

A number of synthetic, calcium-based materials have been used successfully as bone grafts due, in part, to their biomechanical properties. Ceramics are an attractive alternative to natural materials because they are available in virtually unlimited quantities and exhibit a high degree of purity. Some commercially available products have properties closer to cortical bone (Norian; Synthes, Inc.) while others are similar to cancellous bone (Vitoss; Orthovita, Inc.). Others provide no structural capacity *in vivo* and merely act as cement (MIIG; Wright Medical Technology) [82].

Synthetic ceramic products are bioinert or bioactive and provide a scaffold into which the host tissue may grow, but they are not intrinsically osteoinductive. When a ceramic graft is implanted next to healthy bone, osteoid is secreted directly onto the surface of the material without interference from fibrous tissue. This new osteoid is capable of mineralization and subsequent remodeling by the body as the synthetic material degrades over time. The rate of degradation varies from weeks to years depending on the type of ceramic [72]. Ceramics also can be used in combination with other bone graft materials to provide enhanced properties. Surgeons can incorporate drugs or antibiotics into ceramic powders to help fight infection at a defect site [98].

The four classes of synthetic ceramic materials listed in Table 1.4 are most commonly used in orthopedic bone repair [1, 21]. These materials are not always moldable into desired shapes but have been used in a number of applications including: femoral reconstruction, spine arthrodesis, filling of cystic cavities and tibial osteotomies, and treatment of periodontal osseous defects [18].

Table 1.4: Ceramic Bone Graft Substitutes

Bone Graft Material	Important Features	Commercially Available Product
Tricalcium Phosphate	Compressive and tensile strength similar to cancellous bone Gradually removed from the site as new bone is formed Degraded by osteoclasts Porosity and handling characteristics less predictable than other products Faster resorption and less structural strength than HA Structure similar to amorphous biological precursors to bone	Stryker® TCP Putty; Stryker Cellplex TCP Graft; Wright Medical Technology Orthograft; DePuy Vitoss; Orthovita, Inc.
Synthetic Hydroxyapatite	Structure similar to mineralized bone Slow resorption rate of 5-15% per year Much higher compressive strength than cancellous bone Fracture prone in shock loading Predictable and steady rate of absorption and porosity	Apapore; Apatech OpteMX (60% HA); Exactech Biologics Calcitite HA; Zimmer Dental
Coralline Hydroxyapatite	Interconnected pore structure similar to cancellous bone Contains only 20% matrix Faster bone ingrowth and vascularization than other ceramic options 50-80% filling of void within 3 months Does not cause significant stress shielding	ProOsteon; Interpore International Interpore 200®; Interpore International Biocoral®; Biocoral, Inc.
Calcium sulfate	Plaster of Paris Dissolves in 5-7 weeks No structural properties *in vivo* Water soluble	MIIG® 115; Wright Medical Technology Osteoset; Wright Medical Technology DentoGen; Orthogen LLC

Ceramic materials differ in their resorption rate, porosity, crystallinity, and chemical composition, depending on the manufacturer and its end-use form (i.e. large blocks, granules, injectable system, beads, etc.). The structural properties of the various ceramics influence the host tissue ingrowth. The percent porosity in a material indicates the amount of surface area available for cellular attachment and also affects the rate of degradation. In the 1970's Hulbert and colleagues indicated that pore sizes of at least 45-100μm and up to 500μm were optimal for new bone ingrowth, while 100-150μm pores provide for ingrowth of fibrovascular tissue [1, 21, 99]. It has been shown that new bone formation occurs within the internal regions of porous hydroxyapatite (HA) ceramics before forming on the outer surface of the implant. When the ceramic material is mixed with fresh bone marrow, new bone mineral is produced by progenitor cells contained within the marrow that attach to the internal pore surfaces. This sequence of events, 'bonding osteogenesis', has been described by Ohgushi and Caplan and has been found to occur on other ceramic materials including: tricalcium phosphate (TCP), bioglass, and HA/TCP composites [67]. The porous structure of a biomaterial contributes to the material's *in vivo* performance [100].

Ceramic grafts are subject to resorption by chemical dissolution or digestion mediated by osteoclasts [101]. Scaffold resorption rates vary between materials and can have an effect on the regeneration of bone within and surrounding the scaffold [18, 21, 101]. Hing and coworkers determined, in a 12-week rabbit model, that dense calcium sulfate exhibited a rapid rate of resorption through chemical breakdown, while ultra porous tricalcium phosphate was quickly broken down through a combination of cellular resorption and dissolution. Although the mechanisms of resorption differed, the rapid rate was held

accountable for the lack of bone regeneration that surrounded each ceramic material. The ultimate rate of bone regeneration and healing is significantly affected by the rate and process of ceramic degradation; a stable scaffold is necessary to support new bone and vascular growth that will replace the ceramic material over time [18, 21, 101].

Ceramics are weak in tension but strong in compression (Table 1.5) [21]; additionally, they have poor mechanical resistance when used in bulk and have low impact resistance, tensile strength, and fatigue resistance. The mechanical properties of these implants limit their use in weight bearing sites; however, ceramics are sometimes used for hip fracture stabilization and for treating periodontal bone defects [102, 103]. Ceramic bone graft combined with internal fixation devices have successfully promoted repair of non-union and difficult fractures[104].

Table 1.5: Mechanical Properties of Bone Graft Materials [21]

	Cancellous Bone	Cortical Bone	Coralline HA	Porous HA	Calcium Sulfate	Porous TCP
			Strength (MPa)			
Compressive	5.5	162	9.3	60	23	13
Tensile	3	151	2.8	2.5	4.1	-
Young's Modulus (GPa)						
	1	15	1.2	9.2	-	1.6

TCP and synthetic HA are structurally comparable to human bone [1, 10, 11]. Both ceramics are virtually bioinert, eliciting minimal response from the body's immune system [105]. TCP grafts are resorbed as new bone penetrates the graft; HA, however, is less resorbable and can remain at an implant site for many years. Following implantation of TCP, an osteointegrative HA layer forms on the bone-implant interface. Calcium and

phosphate ions interact between the host tissue and in the implanted material to create a strong chemical bond [1, 10]. The precipitation of an HA layer on the surface of these materials in response to the chemical reaction at the surface leads to the adsorption of proteins and other macromolecules. Cellular attachment is enhanced following these surface changes that occur on the ceramic [67]. The porous structure also encourages tissue ingrowth and once the surrounding bone has been incorporated into the implant the mechanical strength is comparable to cancellous bone [1].

1.6.2 Beta-Tricalcium Phosphate

Tricalcium phosphate ($Ca_3 (PO_4)_2$) is widely used for orthopedic applications in its beta form, β-TCP. β -TCP is commercially available in either porous or solid form in granules or blocks. The calcium-phosphate molar ratio has been reported as 1.5 [106, 107]. This material is often used as a graft expander with autologous tissue since the replacement volume of bone is initially less than the amount of β-TCP resorbed within a bone defect [21]. To study the effect of pore size on β-TCP *in vivo* characteristics, von Doernberg and associates implanted eight millimeter diameter cylinders of β-TCP with pore sizes ranging from 150-1220μm into the cancellous areas in the long bones of sheep[108]. Ceramic resorption of the implants occurred within the first 6 weeks following implantation and almost complete bone replacement was seen in radiographs by Week 12. Substrates with a pore size of 510μm had the fastest resorption; however, the resorption rate had a slightly negative effect on bone formation [108]. When mixed with bone marrow aspirate (1:1), the ultraporous β-TCP bone graft substitute Vitoss (Orthovita, Inc.; Malvern, PA), resulted in

new bone formation that matched the density of existing host cancellous canine bone by 12 weeks *in vivo* [100, 109].

An ultraporous form of β-TCP uses particles in the nanometer range to better mimic the structure of naturally occurring cancellous bone. The nanosized particles better mimic the surface of physiological bone and result in improved osteoconduction; better resorption of the smaller particles by osteoclasts is associated with this ultraporous structure [1, 100, 110]. When mixed with bone marrow and implanted subcutaneously in rats, ultraporous β-TCP has been found to produce more bone and osteoblastic cells than DBM [100]. A similar mixture exhibited a 91% spinal fusion success rate after 12 months in humans with degenerative disc disease [111].

1.6.3 Hydroxyapatite

Synthetic HA (C_{10} $(PO_4)_6(OH)_2$) is based on the main mineral component of physiological bone. The ceramic form is sintered at temperatures between 700 and 1300°C to create a porous crystalline structure. Pores are created by the addition of hydrogen peroxide prior to sintering, resulting in bubble formation. This sintering method does not allow tight control of pore size and connectivity [21]. When packaged as a solid block, HA has a higher modulus of elasticity than bone. The block form has a calcium-phosphate molar ratio of 1.67, slightly higher than TCP, and is resorbed at the rate of only 1-15% per year [1, 106]. Large amounts of HA can remain at a defect site for years after initial placement [18]. Examination of HA surface roughness and cellular response to the surface revealed that proliferation, adherence, and detachment strength are all sensitive to the material's topography [112]. Due to its cell affinity, hydroxyapatite has been used

successfully to coat metal implants [21]. Previous studies indicate that when primary or culture expanded marrow stem cells are placed in porous HA implants for treatment of segmental defects in rats or canines, the resulting bone regeneration is greater than in defects treated with the ceramic alone. These data indicate the potential for cellular HA to be used in reconstruction following benign tumor excisions or intra-articular compression fractures [67].

Coralline HA capitalizes on the interconnected, consistent pore size, and permeable structure of marine coral that mimics natural cancellous bone. Coralline HA consists of the crystalline form of calcium carbonate, without any silica or phosphate, arranged in a macroporous structure that facilitates both vascular and host tissue ingrowth [67]. Extreme heat and pressure are used to remove all organic matter from the marine coral, resulting in a sterile material that is packaged as granules or blocks. Experimental and clinical studies have shown this product to be highly osteoconductive and capable of excellent vascular ingrowth. Histological analyses of coralline implants following implantation have shown strong bone adaptation to the calcium carbonate surface. This surface has the ability to support the differentiation of MSCs into bone forming cells [67]. When compared to cancellous autografts, no major differences were seen between the two groups for maintenance of reduction, time required to union, or range of motion for the knee following metaphyseal defect repair of displaced tibial plateau fractures. Coralline HA has been shown to possess improved degradation properties over its synthetic counterpart [1, 67].

1.6.4 Calcium Sulfate

Commonly known as Plaster of Paris, calcium sulfate is thought to act as a conductive matrix for ingrowth of blood vessels and osteogenic cells, when implanted adjacent to a viable periosteum or endosteum [7, 21]. This material dissolves at varying rates and can be mixed with antibiotics to help fight bone infections. Most commonly used as a bone void filler, calcium sulfate completely resorbs and the host tissue is able to regain pre-injury structural properties [18]. Medical grade calcium sulfate is produced with crystals of similar size and shape [113]. This inexpensive material has been used as a bone void filler for over 100 years and has demonstrated success in the repair of ovine metaphyseal defects [114].

1.7 Tissue Engineering Approach to Bone Graft Development

A key challenge in the area of bone repair is the development of tissue-engineered products that can be used to treat bone defects [115]. Bone tissue engineering solutions combine scaffold materials with viable stem or bone cells in products that can be implanted into massive bony defects [67]. A successful tissue-engineered construct would offer a better an alternative to the autologous bone graft that is the 'gold standard' available today. It is widely recognized that osteoblasts are important with respect to bony ingrowth of biomaterials, and the success of a tissue-engineered construct will ultimately depend on how the construct can be incorporated into the host tissue [116]. The incorporation of osteogenic cells and growth factors within a bone graft can improve the substitute viability, but the optimal mix of cells/scaffold/growth factors has yet to be determined [117] and is likely patient and/or application specific. Although the ultimate viability test is in clinical application, the success of modular bioreactors is crucial to the development of tissue test systems with which to model bone cell behavior and bone development [118].

One major bone tissue engineering approach uses the patient's own cells to regenerate lost tissue. These cells can be harvested through needle aspiration or from the removal of bone tissue during surgical reaming [119]. Cells can then be expanded and incorporated into an implantable device or injectable system. It is still unknown whether progenitor cells offer any advantage over terminally differentiated cells for the ultimate success of the device. Schantz and colleagues performed a direct comparison of progenitor and terminally differentiated cells seeded onto three dimensional polycaprolactone scaffolds to repair cranial defects in an animal model. Both cell types formed mineralized bone and exhibited similar osteogenic capabilities [120].

The discovery and development of successful culture techniques for mesenchymal stem cells has been a driving factor behind autologous cellular engineering approaches. MSCs that have been harvested from bone marrow and expanded *in vitro* may be successfully driven toward an osteogenic phenotype [121]; conditioned culture medium can have a similar effect [122]. Cellular differentiation and osteogenic potential of the cultured bone cells is determined using assays and histological staining techniques that check for the expression of the major bone proteins represented in Table 1.1 [123]. Ohgushi and Caplan note that, although MSC densities in bone marrow diminish with age, it is still possible to expand the cells enough to treat older patients using tissue engineering techniques [67].

Other potential drivers for tissue-engineered bone formation include periosteal and alveolar bone cells. These alternative cell sources are easily accessible; procedures for harvesting have been well tolerated by patients, even under local anesthesia [66, 116]. Tests to compare the bone formation potential of these cell types with MSCS have been executed using subcutaneous injections in mice. Periosteal cells were most successful in forming bone

nodules after 12 weeks when delivered within fibrin glue [66]. Periosteal cells have also been successful when incorporated within various polymer constructs and used for the repair of critical-sized calvarial injuries in rabbits [120, 124].

It is also important to note that, although much of the focus is currently on the culture and production of osteoblasts (bone-forming cells), osteoclasts (bone-resorbing cells) have an equally crucial role in the maintenance and success of any tissue-engineered construct. After successful bone formation has been achieved *in vivo*, the construct must be maintained through the bone remodeling process. Remodeling is essential to maintaining the mechanical structure and properties of the native bone tissue [118, 123]. Osteoclasts are the most important cells that will be involved in resorption of the scaffold matrix, but they are only found on about 1% of the bone surface [125]. The discovery of the RANK/RANKL mechanisms has paved the way for successful osteoclast culture *in vitro*, which allows the incorporation of these cells into bone graft systems [69, 125].

The ideal scaffold material must: induce cellular growth and new tissue formation, support vascularization in a timely manner, resorb at a rate consistent with new bone growth, and be readily sterilized [126]. Any of the currently available bone grafts or graft substitutes can potentially be used as tissue-engineered scaffolds. The formation of a mineralized extracellular matrix is the most reliable determinant that a material will induce and support new bone generation [127]. Ceramics, DBM, and polymers are all being considered in bone tissue engineering research due to their various material strengths. The most successful implant may ultimately be a combination of two or more traditional materials. The scaffold will need to facilitate development that mimics the internal bone structure physically, biochemically, and mechanically [33].

Commercially available human cell-based products are still in clinical trials but studies have already provided the 'proof of concept'. Cell/HA ceramic scaffold based treatment has been used successfully for treating long bone segmental defects (4-7cm). When applied with external fixation for stabilization, no major problems were evident up to 6 years post-operatively [128]. HA ceramics seeded with MSCs and implanted *in vivo* have displayed higher compressive strength and energy absorption properties than native tissue following implantation into cortical bone [121, 129]. A modulus mismatch can result in stress shielding and be detrimental to the tissue surrounding the implant site. Due to its high strength and slow rate of degradation, HA is not ideal for all applications, including when treating young patients or when repairing weight-bearing sites. This scaffold material can remain unchanged for many years after successful functional recovery, even with the incorporation of cells [105]. Tricalcium phosphate has a much faster rate of degradation and has been shown to successfully supported the growth of bone tissue *in vitro* [105]. High total porosity and pore interconnectivity are thought to be necessary scaffold characteristics for use in tissue-engineered bone graft product. [105]. The use of MSCs cultured onto the surface of non-bioactive materials in order to improve upon implantation success has also been explored. This "osteogenic matrix coating" technique is most useful in total joint and femur replacement surgeries, but offers a solution to combat loosening of any metal implant [67].

A major area of concern in both the clinic and the laboratory is the development of a treatment option for large bone defects. If a defect is too large (>2cm) there is often not enough autogenic bone available for harvest. The use of allografts and other bone graft substitutes has not been as successful in these critical size defects, mostly due to inadequate vascular tissue growth [130]. Angiogenesis is a necessary precursor for complete

osteogenesis and must be achieved throughout a bony defect in order to facilitate proper bone repair. Creating tissue-based implants with enough mechanical strength for load bearing applications and improving control of stem cell differentiation are additional areas of focus, necessary to improve treatment options.

1.8 Aim of Research

A successful tissue-engineered construct strives to offer a more desirable alternative to the autologous bone grafts that are currently the preferred choice of surgeons. The incorporation of cells and growth factors within a synthetic bone graft may improve its performance; but an optimal, robust mix of cells, scaffold, and growth factors has yet to be determined [14, 131]. The aim of this research was to develop a biologically active bone graft system based on knowledge of bone cells and their inter-relationships. It is hypothesized that osteoclasts play an important role in the recruitment of mesenchymal stem cells (MSCs) to areas of bone resorption and their presence will have an effect on the differentiation of osteoblasts [38, 132]. The natural pattern of bone formation following bone resorption was the basis for the experimental design. Specifically, the goal for the first set of experiments was to determine how the development of osteoblasts from stromal stem cells was affected by: (1) medium collected from osteoclast cultures and (2) direct coculture with osteoclasts. Additionally, it is believed that the aspirate material obtained from the femoral shaft using the Reamer/Irrigator/Aspirator (RIA) device (Synthes, USA; Paoli, PA) is a potential source of mesenchymal stem cells (MSCs) with osteogenic potential. The reamer aspirate may be an untapped source for autologous progenitor cells that could aid in bone formation when incorporated into bone grafts. Hence the goal of the second set of

experiments was to isolate cells isolated from the fat layer of reamer aspirate and examine them for osteogenic potential, using 2-D and 3-D surfaces. Together, the two sets of experiments provide important information toward the construction of a clinically relevant bone tissue engineering device and provide important information toward the assessment of bone tissue engineering devices in an *in vitro* test system setting.

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CHAPTER TWO

EFFECT OF OSTEOCLAST CONDITIONED MEDIUM ON OSTEOBLAST DIFFERENTIATION FROM STROMAL CELLS

2.1 Background

Bone is a dynamic tissue that is constantly renewing itself in order to maintain its internal structure and mechanical properties [1, 2]. Bone tissue remodeling occurs in response to the internal and external stresses that are applied to the body. Osteocytes, osteoclasts, and osteoblasts are the three cell types that interact as part of more than one million bone remodeling units that are at work within the human skeleton. Of these three, osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) have opposing roles, but work together through an interconnected mechanism referred to as "coupling" to continuously remodel the bone tissue (Figure 2.1). The interaction between these two cell types during the coupling process is responsible for keeping the bone mineral new and healthy [3, 4].

Osteoblasts develop from multi-potent mesenchymal stem cells (MSCs) and have been successfully isolated from various parts of the body including the bone marrow, bone tissue, and adipose tissue [5-7]. Numerous researchers have shown that following isolation, MSCs maintained in a laboratory environment can be appropriately stimulated to successfully proliferate, differentiate, and express genes consistent with the osteoblast phenotype (Figure 1.2). These genes include but are not limited to: alkaline phosphatase, osteocalcin, and bone sialoprotein [8-10]. Ultimately, MSCs cultured *in vitro* are able to secrete mineralized matrix when full maturation is achieved, making them an attractive option to consider for use in

orthopedic clinical devices. These osteoblast precursor cells present a potential means of improving the osteoinductive properties of current bone graft materials.

The incorporation of MSCs into tissue-engineered bone grafts is of great interest for use in orthopedic, dental, and craniofacial applications [6, 8, 11]. Korda and coworkers found that significantly more bone was formed around titanium hip stems implanted into sheep when the stems were stabilized with an allograft/MSC mixture compared to those stabilized with allograft alone [12]. The stem cell-graft mixture resulted in a two-fold increase in bone formation at the hip implant site and the test group experienced better overall hip function after twelve weeks as indicated by greater ground reaction force measurement. MSCs have also been used to successfully treat critical sized bone defects in a rabbit model after being cultured on acellular constructs prior to *in vivo* implantation [13].

As the interest in using stem cells for tissue regeneration increases, a myriad of approaches have been used to optimize their culture conditions. *In vitro* test systems are essential to the development of an optimal tissue-engineered bone graft construct [3, 14]. For bone tissue engineering, the *in vitro* culture of osteoblasts, from stem cells, has been the primary focus. Researchers have attempted to increase mineralized matrix production by differentiating osteoblasts through the use of BMPs, growth factors, and various scaffolds [15-17]. The research presented herein was centered on the physiological remodeling order of events: bone resorption by osteoclasts followed by the deposition of mineralized matrix by osteoblasts (Figure 2.1). These events indicate that the cells responsible for resorbing bone mineral may also possess the ability to recruit and/or induce osteoblast development and activation [2, 4].

Little is known about what factors originating from osteoclasts are responsible for bringing osteoblasts to the site of bone resorption; however, exposure to these factors either directly or indirectly could prove to be beneficial in a cell-based bone graft. Incorporating osteoclast-derived factors into a bone graft system, prior to or at the time of implantation, could lower the amount of time needed for native bone cells to proliferate and differentiate at the defect site [2, 4, 18]. Incorporating osteoclasts, or cytokines collected during their culture, into the system could allow researchers to tailor the rate of differentiation of mesenchymal stem cells within the graft.

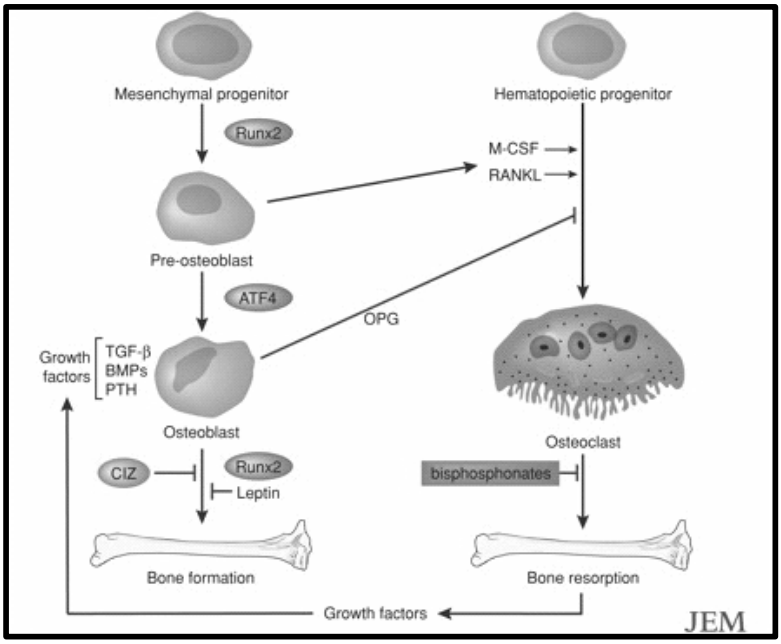

Figure 2.1: Schematic of the interaction between osteoblasts and osteoclasts during the bone remodeling cycle [3].

In the body, cytokines and growth factors released into the blood stream are able to activate surrounding cells; therefore, it is reasonable to believe that these factors are also released into culture medium during *in vitro* cell culture. This 'conditioned medium' theory has been previously used to test interactions between various cell types including: adipocytes and osteoblasts, breast cancer cells and osteoclasts, and calvarial bone cells and osteoclasts [19-21].

Based on this premise, medium collected from osteoclast cultures was incorporated into a culture system for developing bone-forming cells [4, 18]. The objective of this preliminary work was to determine what effect medium taken from differentiating osteoclasts (conditioned medium) had on mesenchymal stem cells being cultured for osteoblast matrix production. Due to the differences in culture conditions, culturing these two cell types separately allowed better characterization of their individual development.

2.2 Methods

Cell Culture & Seeding

All cells were cultured at standard conditions of 37°C and 5% CO_2. Mouse monocytes from the RAW 264.7 cell line (ATCC; Manassas, VA) were seeded into 24-well plates at a density of 3.8 x 10^4 cells per well in α-minimal essential medium (α-MEM; Invitrogen; Carlesbad, CA). RAW 264.7 medium was supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% antibiotic-antimycotic, and 10 ng/ml soluble murine RANKL (Peprotech; Rocky Hill, NJ) to induce osteoclast differentiation. Medium changes were performed every 2-3 days, at which time old medium was collected prior to the addition of new supplemented medium. The collected medium was passed through a 70 micron filter and termed

"conditioned medium". Osteoclast cultures were continuously seeded every 2 days in order to collect fresh conditioned medium from the cells between 2-4 days of culture. The conditioned medium was used immediately, as described below.

Multi-potent mouse marrow stromal cells from the D1 cell line (ATCC) were seeded into 24-well plates at a density of 3.4×10^5 cells per well. Three wells on each plate (OB) were fed 100% α-MEM with osteogenic supplements (10mM β-glycerophosphate and 50ug/ml ascorbic acid; both from Sigma Chemicals; St. Louis, MO). Three additional wells (OC) were fed a mixture of 50% conditioned medium and 50% α-MEM with osteogenic supplements. Medium changes were performed every 2-3 days for the D1 cells over the course of the study.

Days 8, 14, 21, and 26 were selected as the four time points at which to monitor the temporal changes in cell differentiation. At each time point, wells of one plate were rinsed twice with phosphate buffered saline (PBS), and 1.0 ml of distilled water was added to each well. Following addition of water, plates were placed in a -80°C freezer for storage until the conclusion of the study. Quantitative assays were performed on all samples simultaneously after three freeze/thaw cycles.

Qualitative Microscopy

Phase contrast microscopic images were taken of the cells throughout the study prior to medium changes. An Axiovert 135 inverted microscope (Zeiss; Thornwood, NY) fitted with an insight color digital camera was used to capture images using SPOT image acquisition software.

Metabolic Activity

Metabolic activity of the cells was assessed on samples using alamar Blue ™ dye (Biosource International; Camarillo, CA) on Days 8, 14, 21, and 26. Prior to medium change on the specified days, 200 µl of Alamar Blue dye (10% of total medium volume) was added to each well of the Day 26 plate. After gentle mixing, plates were returned to the incubator for a period of 3 hours, during which time the dye was reduced by metabolically active cells to produce a color change from a non-fluorescent blue to shades of fluorescent pink. Following incubation, 200 µl aliquots of media were transferred in triplicate to a black wall, clear bottom 96-well plate (Corning; Corning, NY). Fluorescence values were read using excitation and emission filter values of 544 nm and 590 nm, respectively, on a Fluoroskan Ascent FL fluorometric plate reader (Labsystems; Franklin, MA).

Intracellular Protein Content

The amount of total intracellular protein present in each well was determined using a BCA protein assay kit (Pierce Biotechnology; Rockford, IL). This protein assay produces a color change when bicinchoninic acid (BCA) molecules chelate with Cu^{1+} molecules that result from the reduction of Cu^{2+} ions by protein present in test samples. The resulting purple-colored solution absorbs light at a wavelength of 562 nm. An albumin standard was diluted according to the manufacturer's procedure to create a standard curve. A volume of 25 µl of cell lysate was transferred in triplicate from each test sample to a 96-well plate (Corning). A volume of 200µl of working reagent was added to each well of the 96-well plate. All plates were gently shaken on an orbital shaker and incubated for 30 minutes at

37°C. Absorbance values were read at 562 nm on a MRX Revelation microplate reader and protein values were extrapolated based on the standard curve.

Alkaline Phosphatase Synthesis

An alkaline phosphatase (ALP) assay (Sigma Chemicals; St. Louis, MO) was conducted on samples at the conclusion of the study. A volume of 100 μl of cell lysate from each test sample was transferred to a clean plate containing 500 μl of working reagent, made up of equal parts p-nitrophenyl phosphate and alkaline phosphatase buffer. All plates were incubated at 37°C for 30 minutes, at which time 0.25N NaOH was added to each well to stop the reaction. Samples and standards were transferred in triplicate from each well of the incubated plates for testing within 96-well plates. Absorbance was read at 410nm on a MRX Revelation microplate reader (Dynex Technologies; Chantilly, VA). Reported ALP values were normalized with corresponding intracellular protein values.

TRAP Staining

Two methods were used to stain for tartrate-resistant acidic phosphatase (TRAP) activity by mature osteoclasts. The widely used Sigma-Aldrich TRAP staining results in a dark purple color when TRAP is present in or around a cell (386-A; Sigma). The recommended manufacturer procedure was used for RAW 264.7 cell cultures. The traditional TRAP color change was visible using light microscopy. A second fluorescent-based stain using ELF97 phosphatase substrate (Molecular Probes; Eugene, OR) was combined with a fluorescent nuclear DNA stain, DAPI (1 μg/ml in PBS, 4',6-diamidine-2'-phenylindole dihydrochloride;

Roche Diagnostics; Mannheim, Germany), to visualize TRAP activity and cell nuclei simultaneously. The second method was visible using fluorescence microscopy.

Statistical Analysis

Each data point represents the mean value of n=3 data samples, and error bars denote the standard error of the mean (SEM). The statistical software SAS® was used to compare the effects of medium type and time on the metabolic activity, intracellular protein content, and alkaline phosphatase activity of the mesenchymal stem cells using the Least Squares Means (LSMEANS) command. A significance level of $p < 0.05$ was used for all comparisons.

2.3 Results

Osteoclast Culture

The osteoclast cells were visually observed over the course of the culture period. RAW 264.7 mouse monocytes seeded in 24-well plates proliferated and fused to form large clusters of cells. The observed clusters of cells formed from cultures of RAW 264.7 exposed to soluble RANKL were consistent with reports of multi-nucleated cells that are expected for mature osteoclasts (Figure 2.2) [22, 23]. Both staining methods used for identifying TRAP positive cells confirmed that TRAP expressive cells were present in the RAW 264.7 cultures used in this study for collecting conditioned medium (Figure 2.3).

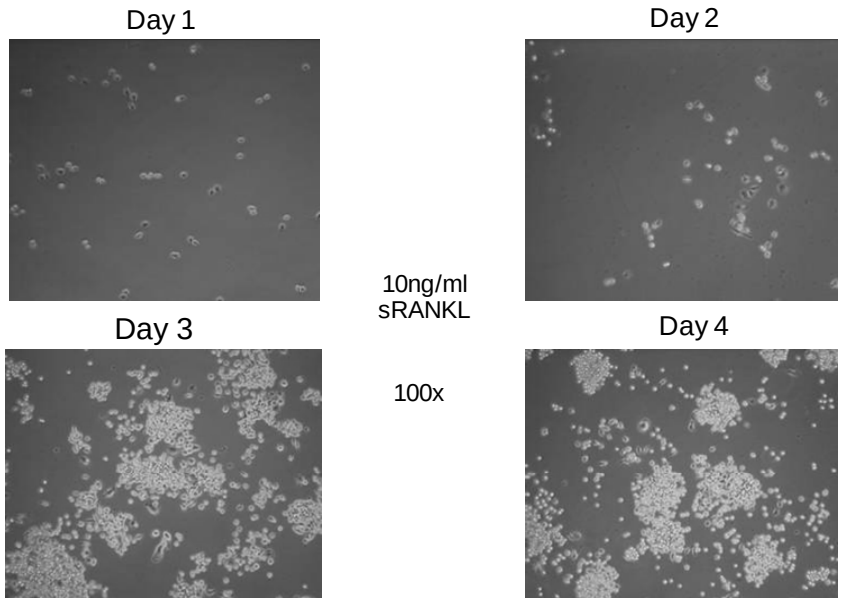

Figure 2.2: RAW 264.7 mouse monocytes in culture medium containing RANKL (10 ng/ml) fused to become multi-nucleated cells. Total magnification for all images is 100x.

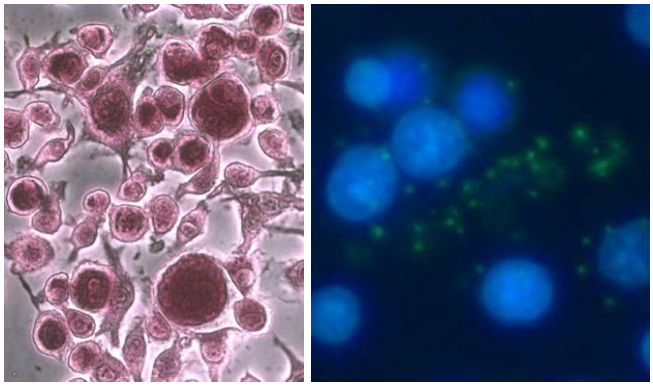

Figure 2.3: Left: Traditional TRAP kit stain produced dark purple areas where TRAP was present surrounding fused cell nuclei. Right: DAPI (blue) stained cell nuclei surrounded by TRAP positive (green) fluorescent ELF-97 stain. Total magnification for all images is 320x.

Conditioned Medium Study

The D1 multipotent cells from both medium test groups looked similar to fibroblasts at Day 2 (Figure 1.4: A.1, B.1). By Day 8, the morphology of the OC-fed cells began to change to a more rounded shape, while the OB fed cells acquired a tightly packed, polygonal shape more consistent with differentiating bone cells (Figure 2.4: A.2, B.2). There were marked differences in morphology at Day 26; the cells of the OC fed group were less dense and included cells with both spindle and rounded morphology, while the OB-fed group had a more uniform, polygonal appearance (Figure 2.4: A.4, B.4).

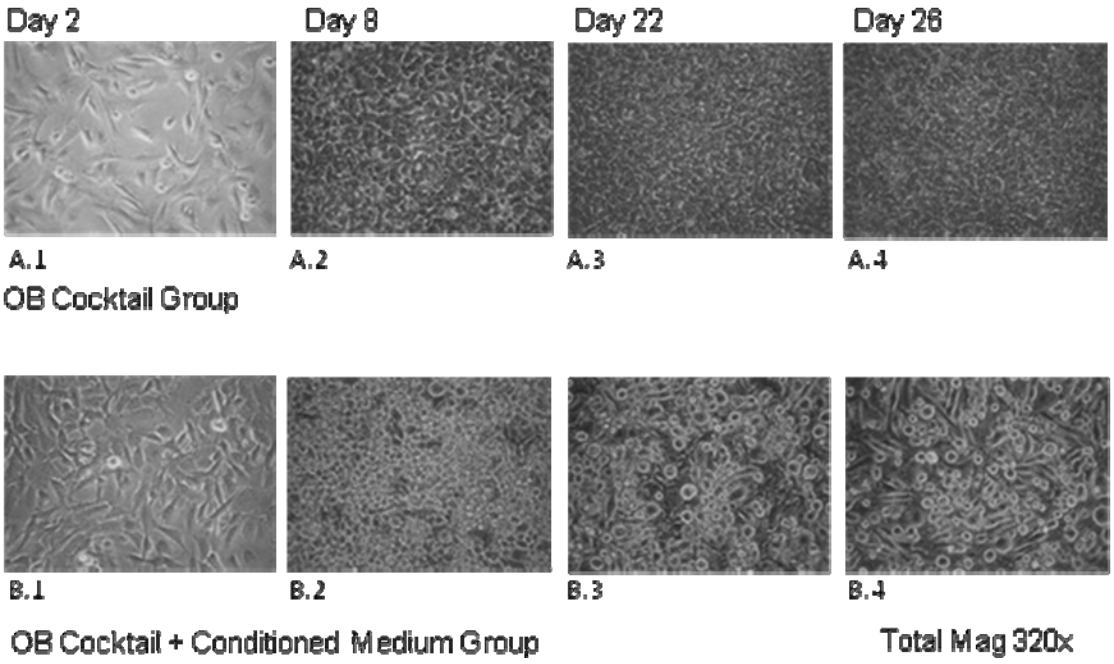

Figure 2.4: Morphology of cells grown in 100% osteogenic medium (Top, A.1-4). Cells grown in osteogenic medium containing 50% osteoclast conditioned medium (Bottom, B.1-4). Total magnification for all images = 320x.

Quantitative Assay Results

Metabolic Activity

The metabolic activity of the stromal cells appeared to be unaffected by the type of medium they were fed (Figure 2.5). There was no significant difference measured between the cells fed 100% osteogenic medium (OB) and the cells fed osteogenic medium with 50% osteoclast conditioned medium (OC) at any time point. Both medium groups exhibited significantly higher levels ($p < 0.05$) of metabolic activity at Day 14 compared to Days 8 and 26.

Intracellular Protein Content

The cells grown in 50% conditioned medium (OC) contained a significantly ($p < 0.05$) higher amount of intracellular protein on Days 8, 14, and 26 compared to cells grown in osteoblast cocktail (OB) alone (Figure 2.6). Both medium groups exhibited a peak in intracellular protein levels at Day 14, which was followed by a significant drop in protein production at Day 21.

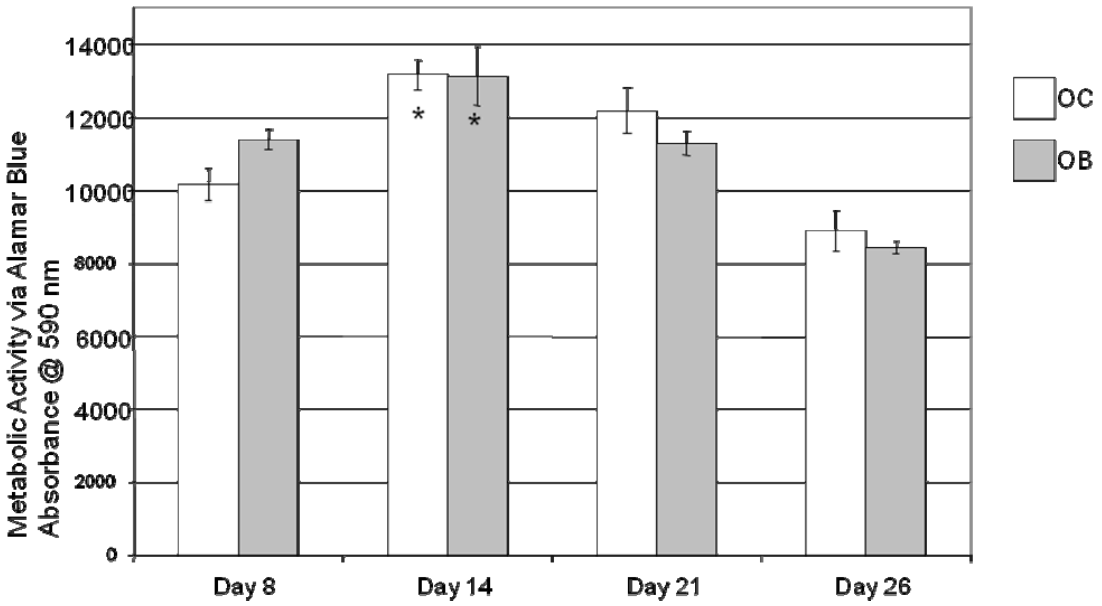

Figure 2.5: Metabolic activity levels as a function of time and medium type. Asterisks (*) denote significantly greater metabolic activity measured for cells at Day 14 ($p < 0.05$) compared to Days 8 and 26. Each data point represents the mean value of n=3 data samples, and error bars denote standard error of the mean (SEM).

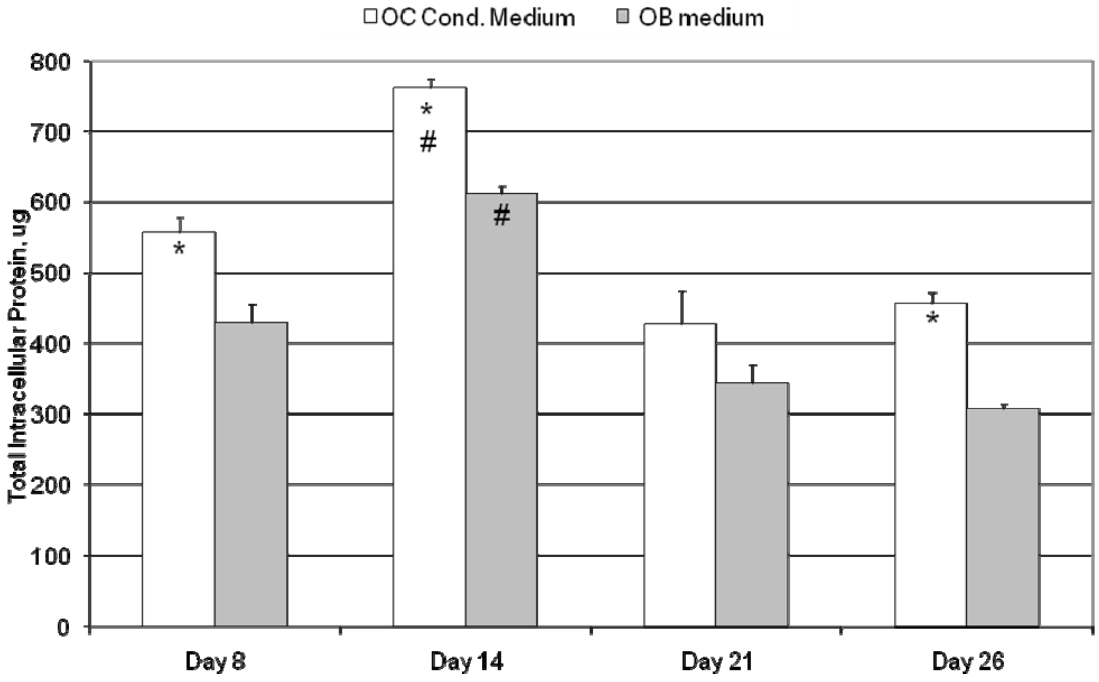

Figure 2.6: Intracellular protein levels as a function of time and type of medium. Asterisks (*) denote significantly higher levels of intracellular protein present in conditioned medium samples compared to samples grown in osteogenic cocktail. Pound signs (#) denote a significant peak in intracellular protein level that occurred at Day 14. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Alkaline Phosphatase Synthesis

Cells grown in OB medium had significantly ($p < 0.05$) higher levels of ALP synthesis compared to the cells grown in conditioned medium at each of the four time points (Figure 2.7). A peak value of ALP was observed at Day 21 in the OB test group.

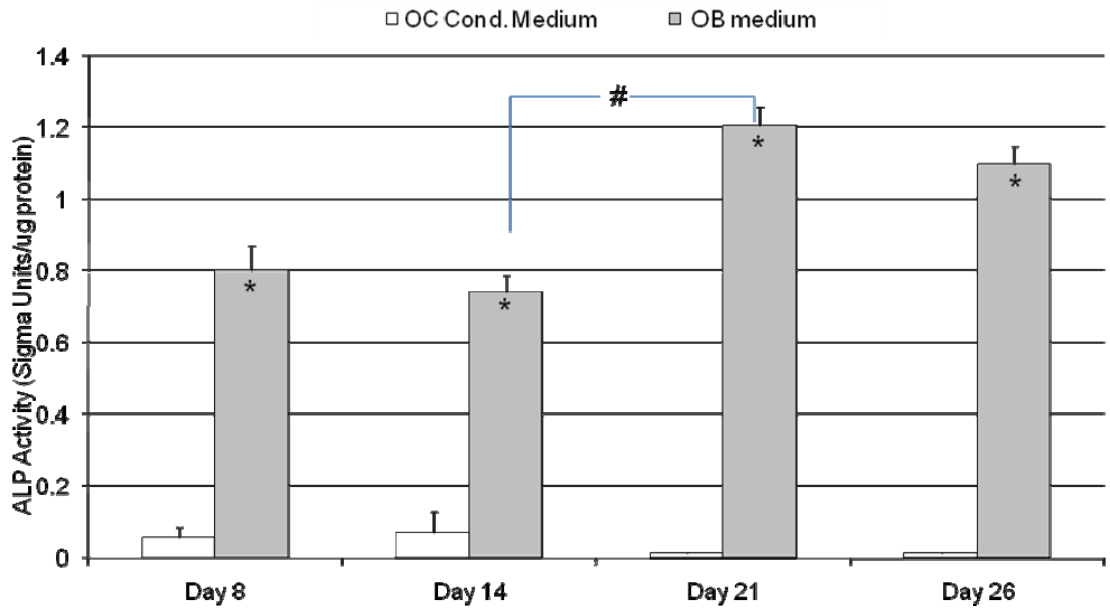

Figure 2.7: Alkaline phosphatase synthesis as a function of time and medium type. Reported values were normalized to intracellular protein levels. Asterisks (*) denote significant differences in ALP levels with respect to medium type ($p < 0.05$). Pound signs (#) denote significant increases in ALP measured between Day 14 and Day 21 ($p < 0.05$). Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

2.4 Discussion

The discovery of the RANK/RANKL pathway paved the way for successful *in vitro* cultures of osteoclasts [24, 25]. An *in vitro* culture method for osteoclasts was tested for its ability to produce multinucleated, TRAP positive cells from a murine monocytes cell line. Previous *in vitro* work involving osteoclast cultures has been conducted by a number of

groups using the murine monocytes cell line RAW 264.7 [26-28]. Following a review of previous two-dimensional culture techniques, the murine cell line seeded at a density of 3.8 x 10^4 cells per well over a maximum of four days was deemed appropriate for use in this conditioned medium study [23, 29, 30]. Soluble RANKL was employed to direct these mononuclear cells to form multinucleated, mature (TRAP positive) osteoclasts. Morphological examination suggested that the cells did achieve this fusion of nuclei as described by Vincent and colleagues [23, 29, 30]. A traditional TRAP stain verified that this osteoclast marker was expressed by the cells and this finding was confirmed using an additional fluorescent TRAP stain [31]. Thus, this culture method could be used with confidence for collecting conditioned medium and in future cellular studies involving osteoclasts.

The term stromal cell is often used interchangeably with mesenchymal stem cell (MSC) when describing the multi-potent cell type that was used in this study. The murine MSCs from the current study were characterized over a 26 day period while being cultured in two different types of medium: 100% osteogenic medium and osteogenic medium mixed with 50 % osteoclast conditioned medium. Qualitative cell morphology images suggested that the differentiation of the MSCs into osteoblasts was inhibited by the presence of osteoclast conditioned medium. Osteoblasts change shape as their function changes; growing larger and more cubical as their level of metabolic activity increases [32, 33]. MSCs cultured in conditioned medium lacked the polygonal morphology and the tight packing behavior that is typically described for differentiated osteoblasts on two-dimensional surfaces. The control group had morphological traits that were much more consistent with what is described for osteoblasts. Results of the metabolic activity assay were consistent with the morphological

changes seen between Days 8 and 26, in that cells had a peak in activity at Day 14. This activity level would be expected to decrease as cells experience a lag in growth size or proliferation. Cells in the conditioned medium group appeared to be fewer in number and smaller in size compared to the control at Day 21. The alamarBlue™ assay can also indicate changes in cell viability, as well as growth, and the two groups may have had different factors contributing to the drop in activity at the later time point.

Osteoblast differentiation has been divided into three distinct phases that begin with active proliferation followed by matrix maturation [8, 34]. An intracellular protein assay performed on test samples indicated that cells grew successfully in each medium type, and reached a peak level of protein at Day 14 regardless of culture conditions. Researchers have shown that a direct correlation can exist between the concentration of total protein and cell proliferation, and the significantly higher levels of total protein recorded for the conditioned medium group may suggest that this group had a greater level of proliferation over the course of the study [35]. A higher level of protein could also be attributed to the lack of cellular spreading and tight packing within the wells, leaving a greater amount of room for cells to proliferate than was available within wells of the control group.

Results of the ALP assay supported this pattern of differentiation. This marker of matrix maturation was present in significantly lower amounts in cultures with the highest levels of total protein. The presence of this surface enzyme, which is expected to be highest between Days 15-28 in stromal cells differentiating into osteoblast cells, suggested that cells grown in 100% osteogenic medium were capable of differentiating along an osteogenic pathway, while cells exposed to conditioned medium did not transition past the initial proliferation phase of differentiation [36, 37].

2.5 Conclusions

The results of this preliminary study demonstrated that we can successfully culture TRAP positive osteoclast-like cells *in vitro* for use in osteoclast conditioned medium studies. Optimal osteoclast culture techniques need to be further examined, including the possible addition of other osteoclastogenic growth factors to promote differentiation [24, 38, 39]. Also, osteoclasts are responsible for resorbing mineralized bone, so culturing these cells on a mineral based material may have a positive effect on their TRAP activity levels *in vitro*.

Significant differences in biochemical markers were observed between stromal cells cultured with and without the addition of osteoclast conditioned medium. Coupled with the distinct dissimilarity in morphological appearance of each cell group, the results of this study suggested that stromal cells exposed to conditioned medium collected under these *in vitro* conditions were inhibited from transitioning along the osteogenic pathway. These results may contribute to the development of medium that could be used to control the differentiation of stromal cells. The ideal combination of cells to include in a cellular-based bone graft is still to be determined and it may be advantageous to include cells at different stages of differentiation [40, 41].

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CHAPTER THREE

THE EFFECT OF OSTEOCLAST ACTIVITY ON THE DIFFERENTIATION OF OSTEOBLASTS FROM STROMAL CELLS

3.1 Background

The link between osteoclastogenesis and osteoblast precursor cells was first observed in a co-culture system of bone marrow hematopoietic stem cells (HSCs) with stromal cells [1, 2]. Most co-culture experimental setups promote cell-to-cell contact between these two cell types, based on the belief that this contact is essential to osteoclastogenesis. Although some studies have indicated that membrane-bound RANKL is more efficient than soluble RANKL in inducing differentiation of osteoclasts, the process of osteoclast maturation has been successfully replicated *in vitro* using a variety of experimental setups [3, 4]. Prior to 1997, work involving osteoclast culture used vitamin D_3 (1α, 25-dihydroxyvitamin D_3) as the main medium supplement to induce the formation of multinucleated TRAP positive cells from monocytes or macrophages [5-7]. The discovery of the RANK-RANKL relationship resulted in a major shift to include soluble RANKL as a major medium supplement to induce osteoclastogenesis [8]. This protein is commonly used in conjunction with other systemic hormones and growth factors such as vitamin D_3 and macrophage colony-stimulating factor (M-CSF); however, no standards presently exist for culturing osteoclasts *in vitro*. The lack of established standards results in a myriad of approaches to culturing osteoclasts, based on various parameters including cost and culture time [2, 9-11].

Osteoclast development from HSCs into multinucleated, mature, TRAP-expressing cells has been examined in detail with regard to what turns these bone resorbing cells 'on' and 'off'. Much of this work has been focused on the development of drugs to treat bone disorders that employ specific soluble factors to regulate osteoclastogenesis. One significant discovery, resulting from an *in vitro* osteoclast culture system, was the identification of a decoy receptor for RANKL, known as osteoprotegrin (OPG) [9, 12, 13]. By interfering with the RANK/RANKL binding, OPG can decrease the activation of osteoclasts, resulting in a decrease in overall bone loss. This mechanism is being explored for the treatment of patients with imbalanced rates of bone formation and resorption.

Due to the focus on regulating bone loss, less work has focused on what effect osteoclasts have on developing osteoblasts. It is well established that bone formation by osteoblasts follows closely behind bone resorption by osteoclasts, but a number of questions remain regarding how mature or differentiating osteoblasts are recruited to the resorption site. Pederson and coworkers described both human and animal model studies that led to the determination that the presence of osteoclasts is essential for normal bone formation, regardless of the level of activity of the osteoclasts. When normal osteoclast are present, but unable to resorb, there was no loss in bone formation seen in human models; however when mice were bred to lack osteoclasts, bone formation was negatively affected [14].

Any effects that osteoclasts have on bone formation may be dependent upon the phase of osteoclast differentiation. Perez-Amodio and Evert reported that when precursors of osteoclasts were seeded in direct contact with osteoblasts, the result was a retraction of the bone forming cells from the growth surface [15]. It was also reported that osteogenic parameters may be affected by the age of osteoclast cultures when bone marrow stromal

were studied in a co-culture model [16]. Preliminary findings from our lab indicated that conditioned medium collected from osteoclasts between 2-4 days of culture inhibited the differentiation of osteoblasts.

The goal of the work presented here was to determine if osteoclast maturation could be divided into distinct phases of differentiation. The development of osteoclasts was examined using a murine monocytes cell line cultured in medium containing either RANKL (20 ng/ml) or RANKL combined with M-CSF (5 ng/ml). The monocytes were cultured on both polystyrene tissue culture plastic and on β-tricalcium phosphate ceramic granules to determine if the growth surface had any impact on cellular activity or TRAP activity. Following the establishment of phases of osteoclast differentiation, a follow-up study was performed to determine what effect conditioned medium taken from each phase would have on the differentiation of osteoblasts from mesenchymal stem cells.

3.2 Methods

Osteoclast Cell Culture & Seeding

Mouse monocytes from the RAW 264.7 cell line (ATCC; Manassas, VA) were maintained at standard culture conditions of 37°C and 5% CO_2. The RAW 264.7 cells were seeded at density of 3.8 x 10^4 cells per well directly onto 24-well plate tissue culture plastic or onto ceramic granules contained within the wells. Osteoclast cultures were tested in three different medium types: plain α-MEM, α-MEM/RANKL (20 ng/ml), and α-MEM/RANKL (20 ng/ml)/M-CSF (5ng/ml). All medium types were supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Medium changes were performed every 2 days.

For the conditioned medium study, RAW 264.7 cells were seeded onto 75 cm^2 tissue culture flasks and maintained in α-MEM supplemented with 20 ng/ml of RANKL. The cells were seeded as needed to ensure cultures were available from all three phases of differentiation for collecting conditioned medium. Conditioned medium was collected from the flasks, filtered to remove any cell debris, and used immediately for feeding osteoblast cultures, as described below.

MouseTRAP ™ Assay

The MouseTRAP Assay (Immunodiagnostic Systems Inc.; Fountain Hills, AZ) was used to measure tartrate-resistant acid phosphatase 5b (TRAP 5b) activity from osteoclast culture medium. TRAP 5b is derived specifically from osteoclasts and can be used as a quantitative indicator of active osteoclast *in vitro*. Medium samples of 500 µl volume were taken from each test well every 2 days and immediately stored at -80°C until the conclusion of the study. The assay was performed according to the manufacturer's instructions following thawing of the samples. Test samples, standards and controls were incubated within an antibody coated plate and any bound TRAP 5b produced a color change in the assay substrate. Absorbance of test samples and controls were detected at 405nm and compared to a standard curve.

Osteoblast Cell Culture & Seeding

Multi-potent mouse marrow stromal cells from the D1 cell line (ATCC) were seeded in triplicate (n=3) onto 24-well tissue culture plates at a density of 3.4 x 10^5 cells per well. Three wells on each plate (OB) were fed 100% α-MEM with osteogenic supplements (10mM β-glycerophosphate and 50ug/ml ascorbic acid, both from Sigma). Conditioned medium

from each osteoclast phase was added to test groups in triplicate. Cells in conditioned medium wells were fed a mixture of 50% α-MEM with osteogenic supplements and 50% conditioned medium from either phase 1, 2 or 3 (P1-P3). Medium changes were performed every 2-3 days for the D1 cells over the course of the study.

Days 7, 13, and 21 were selected as the three time points at which the temporal changes in cell differentiation were monitored. At each time point, wells of one plate were rinsed twice with phosphate buffered saline (PBS) and 1.0 ml of distilled water was added to each well. Following addition of water, plates were placed in -80°C freezer for storage until the conclusion of the study. Quantitative assays were performed on all samples simultaneously after three freeze/thaw cycles.

Metabolic Activity Assay

Metabolic activity of the cells was assessed using the alamar Blue ™ dye (Biosource International; Camarillo, CA) at each media change for the osteoclast cultures. During the conditioned medium study, this assay was performed on Days 7, 14, and 21. Prior to medium change on the specified days, 200 μl of Blue dye (10% of total medium volume) was added to each well of a designated plate. After gentle mixing, plates were returned to the incubator for a period of 3 hours, during which time the dye was reduced by metabolically active cells to produce a color change from non-fluorescent blue to shades of fluorescent pink. Following incubation, 200 μl aliquots of media were transferred in triplicate to a black wall, clear bottom 96-well plate (Corning). Fluorescence values were read at excitation and emission filter values of 544 nm and 590 nm, respectively, on a Fluoroskan Ascent FL fluorometric plate reader (Labsystems; Franklin, MA).

Intracellular Protein Content

The amount of total intracellular protein present in each well was determined using a BCA protein assay kit (Pierce Biotechnology; Rockford, IL). This protein assay produces a color change when bicinchoninic acid (BCA) molecules chelate with Cu^{1+} molecules that result from the reduction of Cu^{2+} ions by protein present in test samples. The resulting purple-colored solution absorbs light at a wavelength of 562 nm. An albumin standard was diluted according to manufacturer's procedure in order to create a standard curve. A volume of 25 µl of cell lysate was taken from each well for testing within a 96-well plate (Corning). A volume of 200µl of working reagent was added to each test sample; plates were gently shaken on an orbital shaker and incubated for 30 minutes at 37°C. Absorbance values were read at 562 nm on a MRX Revelation microplate reader and protein values were extrapolated based on values produced by the standard curve.

Alkaline Phosphatase Assay

An Alkaline Phosphatase (ALP; Sigma) assay was conducted on samples at the conclusion of the study. A volume of 100 µl of cell lysate from each sample well was transferred to a clean plate containing 500 µl working reagent made up of equal parts p-nitrophenyl phosphate and alkaline phosphatase buffer. Plates were incubated at 37°C for 30 minutes, at which time 0.25N NaOH was added to each well to stop the reaction. Samples and standards were transferred in triplicate from each well of the incubated plates for testing within 96-well plates. Absorbance values were read at 410nm on a MRX Revelation microplate reader.

Calcium Deposition Assay

Calcium deposition was evaluated for the D1 stromal cells during the conditioned medium study according to the protocol described by Lonza Walkersville, Inc. (Lonza; Walkersville, MD) [17]. On Days 7, 14, and 21 all culture medium was aspirated and wells were washed with PBS. Following washing, 0.5 ml of 0.5N HCl was added to each well. Cells were scraped from the bottom of the well plate and samples were transferred to an Eppendorf tube for storage. An additional 0.5 ml of HCL was added to each well for rinsing, and the cellular rinse was added to the storage tubes to ensure all cells were collected. Samples were stored at -20^{o}C until the conclusion of the study. Prior to performing the assay, samples were thawed on an orbital shaker for 6-12 hours at 4^{o}C. After shaking, the tubes were centrifuged at 500g for 2 minutes and supernatant with extracted calcium was collected and transferred to a new tube. Using instructions from the Stanbio LiquiColor^{R} Calcio Total Procedure No 0150 (Stanbio Laboratory; Borne, TX), a standard curve was prepared and used to determine the amount of calcium deposition present in each sample.

Statistical Analysis

Each data point represents the mean value of n=3 data samples, and error bars denote the standard error of the mean (SEM). The statistical software SAS® was used to compare test groups using the Least Squares Means (LSMEANS) command. A significance level of $p < 0.05$ was used for all comparisons.

3.3 Results

Osteoclast Culture

Metabolic Activity on Tissue Culture Plastic

The Blue ™ assay revealed that the metabolic activity of RAW 264.7 monocytes cultured on tissue culture plastic appeared to be unaffected by the type of medium they were fed. There was no significant difference measured between the test groups at any time point (Figure 3.1). All medium groups exhibited similar levels of activity throughout the study. After ten days of incubation all three groups expressed a significant peak in activity, which was followed by a decline at Day 12. The presence of RANKL with or without M-CSF did not have an effect on the metabolic activity of the cells when compared to the plain medium.

Metabolic Activity on Ceramic Granules

When cultured on ceramic granules, the RAW 264.7 monocytes had lower levels of metabolic activity throughout the study compared to those grown on tissue culture plastic (Figure 3.2). The results of this assay, for the cells grown on ceramic, displayed considerable similarities to the results from the cells grown on tissue culture plastic, despite the overall lower levels of absorbance. There were no significant differences in activity observed between the types of medium. Furthermore, these cultures also experienced a significant drop in cellular metabolism between Day 10 and Day 12.

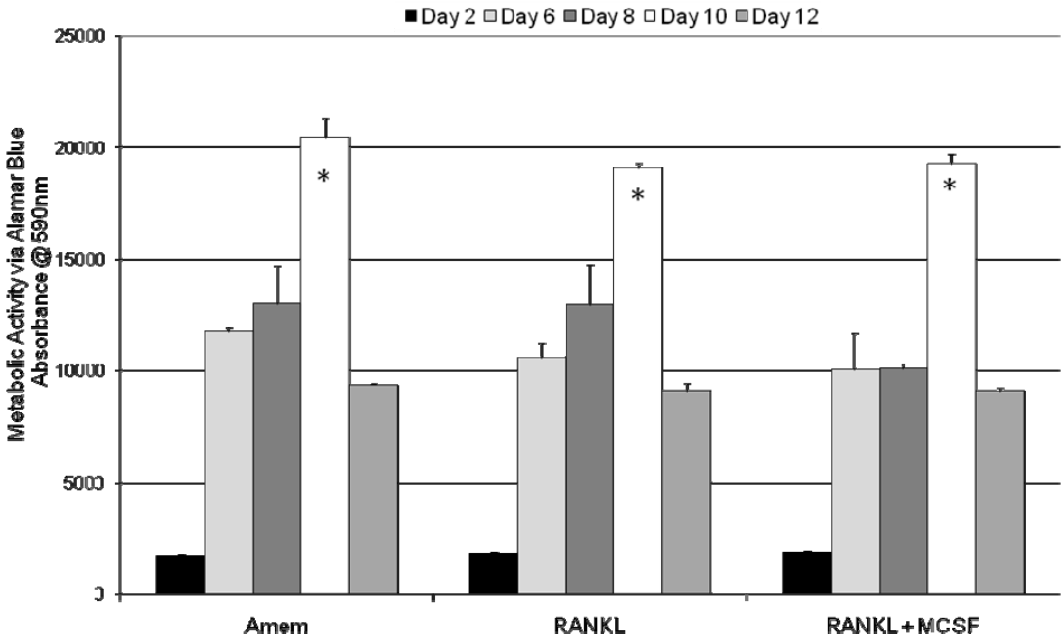

Figure 3.1: Metabolic activity of osteoclasts cultured on tissue culture plastic as a function of time and medium type. Asterisks (*) denote significant peaks ($p < 0.05$) in metabolic activity measured at Day 10 for cells grown in all three types of medium. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

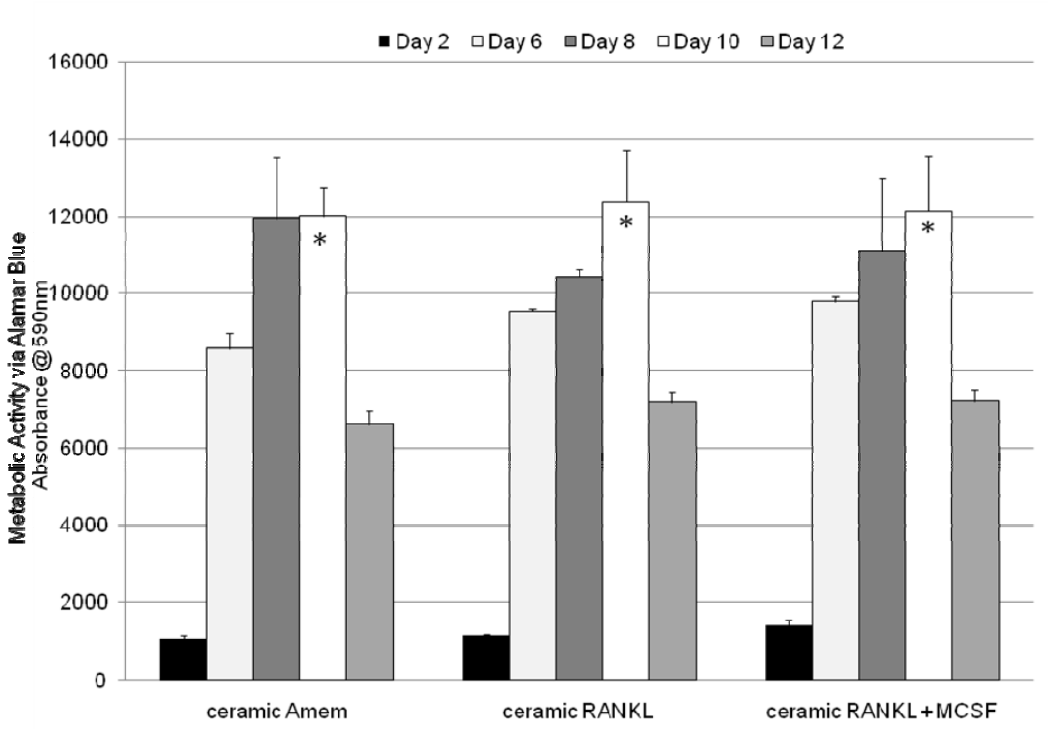

Figure 3.2: Metabolic activity of differentiating osteoclasts cultured on ceramic granules as a function of time and medium type. Asterisks (*) denote significantly greater values ($p < 0.05$) measured at Day 10 compared to Day 12. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

TRAP 5b Activity

The MouseTRAP assay allows users to quantify the amount of TRAP 5b being synthesized by differentiating osteoclasts during their *in vitro* culture. The RAW 264.7 cells were able to successfully synthesize TRAP 5b (Figure 3.3). There were no significant differences in TRAP 5b levels between medium groups, and there was a large amount of variability seen in all culture groups. Cells cultured in RANKL appeared to have three distinguishable phases based on metabolic activity and TRAP activity, regardless of growth

surface. These phases were identified as: Days 2-4 (Phase1), Days 5-8 (Phase 2), and Days 9-12 (Phase 3).

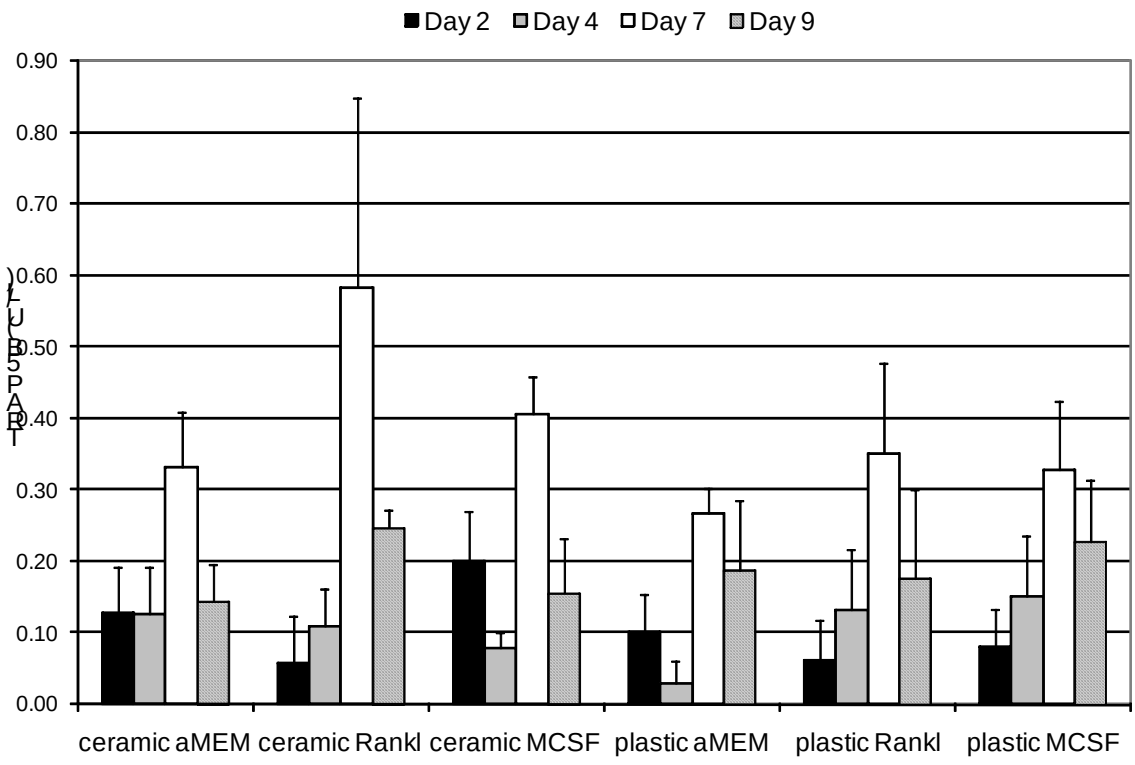

Figure 3.3: Tartrate-resistant acid phosphatase 5b (TRAP 5b) activity by murine monocytes as a function of time and growth medium. TRAP activity was measured for cells grown on ceramic granules and tissue culture plastic. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Conditioned Medium Study

Metabolic Activity

The metabolic activity of the D1 cells appeared to be unaffected by the type of medium they were fed during the initial week of culture, as there was no significant difference measured between the groups at this time point (Figure 3.4). After thirteen days in culture, all of the medium groups exhibited a significant increase in cellular metabolism when compared to the initial time point. Both the plain medium (Plain) and osteogenic medium (OB) groups had slightly higher levels of metabolic activity than the groups fed conditioned medium at the middle time point.

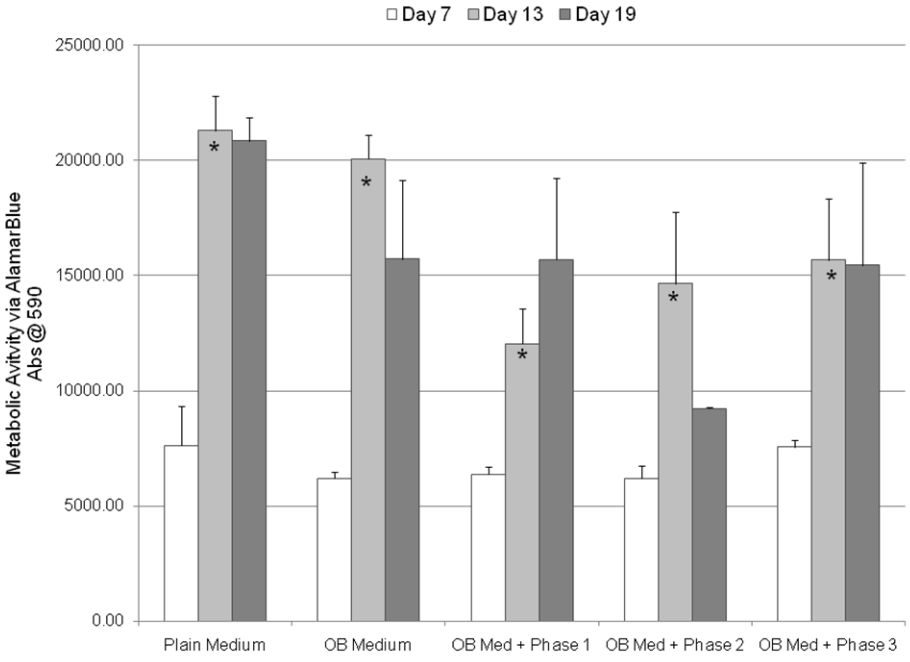

Figure 3.4: Metabolic activity of D1 stromal cells as a function of time and medium type. Asterisks (*) denote significantly greater values ($p < 0.05$) measured at Day 13 compared to Day 7. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Intracellular Protein Content

The result of the BCA total protein assay (Figure 3.5) revealed that murine stromal cells fed conditioned medium combined with osteogenic differentiation medium had significantly higher levels of intracellular protein after thirteen days of incubation compared to groups not fed conditioned medium. This peak in intracellular protein was followed by a significant decrease at the final time point, Day 19, regardless of what phase of osteoclast differentiation from which the conditioned medium was collected.

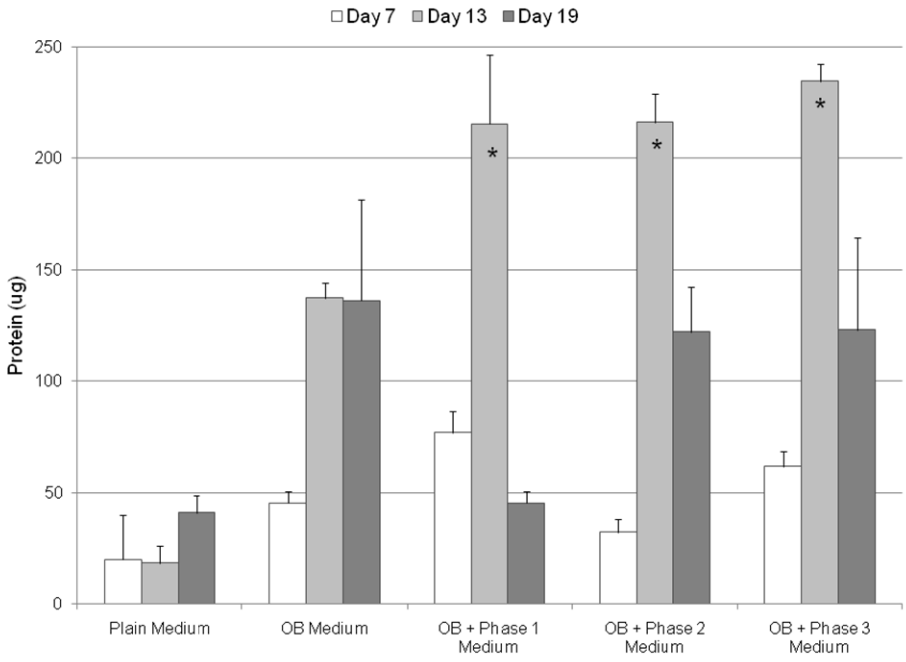

Figure 3.5: Total intracellular protein levels measured for D1 stromal cells as a function of time and medium type. Cultures fed conditioned medium from osteoclasts had significantly (*, $p < 0.05$) higher levels of protein at Day 13 compared to groups not fed conditioned medium. There was no significance difference observed between the three conditioned medium groups. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) measurements were taken from plates frozen on Days 7, 13, and 19 to assess osteoblast differentiation (Figure 3.6). The ALP levels appeared to be hindered by the addition of conditioned medium to the osteogenic differentiation medium. The results of this assay revealed that the D1 stromal cells cultured in 100% osteogenic differentiation medium (OB) expressed significantly greater levels of ALP than either the plain medium control group or the groups fed osteoclast conditioned medium. An increase in ALP was observed in the OB medium group over time.

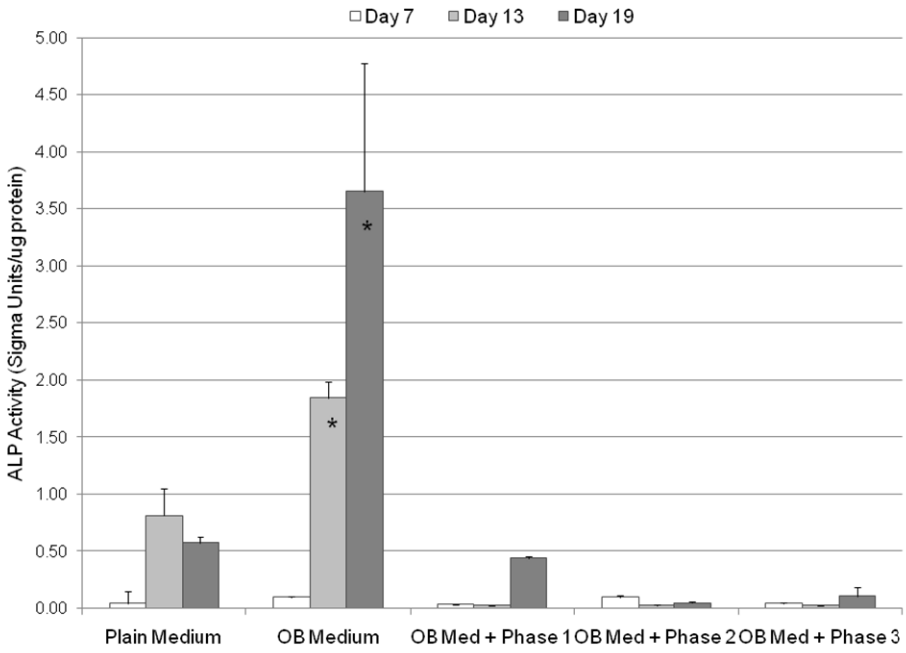

Figure 3.6: Alkaline phosphatase synthesized by D1 murine stromal cells as a function of time and medium type. Asterisks (*) denote significantly higher levels of ALP synthesized observed in the 100% osteogenic medium group at Days 13 and 19 ($p < 0.05$). Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Calcium Deposition

Calcium deposition levels (Figure 3.7) were not significant until Day 19 of the study, at which point the cells cultured in 100% osteogenic medium, Phase 1 conditioned medium, and Phase 2 conditioned medium had significantly greater amounts compared to the plain medium control group. The cells tested in 100% OB medium had significantly higher levels of calcium deposition compared to all other groups at Day 19.

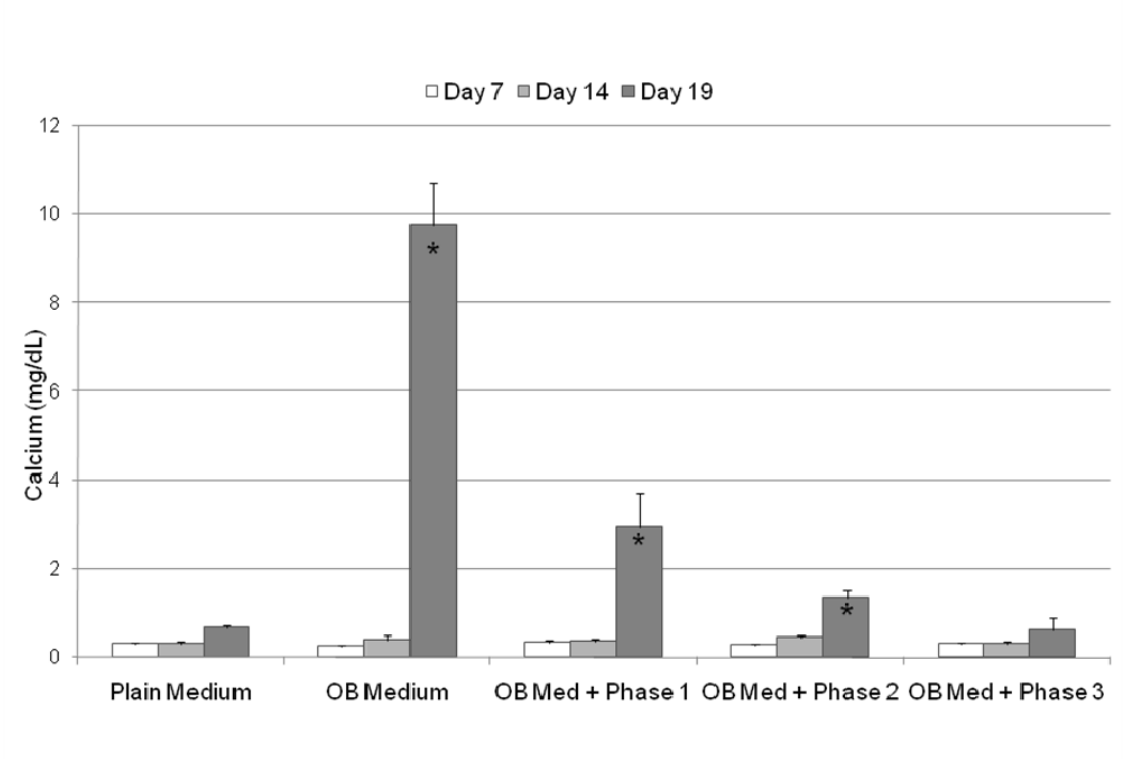

Figure 3.7: Calcium deposition by D1 stromal cells as a function of time and medium type. Asterisks (*) denotes significantly greater ($p < 0.05$) amounts of calcium recorded in OB, Phase 1 CM, and Phase 2 CM groups compared to the plain medium control group on Day 19. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

3.4 Discussion

Medium Supplements & Phases of Osteoclast Differentiation

The lack of standards for cell culture techniques requires each research group to determine the most effective way to perform cellular analysis. There are vast numbers of protocols described in the literature for *in vitro* culture of osteoclasts, many of which employ the use of RAW 264.7 murine monocytes cell line. This cell line has been used extensively in osteoclast studies, though each group has used a different set of parameters to induce differentiation [18]. Sighting the wide range of culture conditions described in the literature, Wittrant and coworkers performed studies to examine the best seeding density for RAW 264.7 cells to examine the biological activities of RANKL and OPG during osteoclastogenesis. They found that a seeding density of 10^4 cells/cm^2 was more appropriate than higher densities for this cell line and was chosen as the seeding density for the work presented here [19]. Another important parameter that varies greatly within the literature is the concentration of RANKL used in osteoclast medium. Concentrations between 5-200 ng/ml have been used, with little explanation given as to why a specific amount was chosen. Okamatsu and colleagues used a low concentration, 10 ng/ml, with success in studies of RAW 264.7 cells, exploring MIP-1γ mRNA expression [20]. The Okamatsu group stated economy as the basis for choosing this concentration, while others have noted that TRAP activity of RAW 264.7 cells increased in a dose-dependent manner [18, 21]. Based on the success of the Okamatsu studies and TRAP staining results obtained during earlier laboratory tests using a minimal amount of RANKL, 20 ng/ml was determined to be an appropriate amount of RANKL to use for characterizing osteoclastogenesis over a twelve day period at a density of 3.8 x 10^4 cells per well for the work described here.

A MouseTRAP enzyme-linked immunosorbent assay (ELISA) was used in conjunction with a metabolic activity assay to characterize osteoclastogenesis using RAW 264.7 cells cultured in the presence of 20 ng/ml of RANKL. TRAP 5b is a form of TRAP that is unique to osteoclasts and its activity was measured within samples of medium taken throughout the course of the study. This bone resorption marker has been used in animal studies as an indicator of local and systemic bone turnover. Lloyd and coworkers found that mice injected with soluble RANKL had increased serum levels of TRAP 5b, with measurement taken using the same MouseTRAP ELISA [22]. These TRAP levels were accompanied by decreases in bone volume, strength, and density. The relationship between TRAP serum levels and bone loss in the mice was indicative of osteoclast activation by the RANKL [22]. In the present study, although not significant, there was an increase in TRAP activity by cells cultured in medium containing RANKL compared to those grown in plain α-MEM. This increase was present in all cultures regardless of growth surface. These data indicated that soluble RANKL was able to activate osteoclasts grown under i*n vitro* conditions.

Peak values of TRAP and metabolic activity at the middle time point were immediately followed by a slump in levels, regardless of culture medium or growth surface. Using these peaks and slumps present within the data, three phases were identified for *in vitro* osteoclast differentiation from RAW 264.7 cells in RANKL: Days 2-4 (Phase1), Days 5-8 (Phase 2), and Days 9-12 (Phase 3). Differentiation phases have been identified for other cell types, most notably osteoblasts. This information has enabled researchers to gain further insight into other cellular mechanisms that occur during these phases, such as gene expression.

Increasing this understanding for osteoclasts and other cell types may lead to better cellular manipulations for use in a tissue engineering approach to bone regeneration [9].

This research also addressed whether the addition of M-CSF would enhance osteoclast activity in RAW 264.7 cells. It has been widely acknowledged that RANKL is sufficient to induce osteoclast differentiation; however, M-CSF has also been identified as a necessary growth factor to induce complete osteoclastogenesis [11, 12, 23]. The data revealed that TRAP activity was not significantly affected by the addition of 5 ng/ml of M-CSF to the osteoclast culture medium. Like RANKL, M-CSF has been used in a wide range of concentrations, and an increase in concentration may result in a synergistic effect of RANKL on osteoclast TRAP synthesis. The effect of M-CSF on osteoclasts may be better measured using factors other than TRAP activity, as it has been reported that M-CSF plays an important role in the proliferation and resorption activity of mature osteoclasts [24, 25].

Conditioned Medium Study

Following the characterization study of osteoclast cultures, a subsequent study was performed using conditioned medium collected from the three phases identified for osteoclasts in the *in vitro* setup. The coupling pattern of physiological bone formation following active resorption suggests that osteoblasts may behave differently when exposed to medium acquired from osteoclasts at different stages of development. The conditioned medium was mixed with osteogenic differentiation medium to test its effect on the differentiation of osteoblasts from the stromal/stem cells. Cellular metabolism was not significantly changed by the type of medium cells were fed, suggesting that cells were capable of proliferating and differentiating over the course of the study. The conditioned medium

did not have any major detrimental effect on the overall activity of the cells, as was evident from the metabolic activity data. An interesting result of this study was the significant increase in activity that occurred in each medium group between Day 7 and Day 13. These results indicated that cells from the D1 cell line experienced an initial lag in activity after seeding on tissue culture plastic, regardless of the growth medium. For groups fed osteogenic medium (with or without CM), this period of activity that was observed between the first and second time point may also coincide with the first of three phases of osteoblasts differentiation that has been described by Owen and coworkers and Deyama and coworkers [9, 26]. This stage has been marked by active proliferation and expression of Type I collagen. The complimentary pattern seen in intracellular protein levels for cells grown in osteogenic medium supports the theory that these groups were experiencing the initial stage of osteoblast differentiation between the first and second time point (Figure 3.5). The initial phase of osteoblast differentiation can be further examined in future studies by testing for the expression of Type I collagen and Runx2, two genes that have been linked to the primary stage of osteoblast differentiation [9, 26-28].

All three conditioned medium groups displayed significantly higher levels of protein at Day 13 compared to groups that were not exposed to any conditioned medium, which may indicate that these cells experienced a period of active growth and/or proliferation during this time. The phase of conditioned medium did not have an effect on this marked increase in protein levels. Furthermore, ALP levels measured at the middle time point for conditioned medium groups were very low, which can also indicate that cells were experiencing a phase of active proliferation. ALP levels would be expected to be very low in cells that were proliferating rather than moving into the second maturation phase of

osteoblast differentiation [9, 28, 29]. Conversely, cells that were fed 100% osteogenic medium had significantly lower levels of intracellular protein levels than CM groups at Day 13, but expressed significantly greater amount of ALP compared to their CM counterparts. The second phase of osteoblast differentiation has been shown to correlate with high levels of alkaline phosphatase activity at approximately Day 14, after exposure to differentiation cocktail as the cells start laying down matrix in the maturation stage [9, 30]. These data indicated that cells in pure osteogenic medium were able to transition out of the proliferation stage.

A calcium deposition assay was employed to further explore differentiation of the D1 cells along the osteogenic pathway. Considered a late stage marker of differentiation, calcium deposition would be expected to occur following high levels of ALP activity when cells reach the final mineralization phase, as was the case on Day 19 with cells grown in 100% osteogenic medium. The fact that the high levels of ALP also occurred at this final time point is an indication that the cultures are made up of a heterogeneous mixtures of cells that can be simultaneously at different stages of maturation. The Phase 1 and Phase 2 conditioned medium groups did have extracellular calcium levels that were greater than the plain medium control group, but they were still significantly lower than the osteogenic medium group that contained no conditioned medium. Again, this small increase in calcium deposition, despite the very low levels of ALP measured for the CM groups, may indicate that there were cells at various stages of development contained within the sample groups. Alkaline phosphatase and calcium deposition values collected via the colorimetric assays that were used in this study are able to give a good indication of what is occurring within the cell cultures; however, to further examine these preliminary results it will be important to

investigate genetic markers specific to each cell type and each phase of differentiation in order to gain a more in-depth understanding of cellular differentiation in our *in vitro* system.

3.5 Conclusions

It can be concluded that RAW 264.7 murine monocytes are able to differentiate into osteoclasts under these culture conditions and that *in vitro* osteoclast differentiation can be divided into different phases based on TRAP 5b activity and metabolic activity. The incorporation of osteoclasts into a cellular based bone graft system has the potential to increase bone formation at the site of injury by recruiting stem cells capable of producing bone matrix in the same manner that bone forming cells are recruited to areas of bone resorption in the bone repair cycle in the body [13, 14]. To test this theory, conditioned medium taken from each of the three phases of osteoclast differentiation was tested on differentiating murine stromal cells. Ultimately, the results of the quantitative assays suggest that osteoclast conditioned medium has a positive effect on the protein levels of D1 stromal cells, but inhibits the overall differentiation of these cells into osteoblasts. These effects are not dependent on the phase of osteoclast differentiation that the conditioned medium is collected from. It has yet to be determined if the *in vitro* culture of maturing bone cells can be managed with the use of medium tailored using various growth factors and cytokines. Kojima and Uemura discuss the importance of improving culture techniques for mesenchymal stem/ stromal cells before any cell-based bone graft system can be applied in a clinical environment. They have attempted to improve MSC culture methods through the use of gene therapy. In the present work we have presented an alternate option that is based on the well known physiological connections between osteoclasts and osteoblasts [31]. This

option has the potential to improve specific aspects of osteoblast differentiation (i.e. protein levels, proliferation) in an *in vitro* culture system.

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CHAPTER FOUR

ANALYSIS OF BONE MARROW ASPIRATE AS AN OSTEOINDUCTIVE BONE GRAFT MATERIAL FOLLOWING REAMING OF THE MEDULLARY CANAL

4.1 Background

Over 2 million bone graft procedures are performed annually worldwide to repair defects caused by trauma or removal of tumors, or to achieve fusion of spinal vertebrae [1]. Bone grafts are the second most transplanted material into the body, surpassed only by blood, with sales that have reached over $1 billion and continue to grow as the global population ages [2, 3]. When possible, transplantation of tissue from the patient's own body (autogenous) to the area in need of repair is the preferred method of reconstruction. Autogenous bone grafts (autografts) are the only clinically available options that possess all four characteristics of an ideal bone graft, specifically, they promote: osteointegration (union with the host bone), osteoconduction (support of boney and vascular ingrowth within the graft structure and onto its surface), osteoinduction (ability to induce differentiation of mesenchymal stem cells into the osteoblast phenotype) and osteogenesis (ability to induce formation of new bone by osteoblastic cells present within the graft) [4-6]. Unfortunately, healthy tissue is not always available for harvest and the surgery required to harvest autograft tissue with conventional methods causes additional pain and possible complications for the patient [2, 7].

Natural and synthetic options have been developed as alternatives to autografts to circumvent the associated issues, but most of these lack the osteoinductive properties

desired by surgeons for optimal fracture repair [8-11]. Allografts are products harvested from cadaver bones that have been preserved by freeze-drying and then vacuum-packed as whole or partial bone, morsels, or chips. Accounting for approximately one third of bone graft procedures in the United States, allografts serve as the preferred substitute to autografts, due to osteointegrative and osteoconductive characteristics that are comparable to the autologous tissue grafts [12]. Autografts and allografts are the most widely used materials, with synthetic bone grafts accounting for only approximately 10% of grafting procedures worldwide [1]. New tissue engineering techniques, coupled with advances in biomaterials research, are helping to improve graft options with the goal of someday replacing the need for autogenous tissue that is the current 'gold standard' today [13].

Bone tissue engineering devices combine a scaffold material with viable stem or mature bone cells in a product that can be implanted into boney defects or around a surgical site [14]. It is widely recognized that the success of a tissue engineered construct will ultimately depend on how it can be incorporated into the host tissue [15]. The incorporation of osteogenic cells and growth factors within a bone graft can improve the graft's viability and desired properties; but the optimal mix of cells/scaffold/growth factors has yet to be determined [16]. One major approach toward solving this engineering problem involves using the patient's own cells to regenerate lost tissue. These cells can be harvested through needle aspiration of bone marrow or from the removal of bone tissue during surgical reaming. Mesenchymal stem cells (MSCs) derived from bone marrow have been successfully cultured *in vitro* but expansion protocols are needed to allow widespread clinical application [17, 18]. In 1997, Jaiswal and colleagues demonstrated a reproducible technique for expanding MSCs isolated from the stroma of human bone marrow aspirate (donor ages 10-

58 years) and differentiating them into osteoblasts [19]. Using medium supplemented with dexamethasone, L-ascorbic acid, and β-glycerophosphate, an *in vitro* system was established for developing osteoblasts from human bone marrow which has seen widespread use in the scientific community. Similar results have been achieved with rat and chick marrow stromal cells using these methods [20, 21]. Assays for cell proliferation, alkaline phosphatase activity, and calcium deposition have been used to analyze MSC commitment into mineral secreting osteoblast-like cells when cultured under these conditions. The long term goal of employing an *in vitro* system with stem cells harvested from various sources is for the development of regenerative treatments for various bone tissue pathologies [19].

It is believed that the aspirate material obtained from femoral shaft reaming prior to surgical repair is a source of mesenchymal stem cells with osteogenic potential that could be used at the surgical site. The aspirate material obtained from the femoral shaft using the Reamer/Irrigator/Aspirator (RIA) device (Synthes, USA; Paoli, PA) consists of three main elements: bone fragments, liquid flow-through, and a fat layer. Bone fragments collected using the RIA device have been used successfully for the treatment of segmental defects and non-unions [22]. Cells isolated from the liquid flow-through have been successfully expanded *in vitro*, and found to possess both osteogenic and chondrogenic potential [23]. The fat layer of RIA aspirate has previously been considered waste and little, if any, work has been done to further examine this component for practical use. Adipose tissue-derived stem cells (ATSCs) have become a topic of great interest, due to the discovery that they possess a level of multipotency similar to that of stromal MSCs [24, 25]. Hence, we hypothesized that cells could be isolated from the fat layer of RIA aspirate and would have the capability to be driven down an osteogenic pathway [26, 27].

The objective of our study was to evaluate the bone fragments and the lipid-rich fat layer of RIA aspirate for the presence of cells that would express osteogenic markers when cultured under conditions that have been established in the literature for *in vitro* differentiation of osteoblasts from mesenchymal stem cells. Using an initial clinical sample, we sought to develop a method to isolate cells from the individual aspirate layers and determine if the isolated cells could be expanded and maintained *in vitro*. Upon developing these methods, an investigation was conducted to determine if the cells held any osteogenic potential for potential incorporation into a cellular based bone graft system.

The study provided preliminary information toward our long term goal of determining if fatty material obtained during reaming of the medullary canal could be incorporated into a bone graft system to improve the system's osteoinductive and osteogenetic capabilities.

4.2 Methods

Institutional Review Board approval was obtained from the Greenville Hospital System University Medical Center and Clemson University prior to the inception of this study. Reaming aspirate was collected from one male patient (age 33 years) following patient consent. The sample was packed on ice in the operating room for transport to a cell culture laboratory. Upon arrival, the floating fat layer was separated from the rest of the sample by decanting it into a separate sterile container. Liquid flow-through was aspirated from above and below the bone fragments and fat layer, respectively, in their individual containers (Figure 4.1). Upon separation, the liquid flow-through was discarded and the remaining two layers were processed.

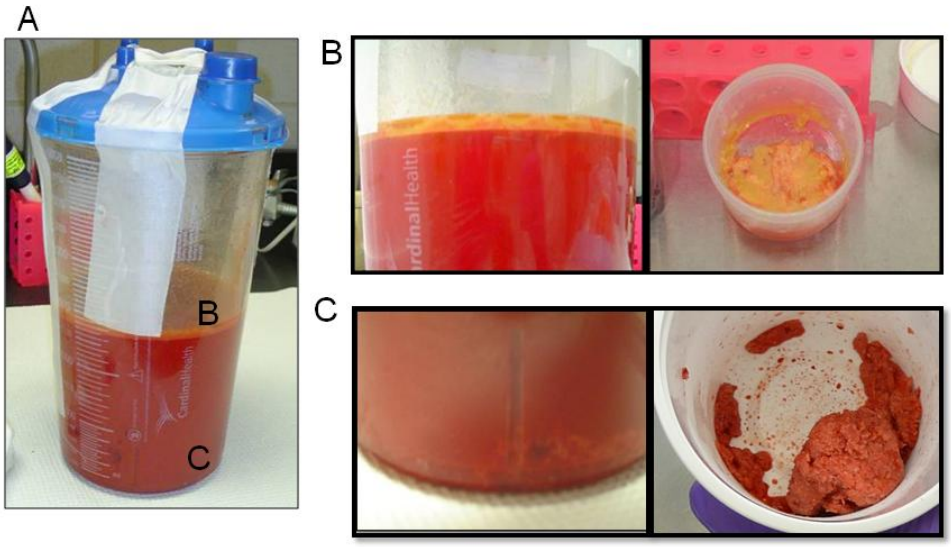

Figure 4.2: Aspirate Sample. A: Bulk aspirate sample before separation of layers. B: Fat layer of aspirate before (left) and after (right) separation. C: Bone fragments of aspirate before (left) and after (right) separation.

Bone & Fat Layer Cell Culture

Bone fragments were rinsed extensively in phosphate buffer saline (PBS) and samples of approximately 8 ml were transferred to culture flasks and maintained at 37°C and 5% CO_2. Five milliliters of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 5% antibiotic/antimycotic was added, but not removed, every two days through Day 8 to promote cellular attachment. Bone fragments were then removed, leaving the attached cells; medium was replaced every two days.

The bulk fatty tissue was rinsed in PBS and divided for further processing. Samples of 3 grams were minced with a scalpel and placed either directly into tissue culture flasks

containing DMEM, or into 50 ml vials containing a Type II collagenase mixture (6 ml supplemented DMEM, 2 mg/ml collagenase, and 2% bovine serum albumin). The vials were maintained at 37°C for 15 minutes to digest the collagen tissue. Vials were inverted at 5 minute intervals. Samples were transferred to a shaker plate and further digested for 45 minutes at 37°C and 250 rpm. Following digestion, samples were passed through 100 and 70 μm filters in succession and centrifuged to form a cell-rich pellet. The pellet was resuspended and expanded in DMEM culture medium. Supplemented DMEM was added, but not removed, every two days through Day 8 from flasks containing explants and resuspended cells. At Day 8, explants were removed and all cultures continued to be fed every two days.

Cell morphology of all cell cultures was examined via light microscopy. Cells from each aspirate layer were frozen at Day 12 for long-term storage in liquid nitrogen. The frozen aspirate cells were thawed and expanded before use in the differentiation study. An established human mesenchymal stem cell line (hMSC) was purchased (Lonza Group Ltd; Switzerland) to serve as a control. The hMSCs were thawed according to manufacturer instructions at the same time as the aspirate cells and all were maintained at standard culture conditions.

Differentiation Study

Cells isolated from the bone and fat layer were tested to determine their osteogenic potential. The human MSC line was used as a control (denoted Stem). Cells from each group were seeded in triplicate onto tissue culture plates at a density of 1,000 cells per cm^2. Test wells (OB) were maintained in MSC Growth Medium (MSCGM, from Lonza)

containing osteogenic supplements (dexamethasone, L-glutamine, ascorbate, and B-glycerophosphate). A second set of control hMSCs were maintained in plain MSCGM (denoted Stem Control).

Days 6, 14, and 21 were selected to monitor the temporal changes in cell differentiation. At each time point, samples were collected for calcium testing and a plate was frozen at -80°C. Metabolic activity (alamarBlue; Biosource International; Camarillo, CA), intracellular protein (BCA; Pierce Biotechnology; Rockford, IL), alkaline phosphatase (ALP; Sigma) and calcium deposition (Ca; Stanbio Laboratory; Borne, TX) assays were conducted on all samples at the conclusion of the study.

Metabolic Activity

Metabolic activity of the cells was assessed on samples using alamarBlue ™ dye (Biosource International; Camarillo, CA) on Days 6, 14 and 21. Prior to medium change on the specified days, 200 μl of alamarBlue dye (10% of total medium volume) was added to each well of the Day 21 plate. After gentle mixing, plates were returned to the incubator for a period of 3 hours during which time the dye was reduced by metabolically active cells to produce a color change from non-fluorescent blue to shades of fluorescent pink. Following incubation, 200 μl aliquots of media were transferred in triplicate to a black wall, clear bottom 96-well plate (Corning; Corning, NY). Fluorescence values were read using excitation and emission filter values of 544 nm and 590 nm, respectively, on a Fluoroskan Ascent FL fluorometric plate reader (Labsystems; Franklin, MA).

Intracellular Protein Content

The amount of total intracellular protein present in each well was determined using a BCA protein assay kit (Pierce Biotechnology; Rockford, IL). This protein assay produces a color change when bicinchoninic acid (BCA) molecules chelate with Cu^{1+} molecules that result from the reduction of Cu^{2+} ions by protein present in test samples. The resulting purple-colored solution absorbs light at a wavelength of 562 nm. An albumin standard was diluted according to the manufacturer's procedure to create a standard curve. A volume of 25 µl of cell lysate was transferred in triplicate from each test sample to a 96-well plate (Corning). A volume of 200µl of working reagent was added to each well of the 96-well plate. All plates were gently shaken on an orbital shaker and incubated for 30 minutes at 37°C. Absorbance values were read at 562 nm on a MRX Revelation microplate reader (Dynex Technologies; Chantilly, VA) and protein values were extrapolated based on the standard curve.

Alkaline Phosphatase Assay

An alkaline phosphatase (ALP) assay (Sigma Chemicals; St. Louis, MO) was conducted on samples at the conclusion of the study. A volume of 100 µl of cell lysate from each test sample was transferred to a clean plate containing 500 µl working reagent made up of equal parts p-nitrophenyl phosphate and alkaline phosphatase buffer. The conversion of paranitrophenylphosphate to para-nitrophenol in the samples produces a yellow color change which is used for quantification by measuring absorbance. All plates were incubated at 37°C for 30 minutes, at which time 0.25N NaOH was added to each well to stop the reaction. Samples and standards were transferred in triplicate from each well of the

incubated plates for testing within 96-well plates. Absorbance was read at 410nm on a MRX Revelation microplate reader. Reported ALP values were normalized with corresponding intracellular protein values.

Calcium Deposition Assay

Calcium deposition was evaluated for the cells during the differentiation study according to the protocol described by Lonza Walkersville, Inc. (Lonza; Walkersville, MD) [28]. On Days 6, 14, and 21 all culture medium was aspirated and wells were washed with PBS. Following washing, 0.5 ml of 0.5N HCl was added to each well. Cells were scraped from the bottom of the well plate and samples were transferred to an Eppendorf tube for storage. An additional 0.5 ml of HCL was added to rinse each well and was subsequently added to the storage tubes to ensure all cells were collected. Samples were stored at -20^{o}C until the conclusion of the study. Prior to performing the assay, samples were thawed on an orbital shaker for 6-12 hours at 4^{o}C. After shaking, the tubes were centrifuged at 500g for 2 minutes and supernatant with extracted calcium was collected and transferred to a new tube. Using instructions from the Stanbio LiquiColor^{R} Calcio Total Procedure No 0150 (Stanbio Laboratory; Borne, TX), a standard curve was prepared and used to determine the amount of calcium deposition present in each sample.

Statistical Analysis

Each data point represents the mean value of n=3 data samples, and error bars denote the standard error of the mean (SEM). The statistical software SAS® was used to compare the effects of medium and time on the metabolic activity, intracellular protein content,

alkaline phosphatase activity, and calcium deposition levels of the isolated cells and the control mesenchymal stem cells using the Least Squares Means (LSMEANS) command. A significance level of $p < 0.05$ was used for all comparisons.

4.3 Results

Cell Isolation from RIA Aspirate

Viable cells were successfully isolated from both the bone and fat elements of the aspirate. Microscopic examination revealed that the cells that grew from the osseous particles, fat pieces, and collagenase treated fat tissue had similar morphology (Figure 4.2). All of the cells grown in this study had morphologies consistent with previously documented MSCs cultured on tissue culture plastic; i.e., a rapidly expanding monolayer of adherent spindle-shaped cells, each marked by a cell body and long thin processes [29-32]. After long-term storage (> 30 days) in liquid nitrogen (-196^{o}C) the cells isolated from both layers of RIA aspirate remained viable. Upon thawing, outgrowth of the cells was not distinguishable from that of the hMSCs, which are typically stored in the same manner.

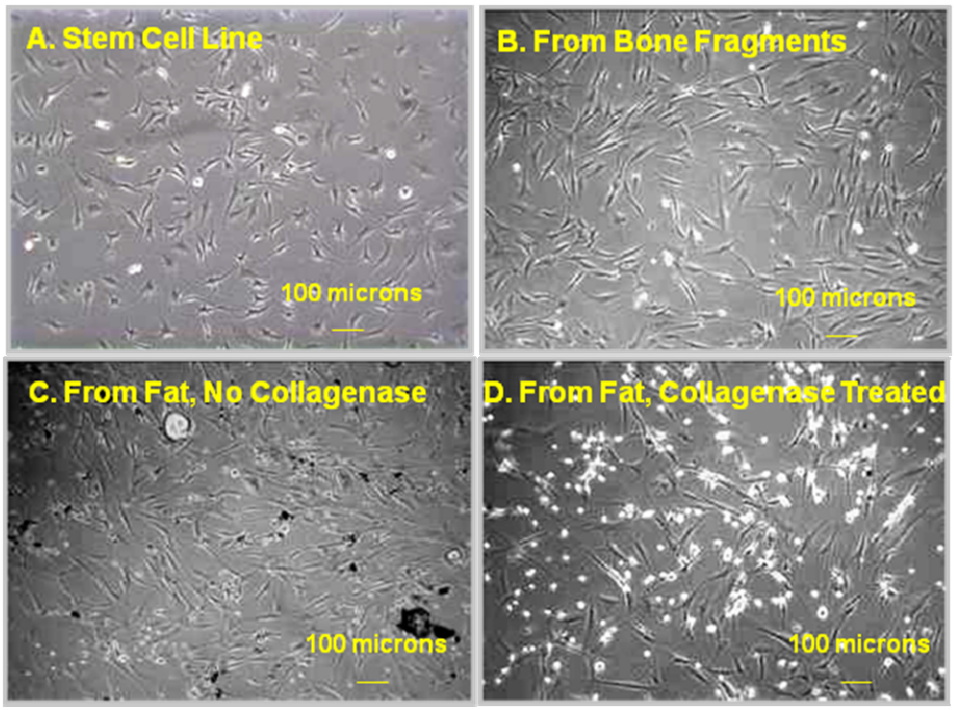

Figure 4.2: Morphology of cells. A: An established human mesenchymal stem cell line used as a control. B: Cells isolated from the bone fragments layer of RIA aspirate. C: Cells isolated from the fat layer of RIA aspirate without the use of collagenase to digest connective tissue. D: Cells isolated from the fat layer of RIA aspirate using a collagenase treatment. Microscopic examination showed that cells grown from osseous particles, fat pieces, and collagenase treated fat tissue had morphology comparable to cells derived from an established mesenchymal stem cell line.

Metabolic Activity

All cell groups showed a significant ($p < 0.05$) increase in metabolic activity over time (Figure 4.3). Significantly higher overall levels of cellular metabolism were recorded for the

hMSCs compared to the aspirate cells on Days 9, 14, and 21; but all the groups had overall increases in metabolic activity with time. A gradual increase was observed over time in all test groups for each of these parameters, and the rate of increase was similar in each group.

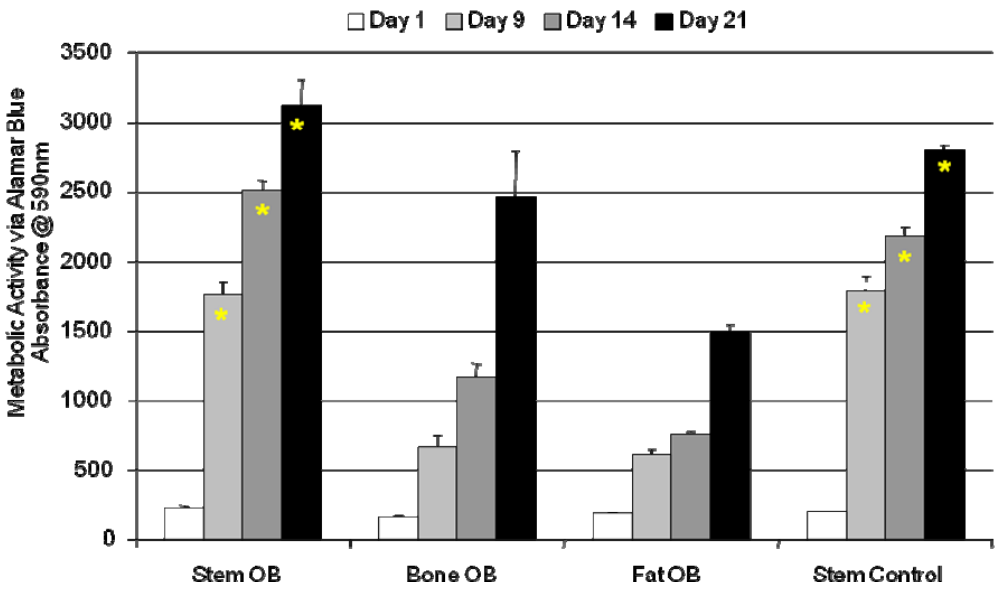

Figure 4.3: Metabolic activity of cells as a function of time and cell origin. Asterisks (*) denote significantly higher levels ($p < 0.05$) of metabolic activity on Day 9, Day 14, and Day 21 compared to cells isolated from aspirate layers. Each data point represents the mean value of n=3 data samples and error bars denote standard error of the mean (SEM).

Intracellular Protein

Total intracellular protein levels (Figure 4.4) increased significantly ($p < 0.05$) for all groups at each time point, with the exception of the hMSC control group maintained in OB

medium on Day 21. The hMSCs maintained in plain medium had the greatest amount of protein overall after 21 days of incubation. Day 6 protein values for cells from aspirate layers were outside the range of the standard curve and were recorded as zero.

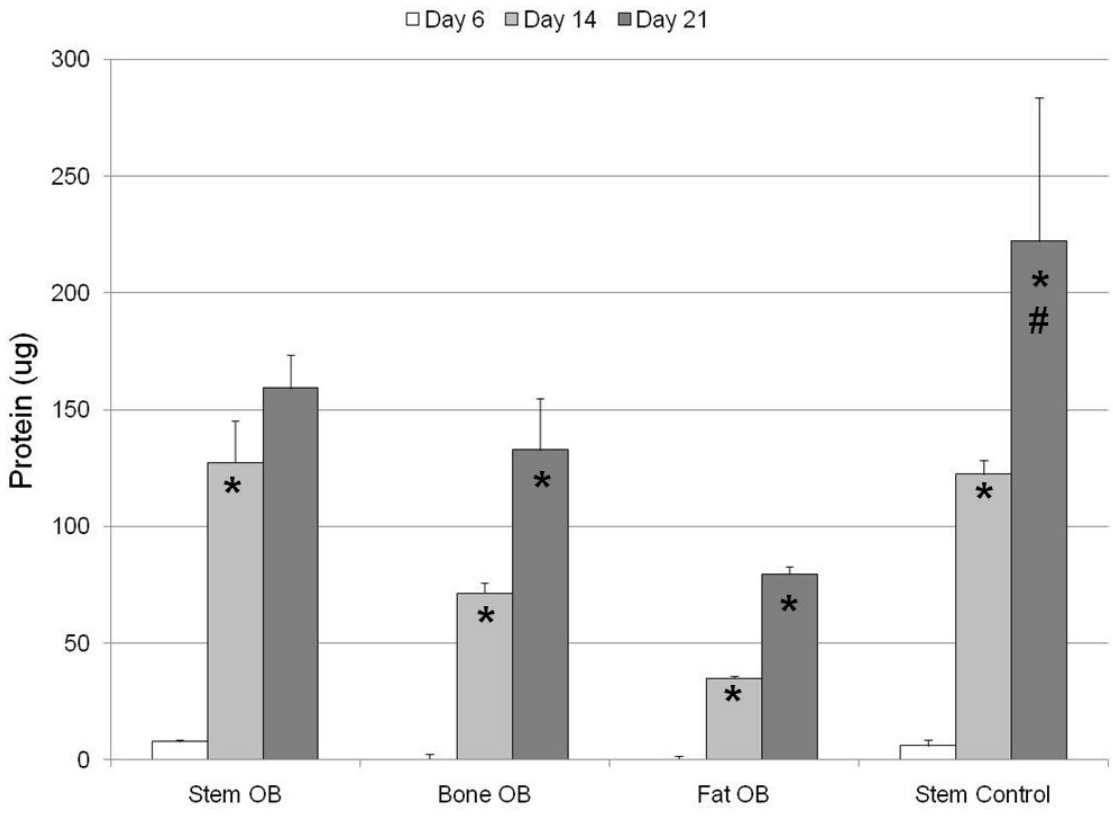

Figure 4.4: Total intracellular protein levels measured for cells as a function of time and cell origin. Asterisks (*) denote significant ($p < 0.05$) increases in protein level over time. Pound signs (#) denote protein levels that were significantly higher on Day 21 compared to all other groups ($p < 0.05$). Each data point represents the mean value of n=3 data samples and error bars denote SEM.

Alkaline Phosphatase Assay

All osteogenic medium (OB) test groups exhibited similar trends in ALP synthesis during the entire culture period (Figure 4.5). After 21 days, all of the cells cultured in osteogenic medium had significantly ($p < 0.05$) greater levels of ALP than those cultured in plain medium. Most notably, there was no significant difference observed between the cells isolated from the aspirate layers and the established hMSC line

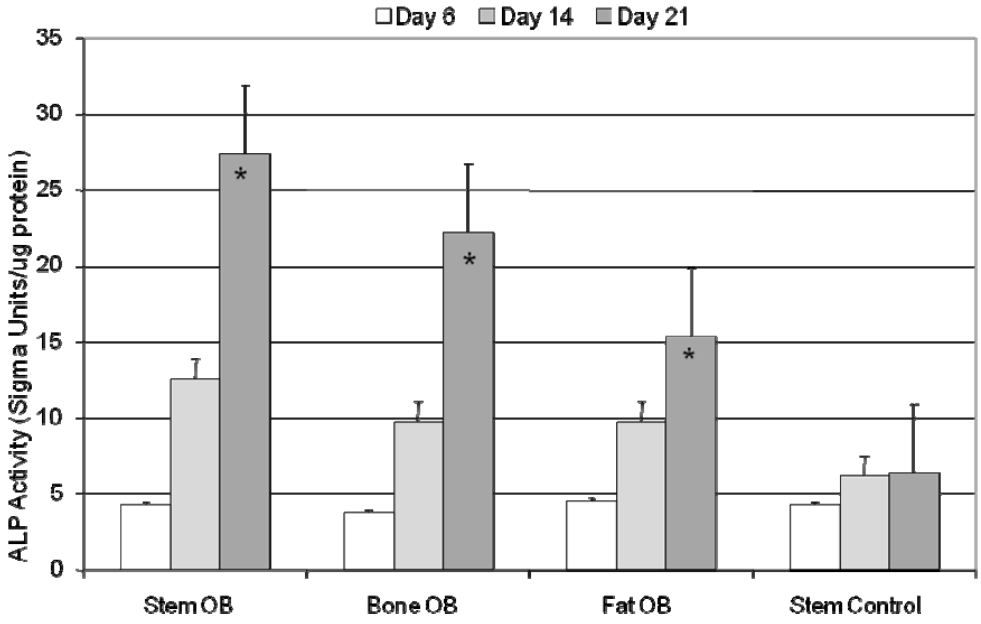

Figure 4.5: Alkaline phosphatase synthesized by cells as a function of time and cell origin. Asterisks (*) denote significantly higher values ($p < 0.05$) of ALP measured in OB medium groups compared to the plain medium groups (Control) after 21 days of incubation. Each data point represents the mean value of n=3 data samples and error bars denote SEM.

Extracellular Calcium Deposition

All groups had a significant increase in calcium (Figure 4.6) between Day 6 and Day 21 ($p < 0.05$); however, the only set of cells to have significantly higher levels of calcium than the plain medium control groups were those isolated from the bone layer of RIA aspirate. This peak level of calcium deposition was observed at Day 21. Cells fed OB medium also had higher levels of extracellular calcium compared to the plain medium group, starting on Day 14; cells isolated from the aspirate bone fragments exhibited the highest levels of this late stage osteoblast marker at Day 21.

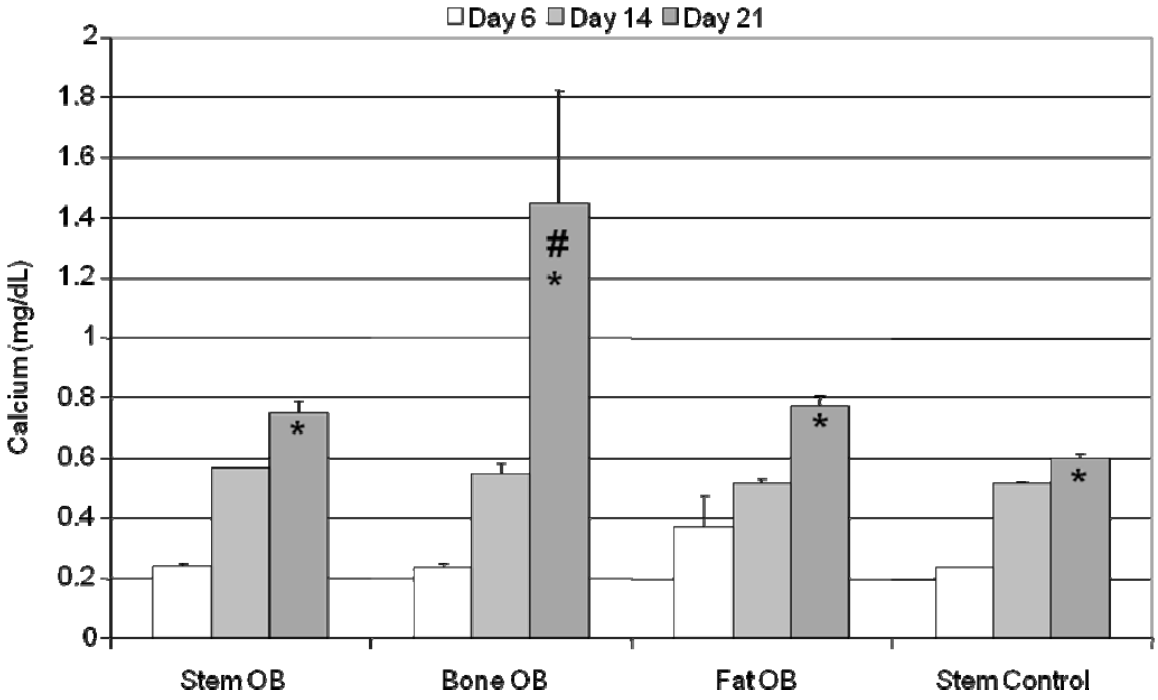

Figure 4.6: Extracellular calcium deposition by cells as a function of time and cell origin. Asterisks (*) denote significant increases in calcium deposition observed between Day 6 and Day 21. Pound signs (#) denote that cells isolated from the bone fragments of RIA aspirate had significantly greater amounts of calcium deposition compared to the control groups at Day 21 ($p < 0.05$). Each data point represents the mean value of n=3 data samples and error bars denote SEM.

4.4 Discussion

To our knowledge, we are one of the first groups to consider the fat layer of RIA aspirate separately from the other layers as a potential source of adult MSCs. The study

described was conducted on the lipid layer of aspirate, which to date has been of low clinical interest, and served to as a proof of concept for using this autologous material in tissue engineering applications. The patient sample described in this work was the first sample from which cells were successfully isolated and enabled the group to establish standard methods for handling and processing future patient samples. Samples from the fatty layer were treated using a technique established by Forest and coworkers for the isolation of preadipocytes from subcutaneous and intramuscular bovine adipose tissue [33, 34]. Isolating cells from bulk fat tissue samples by digesting the connective tissue and filtering the cells has enabled researchers to directly compare different fat depots at the cellular level. This enzymatic digestion technique has been used successfully to isolate cells from bovine bulk fat deposits in order to study the effects of various stimuli on the differentiation of preadipocytes and optimize culture conditions for these cells [33]. In the present study, viable, proliferating cells were successfully isolated from fat layer of the RIA aspirate using the collagenase enzyme.

Explant methods have been used extensively to produce cellular outgrowth from bulk tissue pieces in tissue culture [23, 35, 36]. Both explant and dispersion methods used for processing the bone and fatty layers result in the successful outgrowth of cells. Cellular outgrowth also occurs when autograft bone fragments are packed directly into a bone defect, but this method is limited to small volumes [37-40]. It stands to reason that samples taken from the fat layer of aspirate have similar cellular activity. Hence, combining aspirate fat fractions with other graft material may be a simple and clinically relevant method to enhance graft volume. Dispersion could also be performed in the operating room, in a time efficient manner, to isolate progenitor cells before inclusion into a bone graft system. Although both

methods may result in a mixed cell population, a mixed population may be beneficial in providing a larger number of cells that are undifferentiated and in providing the necessary complex cellular signaling [41-43]. It may be necessary to store cells isolated from a patient during femoral reaming for use in a future surgical procedure and tissue engineering applications, therefore, it is important that the cells isolated from the aspirate layers are able to maintain their ability to proliferate following standard storage. All of the cellular isolation methods outlined in this work produced repeatable outgrowth results.

The reamer aspirate may be an untapped source for autologous pro genitor cells that could aid in bone formation when incorporated into bone grafts. The isolated cells were subjected to conditions widely recognized for inducing osteogenic differentiation in precursor cells [23, 24, 26, 44, 45]. Results of the metabolic activity and intracellular protein assays revealed that all of the cells grew well, regardless of origin or medium type, throughout the 21 day incubation period. The increase of each of these parameters that was observed over time, in all test groups, suggests that all of the cells experienced similar rates of growth. The alamarBlue™ dye and BCA protein assays employed in this study are often used to evaluate cellular proliferation within an *in vitro* culture system [46, 47]. The hMSCs grown in plain medium, for example, had significantly higher levels of intracellular protein and metabolic activity at Day 21. This result suggests that this group proliferated more over the course of the study than the other test groups. While a relative comparison can be made within the study, metabolic activity is determined by measuring the absorbance of medium samples taken after the cells have been given time to metabolize the dye. This measurement alone does not specify any particular cellular activity, such as division or proliferation. Although activity levels in the test groups may be similar, cellular activities may be different

and further examination of specific activities must be conducted. Similarly, the protein assay can also be affected by factors other than proliferation, including the osteogenic medium supplement ascorbic acid, and thus can only be viewed as a relative assessment of growth within the study. A more quantitative analysis of cellular number must be performed in order to validate this increased proliferation conclusion in future work [48].

The activity of the alkaline phosphatase enzyme was used as a marker for osteoblast differentiation in this preliminary study. Synthesis of this enzyme alone does not indicate osteoblast differentiation, but it is an important initial clue for detecting if cells have the capability to proceed along this bone cell pathway [19, 44, 49]. ALP is a middle stage osteoblast differentiation marker and is expected to be highest between Days 15-28 in stromal cells differentiating into osteoblastic cells [50, 51]. Our hypothesis that cells from the fat layer of reamer aspirate could be driven down an osteogenic pathway was supported by the recorded ALP values. ALP activity doubled after two weeks and continued to rise over the three week period. This pattern of ALP synthesis is consistent with other studies involving hMSCs differentiating into osteoblasts [52]. The third phase of osteoblast differentiation, mineralization, would be expected to occur after peak levels ALP are reached during the maturation phase. Cellular calcium deposition would be expected to occur following the peak in the release of ALP, and, indeed, the calcium levels in our study were highest at the final time point. Although the levels of calcium deposition were highest in the cells isolated from aspirate bone fragments, the cells isolated from the lipid layer did display a significant increase over the course of the study. These data may indicate that although the cells from the lipid layer may reach the final phase of osteoblast differentiation, they may do so at a different rate than cells isolated from the autologous bone chips. Additional time

points will allow better characterization of differentiation using calcium, which is considered a late-stage differentiation marker; this marker can be confirmed using histological staining. Alizarin Red S and Von Kossa stains can be employed to visualize mineralized nodules that form *in vitro* [23, 52, 53]. The results of these quantitative assays indicate that all of the cells fed OB medium were induced along an osteogenic pathway using the specified medium supplements and culture conditions. This pathway was confirmed by detecting the bone cell marker alkaline phosphatase and calcium deposition. The combination of ascorbic acid, β-glycerophosphate, and dexamethasone used in this study has been used extensively to study osteogenic differentiation, but an optimal culture system is yet to be established. Researchers have identified a number of factors that affect the terminal differentiation of marrow cells into an osteoblastic phenotype including: the dose of these supplements, time of exposure to these supplements, and the age of the cell population [19-21, 44]. A mix of proliferating and differentiating cells may be more desirable for bone graft applications than a pure population of terminally differentiated cells in order to achieve osteoinduction and osteogenesis at the injury site. The data collected indicate that cells from the bone and fat layer of RIA aspirate may be induced into osteoblast differentiation at differing rates. Incorporating both of these layers into a bone graft system could ultimately lead to an increase in the overall length of time that the graft displays osteoinductive activity.

The results of this pilot differentiation study can be used to determine adequate sample sizes for future work involving this aspirate material in tissue engineering applications. One of the major issues with current bone graft options, such as demineralized bone matrix (DBM), is the inconsistencies that exist within a given batch of the product. Commercially available DBM products, for example, have displayed significant within-batch variability in

mineral composition and growth factors. These differences have been linked to disparities in bioactivity of the products and attributed to inconsistencies observed in clinical results during their use [54, 55]. In contrast, the use of RIA bone fragments has produced relatively consistent clinical results when compared to the iliac crest standard [56, 57]. The pilot study results revealed that the fat layer within-batch variability was on par with the bone layer within-batch variability.

4.5 Conclusions

The results of this pilot study suggest that cells isolated from both the bone and fat layer of RIA aspirate have the potential to differentiate along an osteogenic pathway. The results warrant that further consideration be given to the fat layer of aspirate. Previously considered waste, the lipid rich fat layer of aspirate collected during reaming of the medullary canal may be a source of adult mesenchymal stem cells that can be used to stimulate new bone growth at the site of fracture repair. This potential should be further investigated using the cellular isolation and differentiation techniques described within this work in future studies with multiple patient samples. Osteoblast differentiation can be further examined by measuring the expression of the essential osteoblast related genes such as Runx2, Type 1 collagen, osteocalcin, alkaline phosphatase, and bone sialoprotein [58]. Although an exact map of gene expression is unknown, the three main phases of osteoblast differentiation (proliferation, maturation, and mineralization) have been identified using these gene markers [59]. Gene expression data, coupled with the cellular assays for bone marker expression and histology stains for mineralized nodule formation, generate an improved overall understanding of *in vitro* osteoblast differentiation from precursor cells [23, 47, 51, 60]. *In*

vivo experimentation will be required to determine if the differentiation of osteoblasts from cells isolated from this aspirate source will ultimately translate into new bone formation within a bone defect. By combining the bulk material or cells isolated from the fatty layer with the aspirate bone fragments, the osteogenic potential of RIA autograft may be enhanced.

4.6 References

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CHAPTER FIVE

OSTEOGENIC POTENTIAL OF CELLS ISOLATED FROM THE LIPID-RICH LAYER OF REAMER ASPIRATE

5.1 Background

Stem cells are a unique set of cells that have the capacity for self-renewal and have not yet committed to a specific cell type [1-3]. These cells have been shown to be multi-potent, meaning that they possess the ability to differentiate into a number of different cell types. Bone marrow is widely used as a source for mesenchymal stem cells (MSCs), which exhibit a high degree of plasticity and the ability to produce osteoblasts, chondrocytes, adipocytes, myotubes, and neuronal cells [3, 4]. The application of adult stem cells to bone injury repair is a major area of interest in orthopedic research because of their multi-lineage differentiation potential of stem cells, their relative availability and ease of harvest, and their capacity to undergo multiple replications without losing multi-potent potential [5]. These traits make them an attractive source for cell-based therapeutic approaches. Several experiments have demonstrated that bone marrow derived MSCs can be induced to transform into osteoblasts. These cells are also capable of contributing to bone formation when cultured on a scaffold prior to implantation into the subcutaneous space in mice and rats [6]. Adipose tissue is another potential source of stem cells in the body that may contribute to the formation of bone cells [7]. Cells isolated from bulk adipose tissue have also shown a multi-potency towards bone, cartilage, and neurons, in addition to fat tissue [4, 8]. For these reasons, it is our belief that the fatty portion of RIA aspirate may be an untapped source for progenitor cells that could be used at the site of fracture repair.

A tissue engineering approach to bone repair has emerged, using mature and progenitor cells for defect repair. Incorporating these cells into an established bone grafting material is one strategy being explored that has shown great promise [9]. Traditional materials are often used in this research area, as many are biocompatible, are U.S. Food and Drug Administration (FDA) approved for select applications, and have previous long-term success. βeta-tricalcium phosphate (β-TCP) has been applied clinically as a bone graft substitute in either porous or solid form as granules or blocks. This ceramic contains two of the main minerals components of bone, calcium and phosphate. Porous (60%) β-TCP ceramic was marketed by Synthes, Inc. in a granule form that is indicated for use in the repair of skeletal defects. This material is gradually resorbed by osteoclasts and replaced by new bone [10]. The rate of resorption of β-TCP is often faster than the rate of new bone formation, therefore, it is often used as a graft expander with autologous tissue [11]. When mixed with bone marrow and implanted subcutaneously in rats, ultraporous β-TCP has been found to produce more bone and osteoblastic cells than DBM [12].

The objective of the present study was to evaluate the fat layer of aspirate obtained from the femoral shaft using the Reamer/Irrigator/Aspirator (RIA) device (Synthes, USA; Paoli, PA). After successful isolation, cells from this lipid-rich layer were studied to determine their osteogenic potential and growth potential on a clinically available ceramic bone graft substitute. To further characterize the composition of this unique layer of aspirate, gas chromatography was performed on samples taken from multiple patients to determine and compare the fatty acid content of the lipid samples. A follow-up differentiation study was conducted using cells isolated from this patient and a second patient to examine if cells

isolated from different patients exhibit similar trends in behavior in culture conditions designed to promote osteogenesis.

5.2 Methods

Institutional Review Board approval was obtained from the Greenville Hospital System University Medical Center and Clemson University prior to the inception of this study.. Reaming aspirate was collected from one female patient (age 23 years, #5003) and packed on ice for transport to a cell culture laboratory.

Cell Culture

Samples taken from the fat layer of RIA aspirate were processed according to procedures outlined in Chapter 4 . Briefly, fat samples were minced and placed into a Type II collagenase mixture to digest the tissue. Following digestion, samples were centrifuged to form a cell-rich pellet which was resuspended and expanded in MSC Growth Medium (MSCGM; Lonza Group Ltd; Switzerland). These primary cells were used in the differentiation study after two passages.

Differentiation Study 1

Primary cells isolated from the fat layer were tested to determine their osteogenic potential (Asp). A human MSC line (Lonza) was used as a control (H). Cells from each origin group were seeded at a density of 1,000 cells per cm^2, in triplicate, directly onto either tissue culture polystyrene well-plates or ceramic granules (0.13 grams, Figure 5.1) that covered the bottom surface of the wells. Test wells (OB) were maintained in MSCGM

containing osteogenic supplements (dexamethasone, L-glutamine, ascorbate, and β-glycerophosphate). Control groups were maintained in plain MSCGM.

Days 9, 20, and 29 were selected to monitor the temporal changes in cell differentiation. At each time point a metabolic activity (alamarBlue; Biosource International; Camarillo, CA) assay was performed, samples were collected for calcium testing, and a plate was frozen at -80°C. Samples were frozen until the conclusion of the study, at which point quantitative assays were performed on all samples simultaneously. Intracellular protein (BCA; Pierce Biotechnology; Rockford, IL), alkaline phosphatase (ALP; Sigma), and calcium deposition (Ca; Stanbio Laboratory; Borne, TX) assays were conducted, as previously described in Chapters 2 and 3, on all samples after thawing.

Figure 5.1: Ceramic Granules. The β-tricalcium phosphate ceramic granules of bone void filler, 0.5 - 0.7 mm in size were used in this study (Synthes, USA; Paoli, PA).

Fatty Acid Composition

Gas chromatography was used to determine the fatty acid content of samples from the fatty layer of RIA aspirate from the patient sample used in this study and from three other patient samples. Lipid extracts were collected from approximately 0.1 grams of bulk fatty material and transmethylated according to previously modified procedures [13-15]. Analysis of the fatty acid methyl esters was conducted using a HP6850 (Hewlett-Packard, San Fernando, CA) gas chromatograph equipped with a HP7673A (Hewlett-Packard) automatic sampler. Standards (Sigma, St. Louis, MO; Supelco; Matreya, Pleasant Gap, PA) were used to identify individual fatty acids by comparing retention times. Results are reported as a percentage of total fatty acids.

Differentiation Study 2

Cells isolated previously from the bone and fat layers of one male patient (age 33 years, #5002) and cells isolated from the fat layer of the female patient described above (#5003) were used in a follow-up differentiation study after being stored in liquid nitrogen. The same human MSC line was used as a control. Cells were again seeded at a density of 1,000 cells per cm^2, in triplicate, directly onto polystyrene tissue culture plastic wells. Cells were maintained under the same culture conditions and grown in the same media, i.e. plain MSCG and MSCGM supplemented with the osteogenic growth factors dexamethasone, ascorbate, and β-glycerophosphate. The activity and bone cell marker activity of cells isolated from two different patients were examined at Days 7, 14, and 21. At each time point procedures were performed as described in Chapters 2-4 for Differentiation Study 1 (metabolic activity

assay, calcium testing, and end-point assays). The same quantitative assays were conducted for both differentiation studies.

Statistical Analysis

The data are expressed as the mean value of three data samples, and error bars denote standard error of the mean. Statistical analysis was conducted using the Least Square Means command with SAS statistical software. Differences with p values of less than 0.05 were considered statistically significant.

5.3 Results

Cell Isolation from Patient #5003

Viable cells were successfully isolated from the fatty layer of the RIA aspirate (Figure 5.2B). Morphological examination of these cells was conducted using light microscopy, and cell shape was compared to the established human MSC line. All cells displayed fibroblast-like morphologies representative of undifferentiated mesenchymal stem cells (Figure 5.2A). Cells isolated from the aspirate retained this morphology at initial seeding in OB medium (Figure 5.2C).

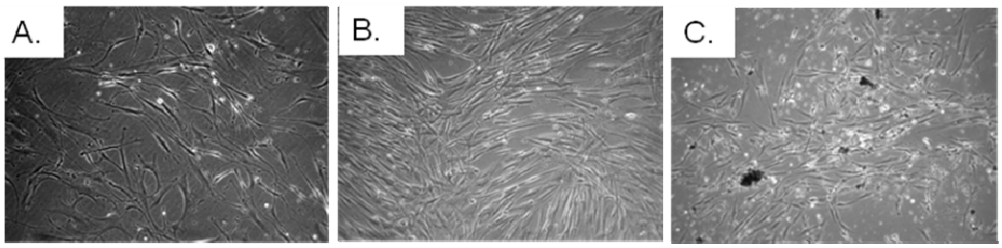

Figure 5.2: Cell Morphology. A: Control human mesenchymal stem cell line in Mesenchymal Stem Cell Base Medium. B: Primary cells isolated from aspirate in Mesenchymal Stem Cell Base Medium. C: Primary cells isolated from aspirate in Osteogenic Medium. Total magnification for all images is 100x.

Differentiation Study 1

Metabolic Activity

No significant difference in metabolism was detected between cells isolated from the RIA aspirate and the MSC line (Figure 5.3). This result was the same whether cells were grown on ceramic or on polystyrene. Additionally, no significant difference was observed when comparing the two culture surfaces or comparing the plain medium and the medium supplemented with osteogenic growth factors.

Intracellular Protein

Cells cultured on the ceramic granules, in both types of medium, had significantly ($p < 0.05$) higher levels of intracellular protein compared to cells grown directly on tissue culture plastic (Figure 5.4). The protein levels also significantly increased for all of the groups between Day 9 and Day 20, a result that is expected for actively growing cells.

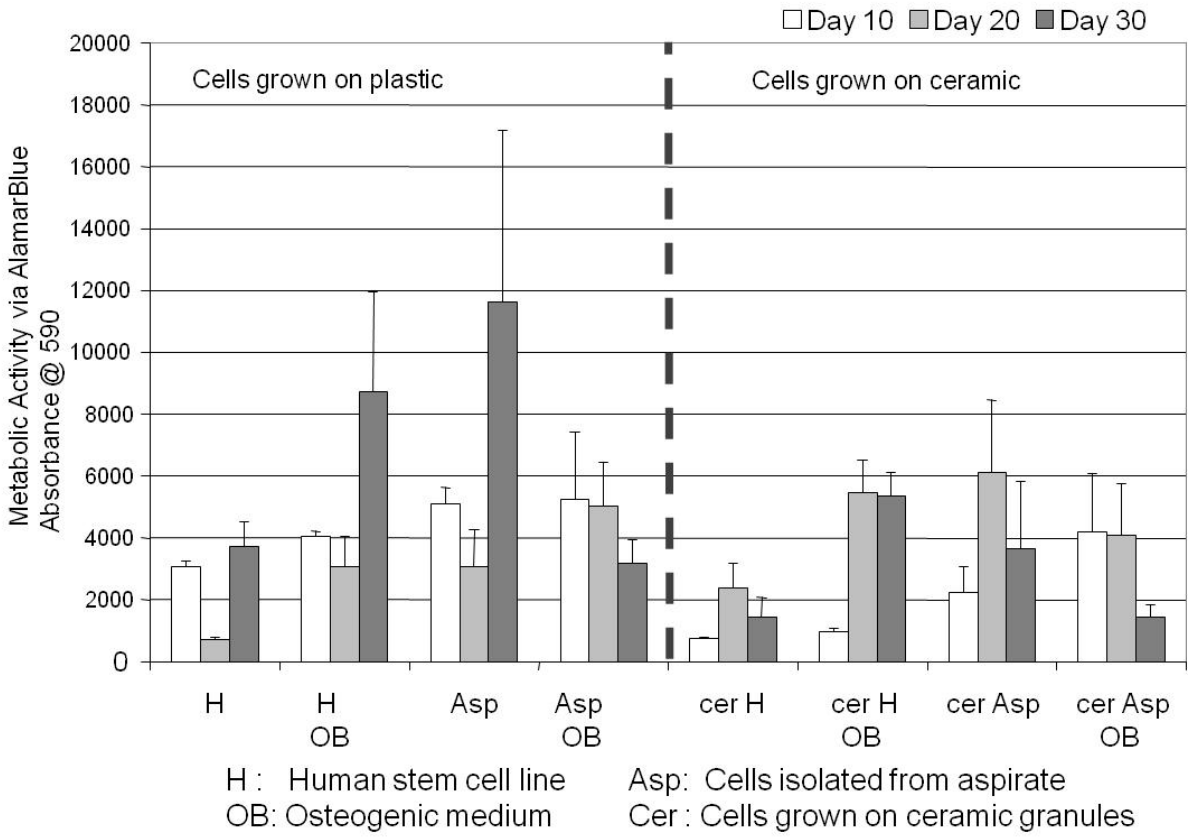

Figure 5.3: Metabolic activity of cells as a function of time and cell origin. No significant difference was observed between cells grown on ceramic granules and cells grown on tissue culture plastic. Metabolism data was similar for cells isolated from the RIA aspirate and the established stem cell line. Each data point represents the mean value of n=3 data samples and error bars denote SEM.

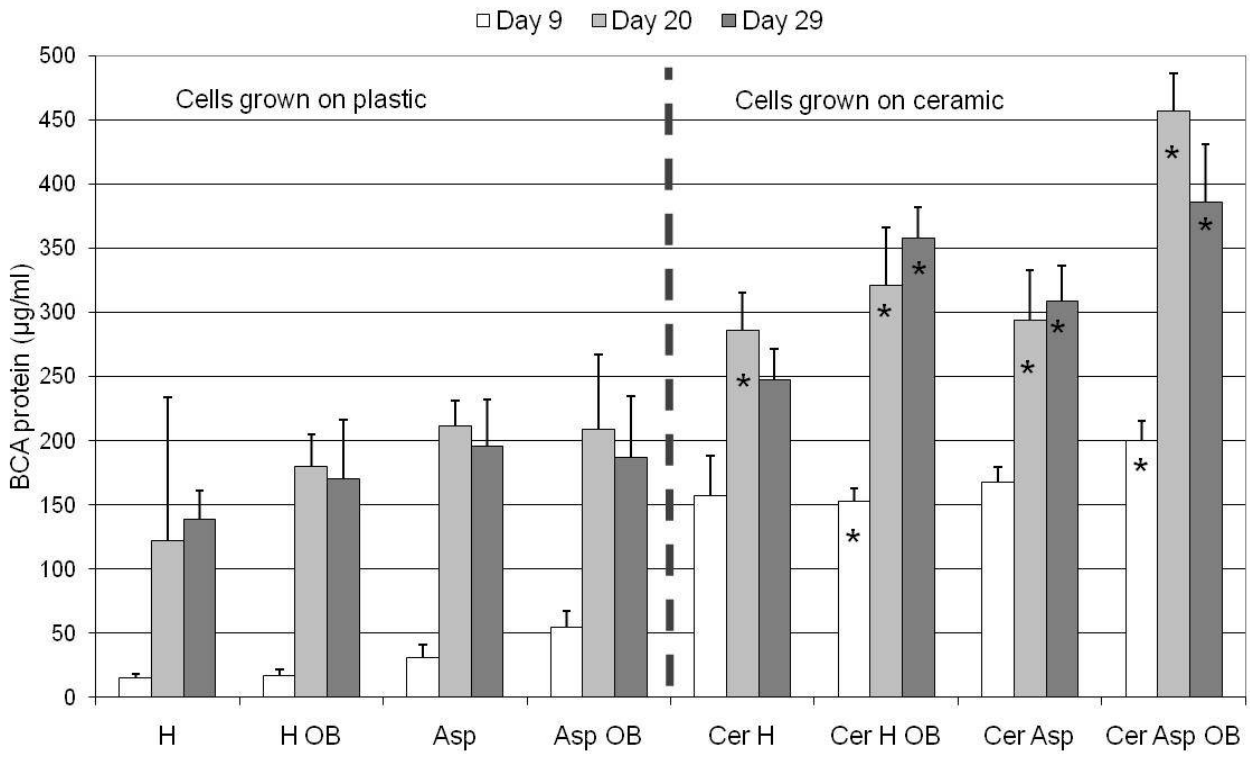

Figure 5.4: Total intracellular protein levels measured for cells as a function of time and cell origin. Asterisks (*) indicate that significantly higher levels of protein were measured in ceramic groups compared to tissue culture plastic groups ($p < .05$). A significant increase in protein was observed for all groups between Day 9 and Day 20. Each data point represents the mean value of n=3 data samples and error bars denote SEM.

Alkaline Phosphatase Assay

An alkaline phosphatase assay revealed data that was contrary to the intracellular protein data. Cells grown on the ceramic granules expressed significantly lower levels of ALP. Cells

cultured on polystyrene and maintained in osteogenic medium had significantly higher levels of ALP than the plain medium, polystyrene groups at Days 20 and 29 (Figure 5.5).

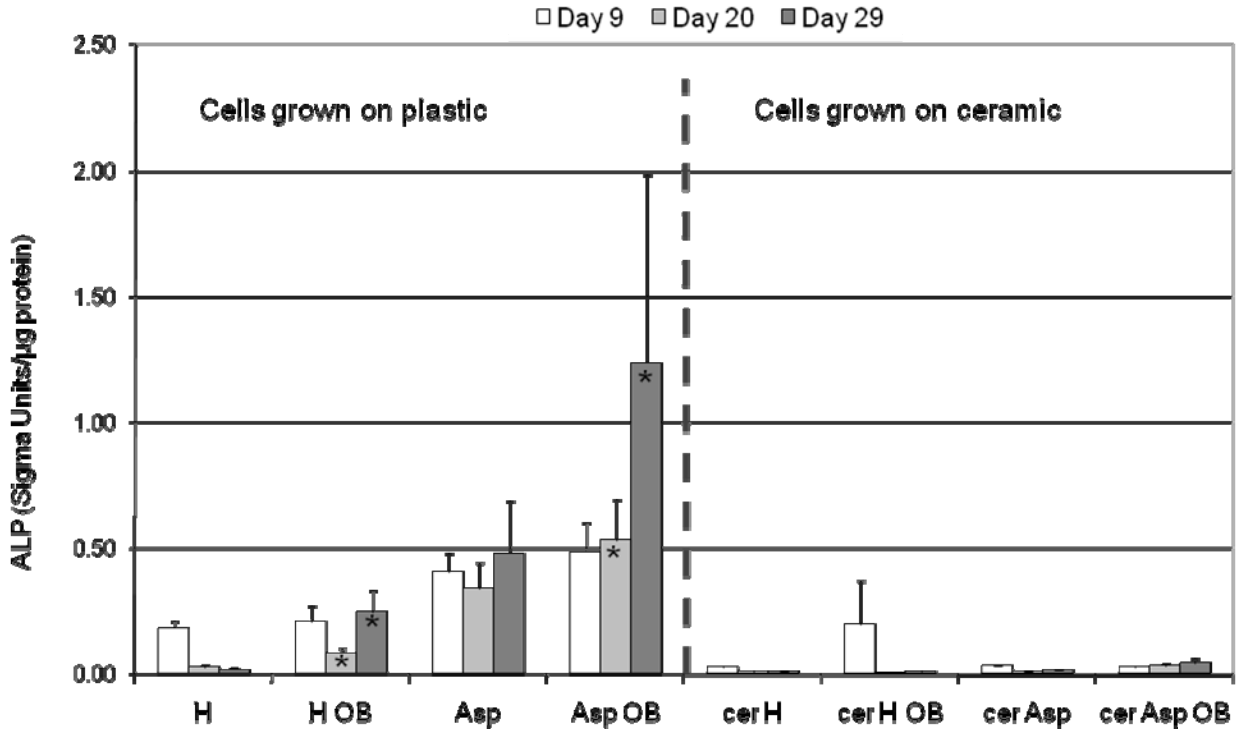

Figure 5.5: Alkaline phosphatase released by cells as a function of cell origin and culture surface. Asterisks (*) indicate that OB medium groups had significantly higher levels of ALP than the plain medium groups at Days 20 and 29 ($p < 0.05$). Significantly lower levels of ALP were expressed by cells cultured on ceramic granules compared to those cultured on tissue culture plastic. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Extracellular Calcium Deposition

Due to the composition of the ceramic granules, the extracellular calcium test was only run on samples collected from cells grown on tissue culture plastic. Levels of calcium deposition were significantly higher for cells isolated from the RIA aspirate and cultured in osteogenic medium at the middle and final time points (Figure 5.6).

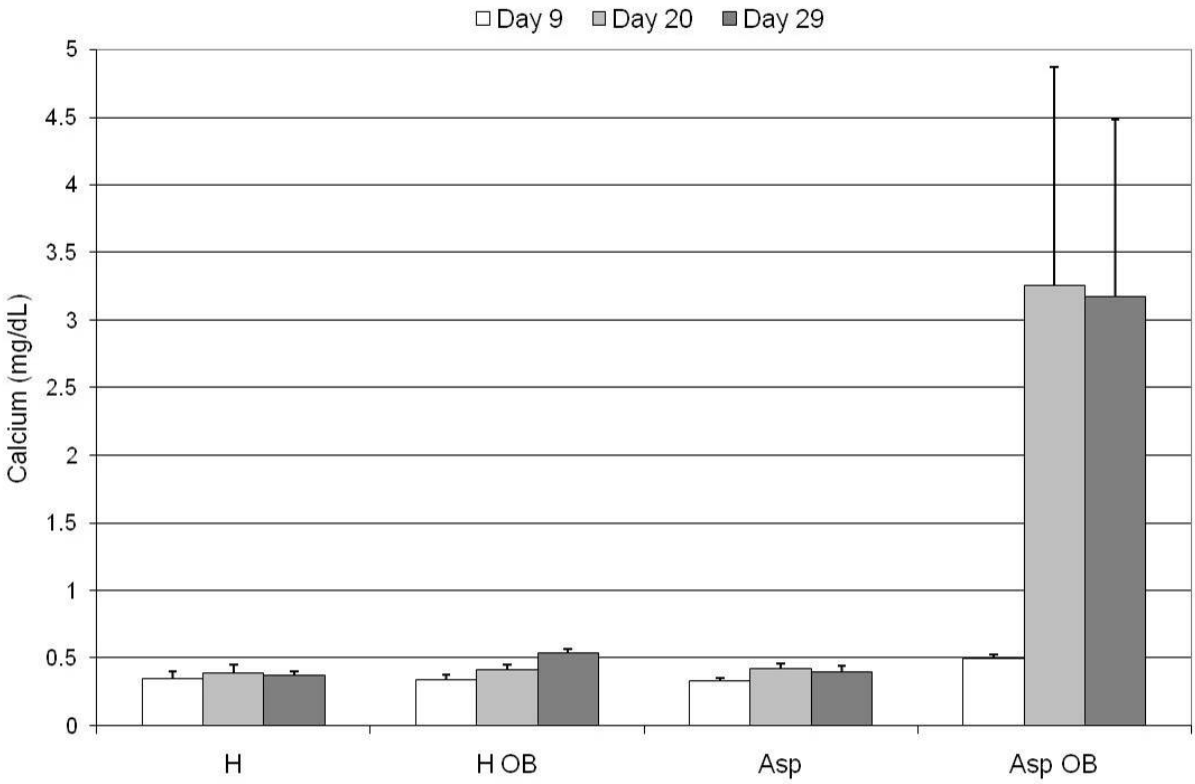

Figure 5.6: Extracellular calcium deposition levels of cells grown on tissue culture plastic. Asterisks (*) indicate that Aspirate OB group had significantly higher levels of calcium than all other groups on Day 20 and Day 29 ($p < 0.05$). Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Fatty Acid Content of Multiple Patient Samples

Gas chromatography analysis exposed the fatty acid content (Figure 5.7) of samples taken from the lipid-rich layer of RIA aspirate of four different patients (#5001-5004). The results of this characterization revealed that the fat layer of RIA aspirate of all four patients had the same four fatty acids present in the highest percentages. In descending order the four most prevalent fatty acids in all patient samples were: oleic, palmitic, linoleic, and stearic.

		Sample Number % of total fatty acid composition			
Carbon #	**Common Name**	**5001**	**5002**	**5003**	**5004**
12	Lauric	0.189	0.173	0.25	0.15
14	Myristic	1.919	1.738	2.30	1.61
14-1	Myristoleic	0.202	0.055	0.24	0.22
15	Pentadecanoic	0.341	0.102	0.14	0.09
16	Palmitic	21.89	23.78	22.14	21.53
16-1	Palmitoleic	2.597	0.528	0.08	0.05
17	Heptadecanoic	0.59	0	0.33	0.33
18	Stearic	8.064	8.668	6.08	3.07
18-1-T9	Trans-9 octadecenoic	0.55	0	0.00	0.00
18-1-T10	Trans-10 octadecenoic	0.669	0	2.12	0.00
18-1-T11	Trans-11 vaccenic	0.701	0	0.00	0.00
18-1-C9	Oleic	37.45	37.16	36.67	39.50
18-1-C11	Cis-11 octadecenoic	1.709	0	1.80	1.27
18-1-C12	Cis-12 octadecenoic	0.567	0	0.36	0.39
18-2 Omega-6	Linoleic	15.23	16.14	16.04	18.59
20	Archidic	0	0.148	0.56	0.00
18-3 Omega-3	Linoleic	0.632	0.75	0.33	0.24
18-2-c9t11	CLA cis9 trans11	0.354	0.379	0.01	0.02
22-5 Omega-5	DPA	0.196	0	0.14	0.04
22-6 Omega-3	DHA	0.189	0	0.01	0.01
	SUM	**94.04**	**89.62**	**89.61**	**87.0999**
	Other	5.958	10.38	10.39	12.9001

Figure 5.7: Fatty acid content of multiple patient samples taken from the fatty layer of RIA aspirate. Four fatty acids were most abundant in all patient samples: oleic, palmitic, linoleic, and stearic (highlighted).

Differentiation Study 2:

Metabolic Activity

Data from the alamarBlue assay showed that all of the test groups had an increase in metabolic activity over the course of the study (Figure 5.8). Cells isolated from the bone particles of patient #5002 had metabolism levels comparable to levels calculated for cells from the human stem cell line. Significantly ($p < 0.05$) higher absorbance values were observed for cells isolated from the fat layer of patient #5003, cultured in OB medium, than were observed for any other group on Day 14 and Day 21.

Intracellular Protein

Values for intracellular protein levels (Figure 5.9) are extracted using a standard curve; therefore, negative values represent levels of protein that were less than could be calculated accurately using the standard equation. Cells from the stem cell line control group and cells isolated from the fat layer of patient #5003 had the highest levels of intracellular protein over time. Visual examination of cells confirmed that these two groups also had an increased number of cells over the course of the study.

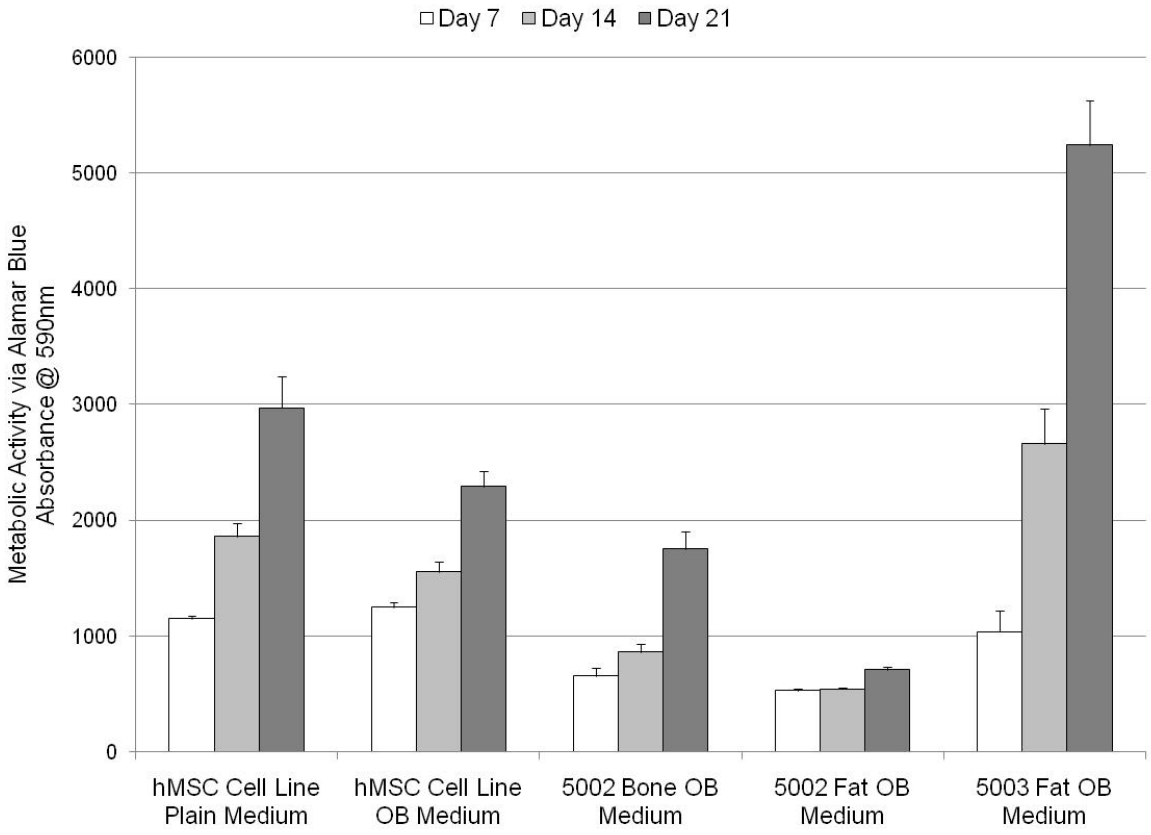

Figure 5.3: Metabolic activity of cells as a function of time and cell origin. Cells Isolated from patient #5003 had significantly (as indicated by asterisks, $p < 0.05$) higher levels of metabolic activity on Day 14 and Day 21 compared to all other test groups. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

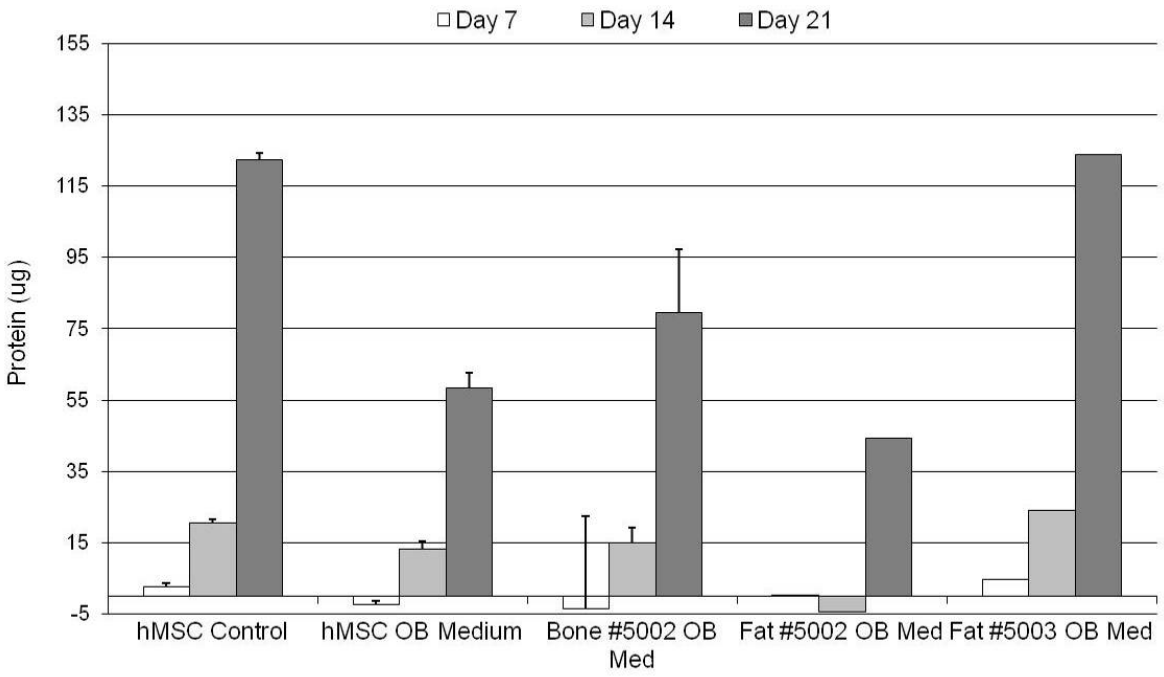

Figure 5.9: Total intracellular protein levels measured for cells as a function of time and cell origin. Each data point represents the mean value of n=3 data samples and error bars denote SEM.

Alkaline Phosphatase Assay

Alkaline phosphatase levels were normalized to intracellular protein values (Figure 5.10). Normalized ALP values show that control hMSCs and cells isolated from the bone particles of patient #5002 had an initial spike in ALP levels, followed by a steep drop at Day 14. The cells isolated from the fat layer of patient #5003 increased in ALP levels over time and were significantly higher than other groups on Days 14 and 21. Negative values reported for ALP are attributed to the protein values used to normalize ALP activity. Non-normalized values show an increase in expression over time for all groups (data not shown).

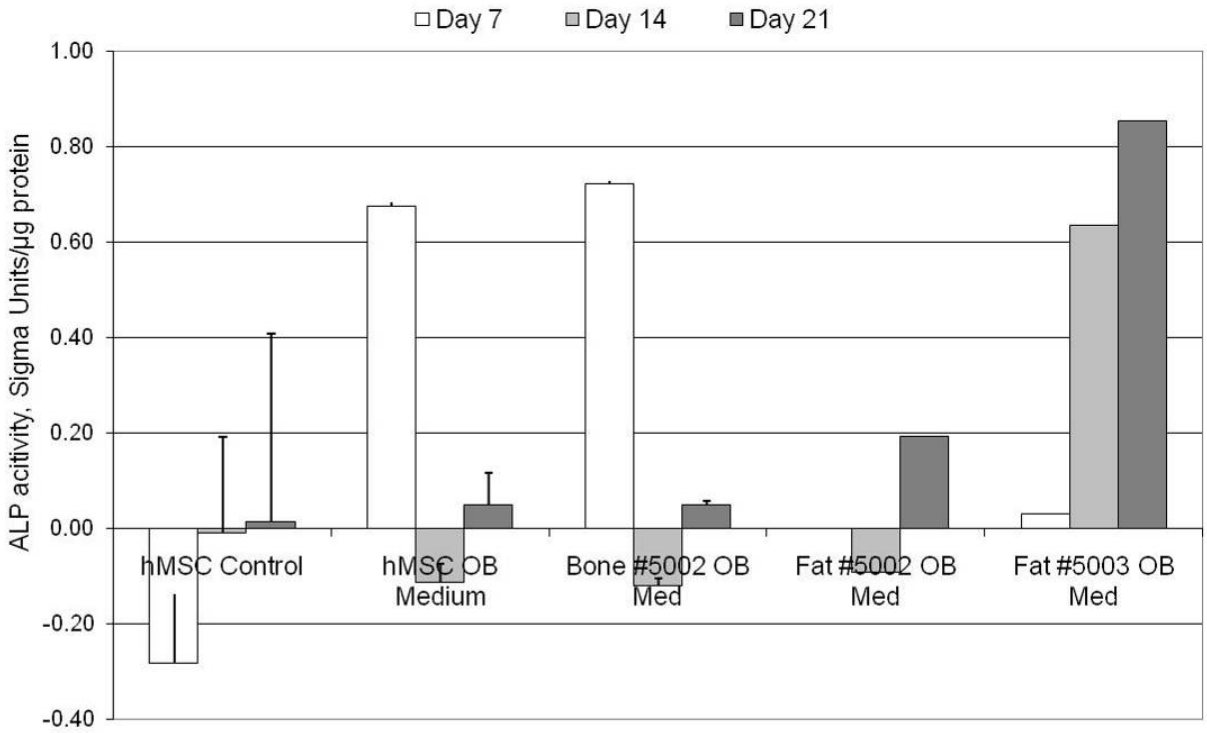

Figure 5.10: Alkaline phosphatase synthesized by cells as a function of time and cell origin. Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Extracellular Calcium Levels

Extracellular calcium deposition (Figure 5.11) was measured after centrifuging the samples to ensure cell debris did not interfere with the results of the test. For the first two weeks there were no significant amounts of calcium present in any of the samples compared to the control group. After 21 days of incubation, cells isolated from the aspirate fat layers of patient #5002 and patient #5003 had significantly greater amounts of extracellular calcium present in their samples compared with all other test groups. With regards to

calcium deposition, it was evident that cells isolated from different patients exhibited very similar levels of this osteoblast indicator.

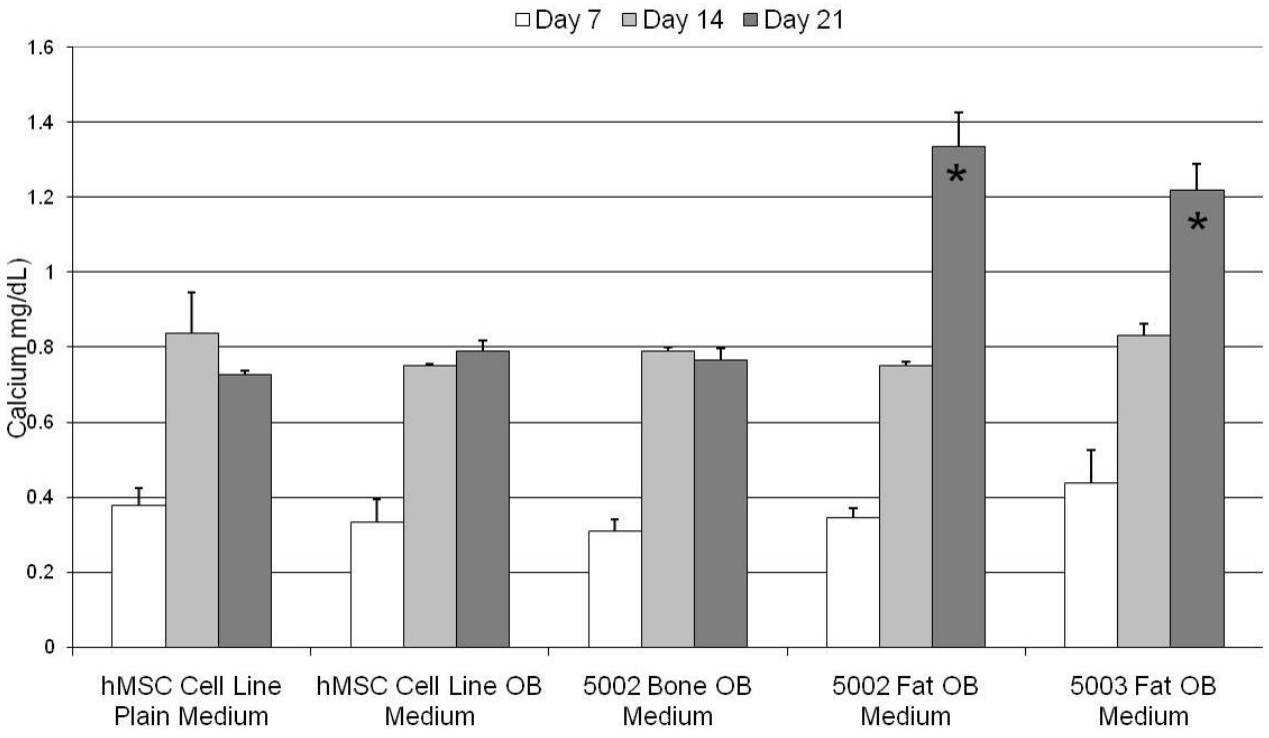

Figure 5.41: Extracellular calcium deposition by cells as a function of time and cell origin. Asterisks (*) denote significant differences in calcium levels compared to stem cell line and cells isolated from bone fragments ($p < 0.05$). Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

5.4 Discussion

Previous research has examined the benefits of using RIA-harvested bone particles as autograft material. Kobbe and coworkers found that the autologous bone granules facilitated healing of a non-union tibial fracture after the more traditional autograft collected from the iliac crest had failed in a human patient [16]. In a sheep study, re-implantation of these particles resulted in callous formation within a tibial defect that had significantly increased volume stiffness and strength compared to callus formed in the absence of RIA bone particles [17]. Minimal characterization has been conducted on the bone particles, which are reamed from cancellous bone, but they have been found to possess higher levels of growth factors connected to bone formation than material taken from the iliac crest [16]. Additionally, Porter and colleagues have had success growing fibroblastic cells from explants taken from the bone layer of aspirate [18]. We have been able to achieve similar cellular outgrowth from the osseous particles and believe that our group is one of the first to also successfully isolate cells from the fatty layer of RIA aspirate. Our isolation procedure has produced consistent results. We were able to successfully isolate cells from the fatty layer of RIA aspirate using the same techniques with multiple patient samples. Cellular outgrowth was achieved from the fat tissue with and without the use of enzymatic digestion.

Further evaluation of the fatty layer revealed that the bulk material is mainly composed of palmitic, stearic, oleic and linoleic fatty acids. These four accounted for more than 80% of the total fatty acid content. This fatty acid composition did not seem to be affected by the patient's sex or age. The most abundant fatty acids in our samples, oleic and palmitic, have also been identified as the most abundant fatty acids in subcutaneous human adipose tissue and in bone marrow aspirates [19, 20]. Similar fatty acid composition have been

reported for subcutaneous fat sample taken from various areas of the body [19]. The significance of the fatty acid composition within the RIA aspirate and within the bone marrow is being explored to determine if the major fatty acids have any role in bone cells development. Deshimaru and coworkers report that oleic acid promotes the differentiation of osteoblast when bone morphogenetic protein-2 (BMP-2) is present in cultures of a mouse mesenchymal cell line [20]. Oleic acid has also been attributed to increased osteoblast expression of Type I collagen and fibronectin in *in vitro* studies using human osteoblast-like cells [21]. Conversely, studies have found that infants fed formula supplemented with oils rich in palmitic acid have reduced levels of bone mineral density after 3 and 6 months [22]. The results presented here warrant further investigation into the role of fatty acids in bone formation.

Cells isolated from the aspirate fat layer grew successfully on three-dimensional ceramic substrates. Any of the materials currently in use as bone grafts or graft substitutes can potentially be used as scaffolds for tissue-engineered constructs. The ideal scaffold material must possess a number of important properties including: ability to induce cellular growth and new tissue formation, ability to support vascularization in a timely manner, ability to resorb at a rate consistent with new bone growth, and ability to be readily sterilized [23]. Results of the metabolic activity and intracellular protein assays indicated that the β-TCP granules supported growth of all of the cells used in the study and could be combined with RIA aspirate autologous material to form a graft expander.

Cells isolated from the fatty portion of the bone marrow have the capability to mature along both the adipocyte and osteoblast cell lineages [3]. Although levels of ALP were low in this preliminary study using cells from the fat layer of RIA aspirate, the β-TCP material

has been shown to successfully support the growth of bone tissue *in vitro* [24]. Cells will not transition along the osteogenic pathway when they are actively dividing; the textured surface and increased surface area of the ceramic granules may have stimulated higher levels of proliferation., and, therefore, delayed differentiation and release of the marker for maturing osteoblasts. This active growth surface may have contributed to the lack of ALP measured in our studies. Future work will focus on better quantifying cellular growth.

The ideal conditions for storage and expansion of cells for use in tissue engineering applications is an area of great interest. Liu and colleagues determined that liquid nitrogen was a reliable storage method for stem cells derived from human lipo-aspirate. Their group found that this preservation method had no detrimental effect on the ability of the adipose-tissue derived cells to proliferate, express osteogenic genes (ALP and osteocalcin), or deposit calcium after storage. Their cells were cultured in osteogenic medium comparable to that used in our RIA aspirate studies [25]. Results from our second differentiation study, using cells isolated from two different patients, revealed that the aspirate cells may have experienced an initial lag in growth following long-term storage in liquid nitrogen. Cells were seeded at a low density in order to avoid cell lifting during the 21 days of culture, and cells were fed differentiation medium at Day 0, which may have contributed to a lag in proliferation which can result in low intracellular protein values. Also, cells from patient #5003 were frozen for a shorter period of time than the other cells. Data from this test group at the initial time points were more consistent with data gathered in previous differentiation experiments.

It is important to note that RIA aspirate samples do not always contain distinct layers of fat and bone particles. This was the case with patient #5003. The RIA system can be set up

to filter bone particles out of the aspirate; if this step is not executed there may be some mixing of the bone and fat layers. Although the fatty layer was very distinguishable in the sample from patient #5003, it was not separated from the bone particle with a filter, as was accomplished with the sample from patient #5002. The higher levels of protein and ALP activity from the fat layer of patient #5003 may be a result of a synergistic effect that may occur when there is mixing of these two aspirate layers, and cells are isolated from the resultant mixture. Synthesis of the osteoblast protein ALP in greater amounts by the cells isolated from the fatty layer of aspirate than by cells from the purchased cell line indicated that the aspirate cells may have increased osteogenic potential. The calcium deposition assay results supported this conclusion. An important observation of the second differentiation study was the similarities that were displayed for the cells isolated from different patients. This was particularly evident in calcium deposition data. A major drawback of current bone graft substitutes is the inconsistent results that have been recorded with their use, and these data indicate that the cells isolated from the fat layer aspirate may be capable of more consistent mineral deposition [26, 27].

This preliminary study supports the hypothesis that the fatty portion of RIA aspirate may be an untapped source for progenitor cells that could be beneficial to bone formation if introduced at the site of fracture repair. Gaining a better understanding of the potency potential of cells from this novel source of autologous material will aid in the development of a cell-based bone graft system that may be used in place of the traditional autograft or the less osteoinductive bone graft substitutes.

5.5 Conclusions

The results of this study suggest that cells isolated from the fat layer of RIA aspirate proliferate on ceramic bone void filler and have the potential to differentiate along an osteoblast pathway. Generally considered waste in the operating room, the lipid-rich fat layer of aspirate may be a source of mesenchymal stem cells that could be used to stimulate new bone growth, alone or in conjunction with currently available synthetic bone graft material. The fatty layer could also be easily combined with the boney layer of RIA aspirate, and has the potential to enhance osteogenic growth at the site of repair.

5.6 References

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CHAPTER SIX

EFFECT OF OSTEOCLAST COCULTURE ON THE DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS GROWN ON CERAMIC BONE GRAFT GRANULES

6.1 Background

The physiological connection between osteoclasts and osteoblasts, as discussed in Chapter 1, has been closely examined using *in vitro* models. These models have consisted of a variety of configurations, with and without direct contact between the two bone cell types. Successful co-culture experiments have been conducted on two-dimensional cell culture plates and on three dimensional substrates. Initial studies performed in our laboratory were conducted with the two cell types cultured in separate vessels in order to evaluate the effects of osteoclast conditioned medium on mesenchymal stem cell (MSC) maturation to osteoblasts (Chapter 2). Results of this work suggested that the differentiation of MSCs into osteoblasts can be affected by the presence of osteoclast conditioned medium [6]. Briefly, exposure to conditioned medium had a positive effect on intracellular protein levels of MSCs, but inhibited activity of the bone cell marker alkaline phosphatase (ALP). Pederson and colleagues also concluded that osteoclast conditioned medium had no impact on the activity of ALP by mesenchymal stem cells; however, cellular migration and mineralization were positively influenced by the conditioned medium [13].

Osteoclast conditioned medium used in these studies was collected from monocytes differentiating into osteoclasts on two dimensional polystyrene surfaces; however, in normal

physiological conditions osteoclasts actively resorb three-dimensional bone mineral prior to the deposition of new bone matrix by osteoblasts[1, 12]. To better model this physiological environment, ceramic scaffolds have been employed to mimic the mineralized component of bone tissue for studies examining osteoclast resorption. Loomer and coworkers designed a co-culture setup based on the theory that osteoclasts may influence bone formation through excretion of cytokines [10]. The experimental configuration consisted of osteoclast cells seeded onto mineral coated discs (to promote resorption) that were suspended above growing stromal cell and separated by a mesh. The porous mesh allowed fluid and cytokine exchange without direct cellular contact. Loomer's group concluded that the simultaneous 'cross-talk' that occurred between the two bone cell types resulted in the inhibition of both osteoclast resorption and osteoblast maturation. Initial studies performed in our laboratory employed a similar design, with osteoclasts suspended in co-culture with MSCs; however, successful osteoclast proliferation was not achieved. It is believed that the setup had inadequate area for proper osteoclast development; this deficiency may have been a contributing factor to the lack of resorption observed in the Loomer study. Tortelli and colleagues designed a co-culture system of osteoblasts and osteoclasts seeded simultaneously onto resorbable tricalcium phosphate discs. The three-dimensional culture condition was found to be advantageous to differentiation and mineral deposition of the osteoblasts and it also increased osteoclast development [18]. Recent studies have proposed that osteoclast resorption can induce osteoblast differentiation. Spence and coworkers concluded that osteoclasts 'condition' the surface being resorbed with a bioactive layer that positively affects the response by osteoblasts [16].

Based on the works described above, it is our belief that the act of resorbing mineral may enable osteoclasts to induce osteogenesis. The commercially available β-tricalcium phosphate ceramic bone graft substitute, chronOS (Synthes, Inc.), contains two of the major minerals found in bone and is already used clinically for fracture repair. Furthermore, the resorption of chronOS has been attributed to osteoclasts *in vivo*. These attributes, coupled with the availability of the material, made chronOS a desirable substrate to use within this study. The purpose of our study was to determine if osteoblast differentiation is stimulated when mesenchymal stem cells are co-seeded with osteoclasts on a three-dimensional resorbable surface.

6.2 Methods

Cell Seeding

Murine monocytes, which are indicated for use as osteoclast precursor cells (RAW 264.7; Sigma) were seeded (100,000 cells per well) onto 0.13 grams of ceramic granules (chronOS; Synthes) contained within 12-well culture plates. The well plates were not tissue culture treated. To produce osteoclasts, soluble RANK-ligand (RANKL) was mixed with serum-containing medium at a concentration of 30 ng per ml. A volume of 2 ml of the solution was added to each well. Culture medium was changed every 48 hours for the duration of the study. After four days in culture, human MSCs (hMSCs; PT-2501; Lonza) were added to the wells (70,000 cells per well) containing the ceramic granules pre-seeded with RAW 264.7 cells. Four additional groups of hMSCs were seeded onto granules contained within 12-well non-tissue culture treated plates, as outlined in Table 6.1. Manufacturer recommended mesenchymal stem cell growth medium (MSCGM) was used as 'plain medium'. MSCGM

supplemented for osteogenesis (10mM B-glycerophosphate, 50ug /ml ascorbic acid, and 0.1 μM dexamethasone) was used as osteogenic medium (OB). Medium was removed and replaced every 48 hours for all test groups. All plates were kept under the standard culture conditions of 37°C and 5% carbon dioxide (CO_2).

Table 6.1: Description of Study Test Groups Cell Types and Medium used within Each Test Group		
Test Group	**Cells**	**Culture Medium**
Plain	Human mesenchymal stem cells	MSCGM : Plain mesenchymal stem cell growth medium
OB	Human mesenchymal stem cells	OB medium: MSCGM supplemented with 10mM B-glycerophosphate, 50ug/ml ascorbic acid and 0.1 μM dexamethasone
OBR	Human mesenchymal stem cells	OB medium supplemented with 30 ng per ml soluble RANKL
OBOC	Osteoclast precursor cells co-seeded with hMSCs	OB medium supplemented with 30 ng per ml soluble RANKL
preOC	Osteoclast precursor cells seeded four days before addition of hMSCs	OB medium supplemented with 30 ng per ml soluble RANKL

Cell Tracker™ Probes

CellTracker™ fluorescent probes (Molecular Probes; Eugene, OR) were used to visualize each cell type with fluorescent microscopy. These trackers pass through cellular membranes and are retained by living cells. The probes remain visible for approximately 72 hours. CellTracker™ Green CMFDA (cat. Number C2925) was applied at a concentration of 20 μM to RAW 264.7 osteoclast precursor cells; the Red CMTPX (cat. Number C34552) was applied at a concentration of 20 μM to the hMSCs. The manufacturer staining protocol was used in this study. Briefly, the dyes were diluted to a final working concentration of 20 μM in serum-free medium and warmed to 37°C. At cell seeding time, the dye solution was added to a monolayer of cells growing in a tissue culture flask. After 45 minutes of incubation at 37°C, the dye solution was replaced with warm serum-containing MSCGM and

allowed to incubate for an additional 30 minutes. Following the final incubation, cells were rinsed with phosphate buffered saline (PBS) solution, trypsinized to release them from the surface of the culture flasks, and seeded onto ceramic granules as described in the previous section. Images of the cells were taken using a Zeiss Axiovert 40 cFL microscope (Carl Zeiss MicroImaging, Inc.; Thornwood, NY). Digital images were captured using an Axiocam mRc5 camera and AxioVision Rel 4.6 software, both from Zeiss.

LIVE/DEAD® Viability Assay

Cell viability was assessed on Day 8 and Day 17 via fluorescence microscopy. Cells cultures were exposed to cell-permeable calcein acetoxymethyl (calcein AM; Molecular Probes) and ethidium homodimer-1 (EthD-1; Molecular Probes). Green-emitting calcein AM (excitation 485 ±10 nm) is retained within live cells while EthD-1 produces red fluorescence (excitation 530± 12.5 nm) in damaged or dead cells after binding to nucleic acids. After rinsing with PBS, a solution of 20 μl EthD-1 and 5 μl calcein AM per 10 ml of PBS was added to the wells designated for the LIVE/DEAD® assay. After 30 minutes of incubation at 37ºC, cells were imaged using a Zeiss Axiovert 40 cFL microscope (Carl Zeiss MicroImaging, Inc.) with an Axiocam mRc5 camera and AxioVision Rel 4.6 software, both from Zeiss.

PicoGreen Assay

The Quant-iT™ PicoGreen® dsDNA assay kit from Molecular Probes (P7589; Molecular Probes) was used to quantify the amount of DNA present in the test samples. This method employs a fluorescent nucleic acid stain that is excited at 480 nm and

specifically targets double-stranded DNA contained within cell nuclei. Cell quantities were calculated using a standard curve generated by plotting the fluorescence emission intensity (520 nm) versus DNA concentration of a set of known standards. On Days 1, 4, 8, and 16, samples were frozen in 1ml of 1X TE Buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) for use in PicoGreen® analysis. At the conclusion of the study, a working aqueous solution of Quant-iT™ PicoGreen® reagent diluted (1:200) with 1X TE buffer was added, at a quantity of 200 µl, to each well of a black 96-well plate containing 2µl aliquots of thawed test samples. After 5 minutes of incubation, fluorescence intensity values of the samples were read with a Fluoroskan Ascent® FL fluorometric plate reader (Labsystems; Franklin, MA). Excitation and emission values used for reading were 480 nm and 540 nm, respectively. All samples were tested in triplicate and results are reported as the calculated mean of n=3 samples.

Metabolic Activity

Metabolic activity of the cell cultures was assessed via the alamarBlue assay, as described previously in Chapter 2. The assay was performed every four days, beginning on Day 2, in order to ensure that residual dye was removed from the wells and that each reading was conducted on culture medium that was in culture for two days. All samples were tested in triplicate and results are reported as the calculated mean of n=5 samples.

Alkaline Phosphatase Assay

Designated plates were frozen on Days 4, 9, and 17 for use in this end-point assay. After three freeze-thaw cycles, the alkaline assay was performed at the conclusion of the study as described previously in Chapter 2. All samples were tested in triplicate and results are

reported as the calculated mean of n=5 samples. Reported values were normalized to intracellular protein levels.

RNA Isolation

RNA isolation was performed using the "Purification of Total RNA from Animal Cells Using Spin Technology" protocol described in the QIAGEN RNeasy® mini kit manual (QIAGEN; Valencia, CA). On Days 4, 9, and 17, RNA isolation was performed on five samples within each test group. Buffer RLT from the kit was mixed with 10 µl per ml of β-mercaptoethanol (β-ME) prior to use. A volume of 350 µl of Buffer RLT was added to all wells containing test samples, to lyse the cells. After addition of Buffer RLT, samples were placed on an orbital plate shaker for 2.5 hours before continuing with the isolation procedure. This modification to the manufacturer procedure was developed to increase the efficiency of RNA isolation from cells attached to ceramic granules. Sample lysates were homogenized using QIAshredder spin columns supplied in the kit, by centrifuging for 2 minutes at full speed (14,000 RPM). An equal amount of 70% ethanol was added to each homoginized lysate (350µl) and mixed thoroughly. Following transfer to an RNeasy® spin column, samples were subjected to a series of centrifuge cycles with appropriate buffers (Appendix A). RNase free water (50 µl) was used to elute the RNA from the spin columns, and capturing the eluent and RNA in collection tubes. RNA samples were stored at -80ºC until analysis.

RNA Analysis

RNA sample quantities and integrity were checked using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay Kit (Agilent Technologies; Palo Alto, CA) prior to conducting RT-PCR analysis. Isolated RNA samples were processed according to the manufacturer's protocol on RNA 6000 Nano LabChips® (Appendix B). RNA concentration values were calculated on the bioanalyzer using Agilent 2100 Expert Software and reported as ng per ml. Samples with an RNA Integrity Number (RIN) of 6 or greater were used for PCR analysis.

Real-Time Quantitative RT-PCR

The following phenotype-specific genes were chosen to identify osteoblast maturation: runx2, alkaline phosphatase (ALP), and bone sialoprotein (BSP). Forward and reverse primers (5'→3') were chosen for each gene of interest from published studies that have determined genetic expression using the same cells lines employed in the present work (Table 6.2). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. All custom 25nmole DNA oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA).

Table 6.2: Primer Sequences Used in SYBR Green PCR

Gene	Forward Primer Sequence 5' to 3'	Reverse Primer Sequence 5' to 3'	Reference
Runx2	GACGAGGCAAGAGTTTCACC	ATGAAATGCTTGGGAACTGC	[8]
ALP	TGGAGCTTCAGAAGCTCAACACCA	ATCTCGTTGTCTGAGTACCAGTCC	[9]
BSP	AATGAAAACGAAGAAAGCGAAG	ATCATAGCCATCGTAGCCTTGT	[9]
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT	[8]

Real-time, one-step RT-PCR was conducted with the Quantifast™ SYBR® Green RT-PCR kit (cat. No. 204154; QIAGEN; Valencia, CA) using the reaction mix according to volumes listed in Table 6.3. All primers were diluted to a final concentration of 30 pmol/μl and RNA samples were diluted to 10 ng /μl. No-template and minus-RT control samples were mixed using RNase-free water instead of template RNA and RT mix, respectively. Both controls were included in each set of PCR samples. All kit components (Master Mix, template RNA, RNase-free water and RT Mix) were stored at -20ºC and maintained on ice during the procedure. RNA samples were thawed on ice before aliquoting into individual PCR vessels. All vessels were vortexed before being placed in the real-time cycler.

All PCR reactions were conducted on a Rotor-Gene RG-3000 (Corbett Research; Sydney Australia) using Rotor-Gene 5.0.28 software (Corbett Research). The real-time cycler was programmed as outlined in Table 6.4. A melting curve analysis was performed with the software during each run to verify the primers' specificity to the target genes.

Table 6.3 SYBR Green Reaction Setup

Component	Volume/Reaction (μl)	Final Concentration
2x QuantiFast SYBR Green RT-PCR Master Mix	12.5	1x
Forward Primer	1	1μM
Reverse Primer	1	1μM
QuantiFast RT Mix	0.25	100ng/reaction
Template RNA	10	
RNase-free water	0.25	
Total reaction volume	**25 μl**	

Table 6.4: Real-time Cycler Conditions			
Step	**Time**	**Temperature (ºC)**	**Additional Comments**
Reverse transcription	10 min	50	Hold
PCR initial activation step	5 min	95	Hold
Two-step cycling			
Denaturation	10 s	95	
Combined/annealing extension	30s	60	Data acquired during this step
Number of cycles	40		

RT-PCR Analysis

The mRNA levels for each gene of interest were calculated using the comparative C_t method, also known as the delta delta C_t ($\Delta\Delta C_T$) method. Means ± standard error of the mean were calculated from five samples (n=5). The threshold for determining C_t values was taken from the linear region of the dRNA vs. cycle number plot, using a linear scale. The GAPDH C_T values were used as the reference in the equations:

$$\Delta\Delta C_T = \Delta C_{t,\,sample} - \Delta C_{t,\,control} \qquad \text{(Equation 6.1)}$$

$$\Delta C_{t,\,sample} = \Delta C_{t,\,target} - \Delta C_{t,\,reference} \qquad \text{(Equation 6.2)}$$

$$\Delta C_{t,\,control} = \Delta C_{t,\,target} - \Delta C_{t,\,reference} \qquad \text{(Equation 6.3)}$$

Day 9 plain medium (Plain) values were used to calculate the ΔC_T control values. Final relative gene expression ratio data were determined using the equation:

$$\text{Relative Expression Ratio} = 2^{-\Delta\Delta CT} \qquad \text{(Equation 6.4)}$$

MouseTRAP™ Assay

An enzyme-linked immunosorbent assay (ELISA) specific to osteoclast secreted tartrate-resistant acid phosphatase isoform 5b (TRAP) was used to examine osteoclast activity. Samples for use in the ELISA were taken during medium changes from the test groups that contained RAW 264.7 cells OBOC and preOC. Medium samples were stored at -80ºC until the conclusion of the study. The protocol and reagents supplied with the MouseTRAP™ assay (Immunodiagnostic Systems Ltd; Fountain Hills, AZ) were used in this study. The Anti-MouseTRAP Antibody was reconstituted in 10.5 ml of distilled water and added (100µl) to appropriate wells of the Antibody Coated Plate. The plate was then incubated for one hour at room temperature, on a shaker plate(IKA Vibrax VXR; IKA Works; Wilmington, NC). Following incubation, the plate was washed manually with the Wash Buffer solution. To wash, 250µl of Wash Buffer were added to each well of the plate with a multichannel pipette & then decanted. This wash was repeated four times. Calibrator and Control solutions were added (100 µl) to the designated wells of the Antibody Coated Plate, in duplicate. Aliquots of the collected medium samples (25µl) were also added to the plate in duplicate, mixed with 0.9% NaCl (75 µl per sample). The assay Releasing Agent was added to all wells of the plate (25 µl), followed by another hour of incubation at room temperature on a shaker plate. A volume of 100 µl of fresh Substrate solution (2 Substrate Tablets in 10 ml Substrate solution, all supplied) was added to each well after washing (x4). After addition of the Substrate, the plate was sealed with an adhesive cover and placed in a cell incubator for 2 hours at 37ºC. Next, a Stop Solution was added to all of the wells (25 µl) and the absorbance of each sample and control was measured at 405 nm after mixing. A standard

curve was developed using the Calibrator solutions and was used to determine the concentration of TRAP in the test samples.

Statistical Analysis

Each data point represents the mean value of n=3 data samples, and error bars denote the standard error of the mean (SEM). The statistical software SAS® was used to compare the effects of medium and time on the metabolic activity, intracellular protein content, alkaline phosphatase activity, and calcium deposition levels of the isolated cells and the control mesenchymal stem cells using the Least Squares Means (LSMEANS) command. A significance level of $p < 0.05$ was used for all comparisons.

6.3 Results

Cell Tracker™ Probes

Both the RAW 264.7 osteoclast precursor cells (green) and hMSCs (red) were visible on the ceramic granules after uptake of the Cell Tracker Probes. The RAW 264.7 seeded prior to the addition of hMSCs were still present at the time that the stem cells were introduced. Co-seeding was successfully achieved with the two cell types and images of both cell types attached to the ceramic granules were captured using fluorescent microscopy (Figure 6.1). Bothe cell types were still present at 72 hours; however, the green probe was faded and less visible in the images.

LIVE/DEAD® Viability Assay

All five test groups had viable cells covering the ceramic granules through the 17 day study (Figure 6.2). Cultures of hMSCs fed osteogenic medium supplemented with RANKL (OBR) had larger number of dead cells (red) present at Day 17 than the other test groups.

PicroGreen® Cellular Proliferation Assay

The number of cells calculated for each of the test groups increased significantly ($p < 0.05$) between Day 1 and Day 16 of the study (Figure 6.3). As expected, the data indicated that the group pre-seeded with osteoclasts (preOC) contained a greater number of cells compared to all other groups 24 hours after the addition of the mesenchymal stem cells. The addition of RANKL to osteogenic medium had a significant ($p< 0.05$) effect on the proliferation of hMSCs after eight days in culture, when osteoclasts were not present. The OBOC group had a significantly ($p<0.05$) greater number of cells present on Day 4 and Day 8 compared to all other groups; however, no significant differences were detected between the groups at Day 16. Both groups that contained osteoclasts displayed a significant increase in proliferation between Day 4 and Day 8 of osteoclast culture (corresponding to Day 1 and Day 4 of preOC group), which was followed by a gradual decrease.

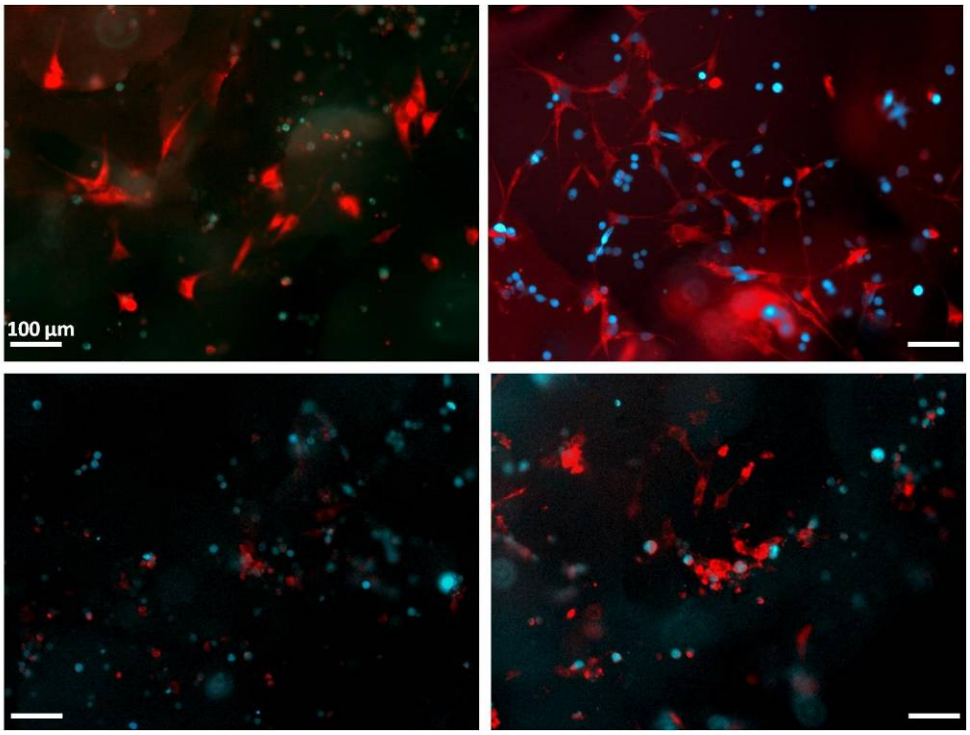

Figure 6.1: Cell Tracker™ Probes Images

Living human mesenchymal stem cells (MSC, red) and RAW 264.7osteoclast precursor cells (OC, green) in culture on ceramic bone graft granules. Top left: OC cells (green, Day 4) 24 hours after addition of MSCs (red). Top right: MSCs and OCs 24 hours after simultaneous seeding. Bottom: MSC and OC seeded 72 hours after simultaneous seeding. Scale bar is 100µm

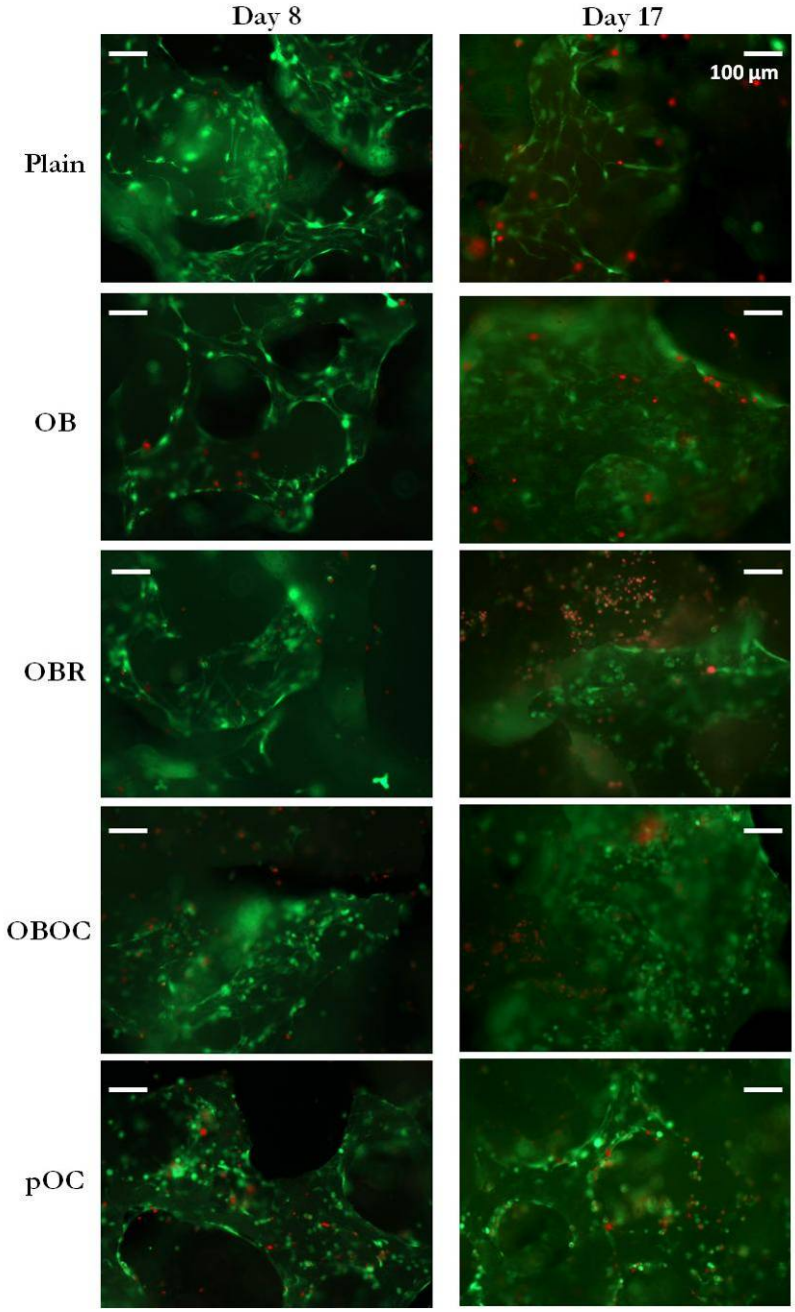

Figure 6.2: LIVE/DEAD Cell Viability Images. Viable/live (green) and damaged/dead (red) cells stained on ceramic granules on Day 8 (left) and Day 17(right). Scale bars are 100µm

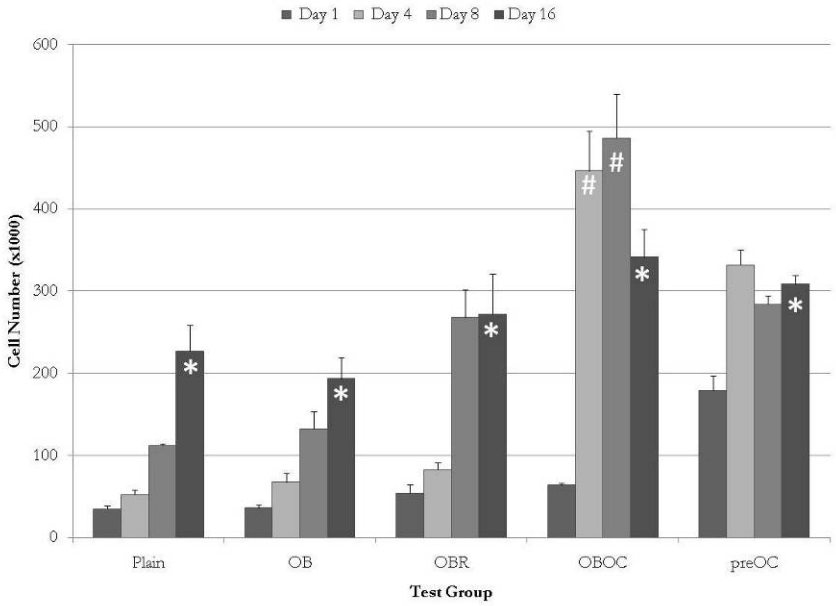

Figure 6.3: PicoGreen® Cellular Proliferation Assay. Calculated cell number as a function of time for each test group. Asterisks (*) highlight that significantly greater number of cells were present in all samples on Day 16 compared to Day 1. The OBOC group had significantly greater number of cells on Days 4 and 8 compared to all other groups (#, $p < 0.05$). Each data point represents the mean value of n=3 data samples, and error bars denote SEM.

Metabolic Activity

Four of the test groups had significant increases in metabolic activity (Figure 6.4) between Day 2 and Day 17: Plain, OB, OBR, and OBOC. As expected, a high level of metabolic activity was recorded for the pre-seeded osteoclast group two days after the addition of the hMSCs. This initial activity was followed by a significant decrease in activity within the preOC group. The pre-seeded group also had a spike in activity on Day 17,

returning to a level that was almost equivalent to Day 2 values. There was no significant difference in metabolic activity between the five test groups.

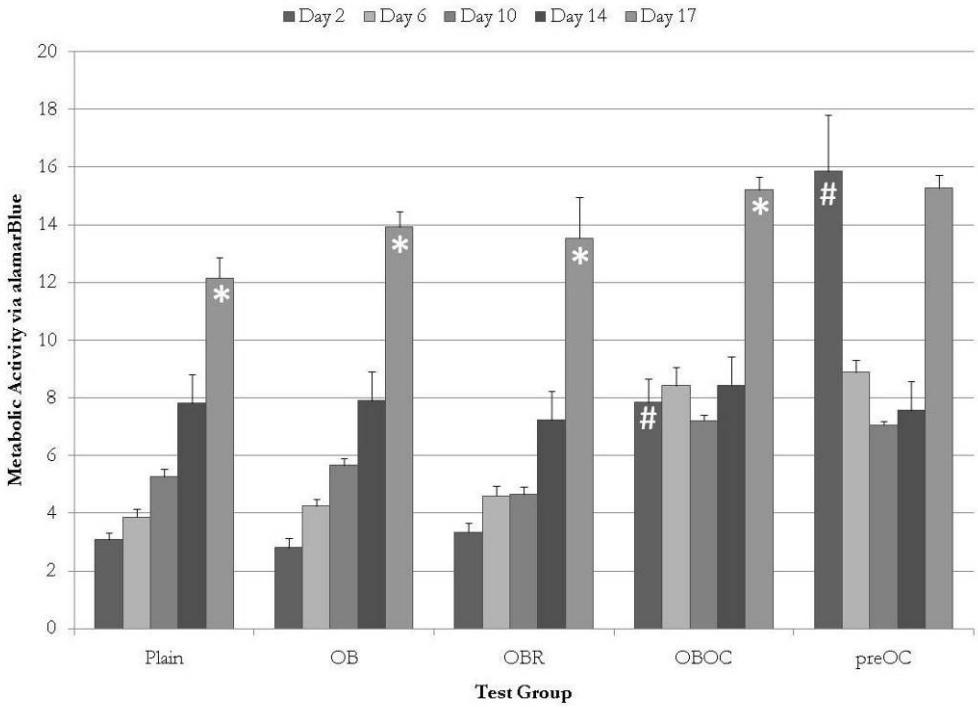

Figure 6.4: Metabolic Activity via alamarBlue™. Metabolic activity levels as a function of time and culture condition. Asterisks (*) denote a significant increase in activity occurred between Day 2 on Day 17 in four of the test groups. The groups with both cell types (OBOC, preOC) had significantly greater levels of activity compared to the single-cell type groups at Day 1 (#, $p < 0.05$). Each data point represents the mean value of n=5 data samples and error bars denote SEM.

Alkaline Phosphatase Assay

Results of this colorimetric assay (Figure 6.5) showed that the groups given osteogenic medium had significantly higher levels of ALP activity on Day 17 compared to Day 4 ($p < 0.05$). At Day 17, the level of ALP was significantly greater in the OB group compared to the other culture conditions. Interestingly, the two groups that contained osteoclasts (OBOC and preOC) had the lowest levels of ALPdetected throughout the course of the study.

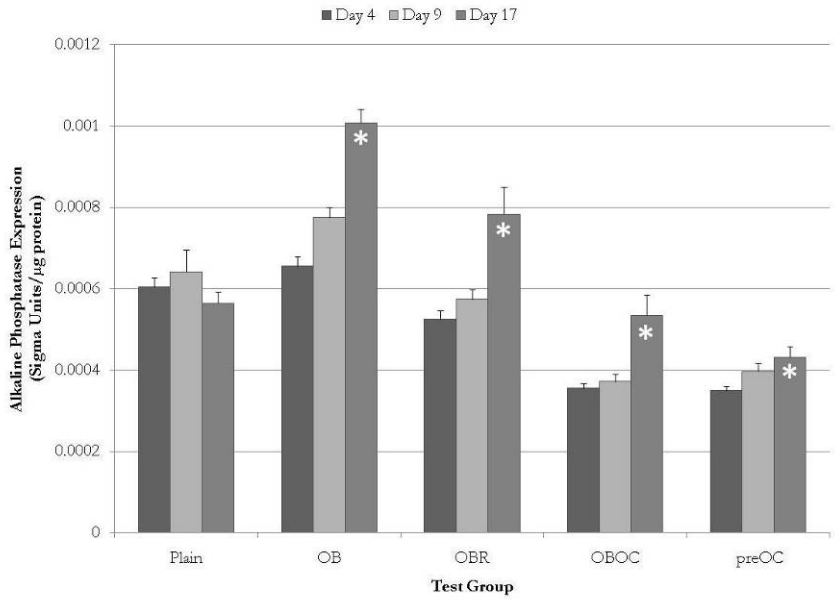

Figure 6.5: Alkaline Phosphatase Activity

Alkaline phosphatase activity as a function time. Asterisks (*) highlight that osteogenic medium groups had significantly higher levels of ALP on Day 17 compared to Day 4 ($p < 0.05$). Groups that contained osteoclasts (OBOC, preOC) had lower levels of ALP overall than the other groups which contained only hMSCs. Each data point represents the mean value of n=5 data samples and error bars denote SEM

RT-PCR Gene Expression

The RNA isolation procedure did not yield enough RNA to test all of the samples with RT-PCR. Gene expression data was collected for all test groups on Day 9, but was only available for three of the groups on Day 17. Reported data values are relative to Day 9 Plain medium test samples that were used as the control in Equations 1-4. The highest value of Runx2 expression was recorded in the hMSC samples that were given osteogenic medium (OB) for 9 days. This group had significantly ($p < 0.05$) higher levels compared to the hMSCs that were co-seeded with osteoclasts (OBOC). The samples that contained osteoclasts expressed the lowest levels of Runx2. No other significant difference in Runx2 expression was observed between the groups.

Calculated levels of ALP expression were not significantly different between the groups on Day 9. Levels for this marker were highest on Day 9 for the hMSCs cultured in osteogenic medium without osteoclasts. After 17 days in culture, both groups containing hMSCs in culture with osteoclasts (OBOC and preOC) had significantly ($p < 0.05$) greater levels of ALP expression compared to hMSCs cultured in the absence of osteoclasts (OBR).

Levels of BSP followed a similar pattern as alkaline phosphatase; cells grown without osteoclasts had significantly higher levels ($p < 0.05$) at Day 9 while cells in co-culture expressed significantly higher levels of BSP after 17 days in culture.

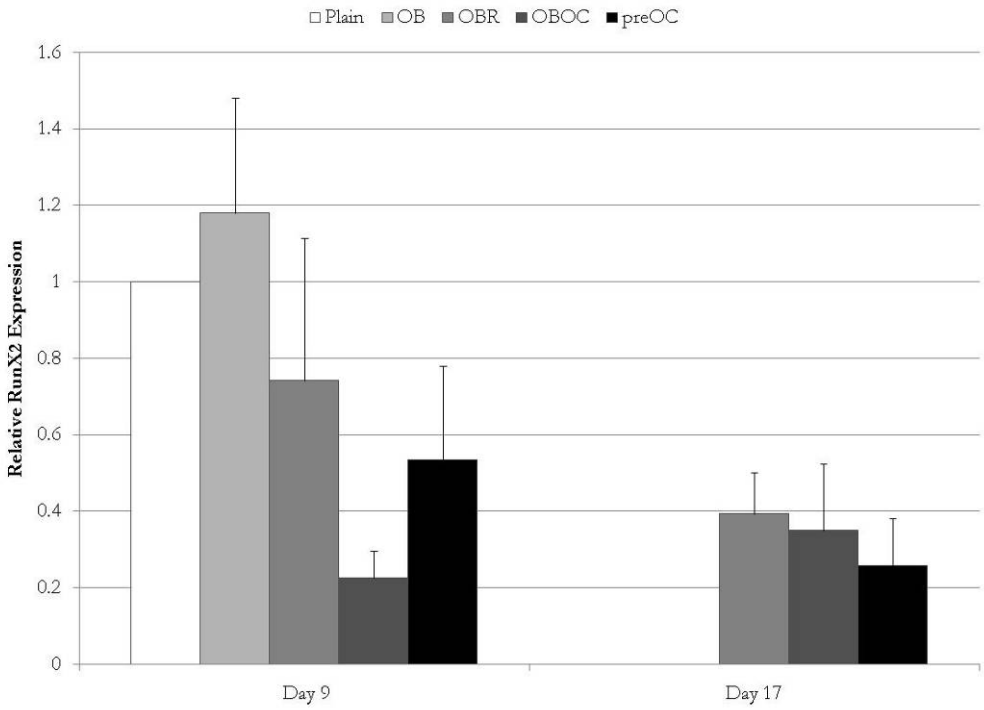

Figure 6.6: Runx2 expression as a function of culture condition and time for hMSCs. All levels are relative to calculated expression of hMSCs cultured in plain medium (Plain) for 9 days. Each data point represents the mean of five values, and error bars denote SEM.

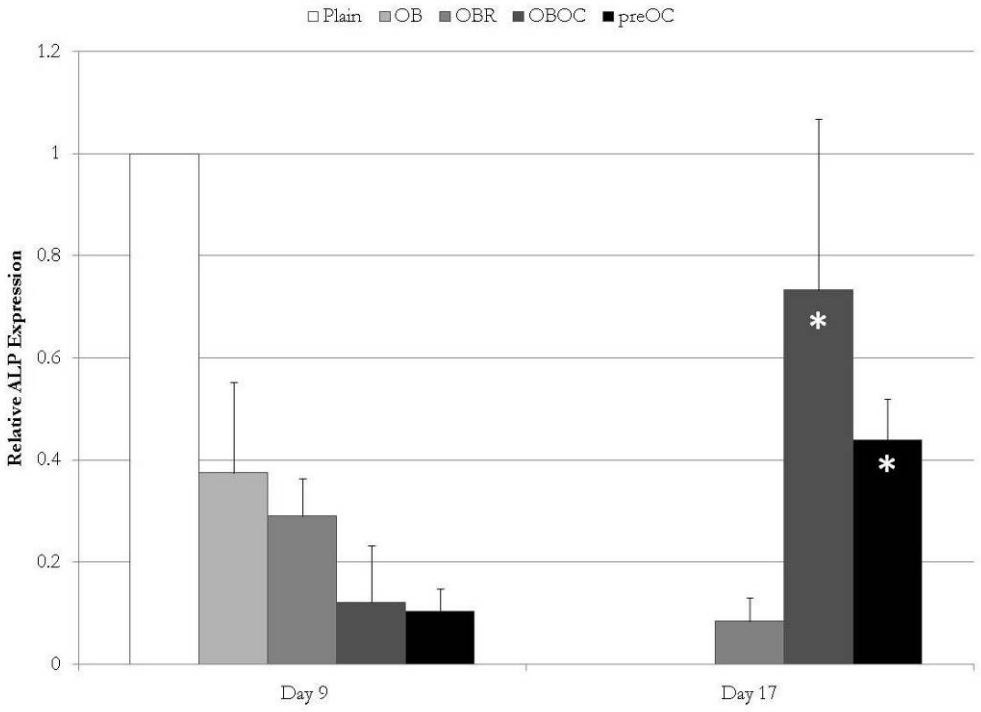

Figure 6.7: Alkaline phosphatase expression as a function of culture condition and time for hMSCs. All levels are relative to calculated expression of hMSCs cultured in plain medium (Plain) for 9 days. Asterisk (*) denotes significantly greater levels of ALP expression compared to the OBR (no osteoclasts) group on Day 17. Each data point represents the mean of five values, and error bars denote SEM.

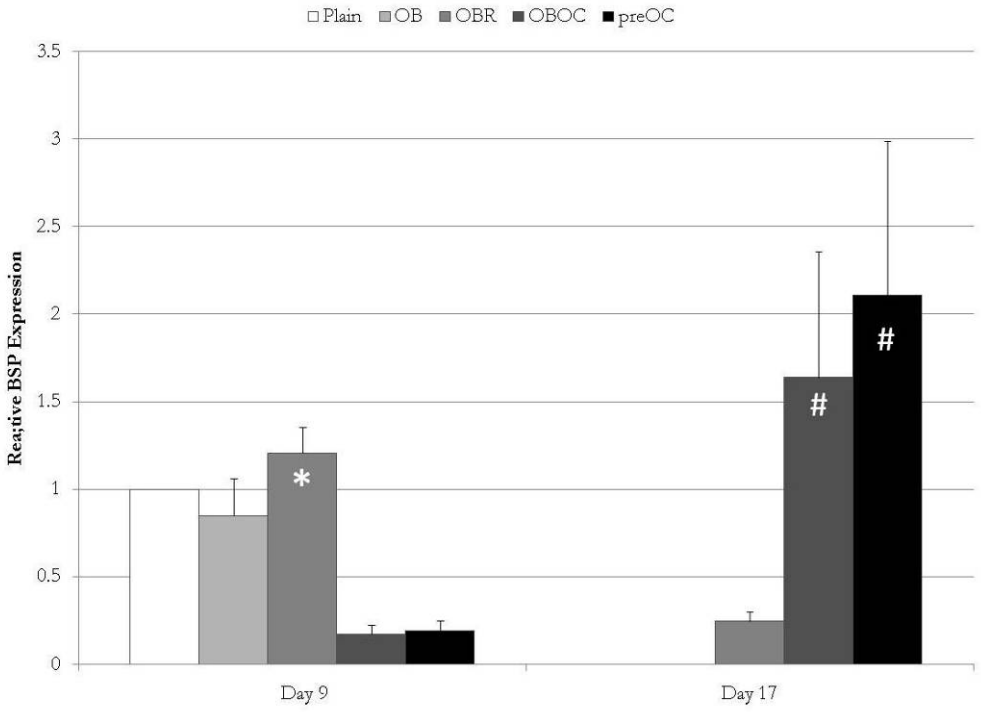

Figure 6.8: BSP expression as a function of culture condition and time for hMSCs. All levels are relative to calculated expression of hMSCs cultured in plain medium (Plain) for 9 days. Asterisks (*) highlight the significantly greater levels of BSP expression for hMSCs grown alone in osteogenic medium with RANKL compared to cells grown in co-culture. Pound signs (#) highlight that the co-culture groups had significantly greater levels of expression after 17 days than the OBR group. Each data point represents the mean of five values, and error bars denote SEM, $p < 0.05$.

TRAP Activity

The concentration of TRAP released into the culture medium samples by osteoclasts is represented in Figure 6.8. Both sets of conditions, osteoclasts pre-seeded and those

seeded simultaneously with mesenchymal stem cells, expressed similar levels through 8 days. After Day 8 the cells pre-seeded on the ceramics had levels outside the detectable range of the standard curve (negative values). Conversely, the TRAP levels of osteoclasts seeded at the same time with MSCs steadily increased through Day 17.

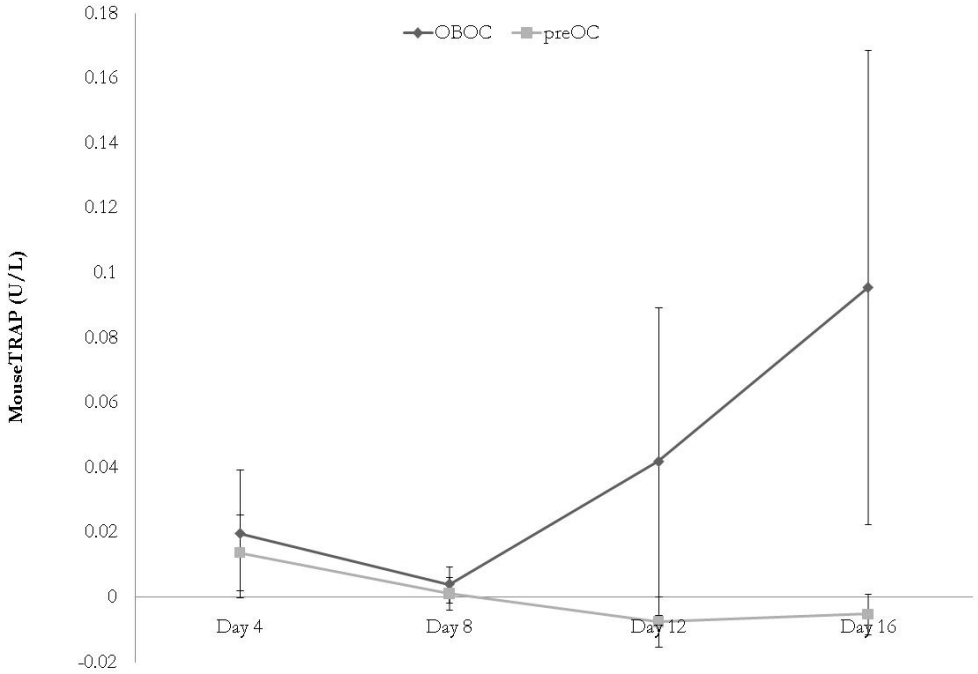

Figure 6.8: TRAP activity by osteoclasts as a function of time.

Osteoclasts seeded simultaneously with mesenchymal stem cells (♦, OBOC) exhibited a steady increase in TRAP levels after eight days in culture. Osteoclasts pre-seeded on ceramics four days before the addition of mesenchymal stem cells exhibited a decrease in TRAP released over the course of the 17 day study (■, preOC). No significance was measured between the two groups ($p=0.08$) Each data point represents the mean of $n=5$ values.

6.4 Discussion

In the present study, an *in vitro* co-culture model was used to examine the effects that osteoclasts have on the differentiation of osteoblasts. This work was conducted with cells seeded onto a clinically available ceramic bone graft substitute that is resorbed by osteoclasts *in vivo*. Preliminary work was conducted with a different co-culture setup that did not result in active osteoclast growth. Fluorescent CellTracker™ Probes (Molecular Probes) and digital imaging enabled us to ensure that RAW 264.7 osteoclast precursor cells were present on the ceramics before hMSCs were added to the preOC group. The probes also made it possible to ensure that both cell types were able to attach to the ceramic substrates when seeded simultaneously in the OBOC group (Figure 6.1). After 72 hours, the probes were no longer visible and the LIVE/DEAD assay was used to confirm that cells in all of the test groups remained viable, proliferated, and remained attached to the ceramic granules throughout the duration of the study (Figure 6.2). Work conducted with cells grown in a monolayer on two-dimensional tissue culture plastic surfaces has been limited by the tendency of the cells to proliferate to confluence and then detach from the surface. Seeding density is often a limiting factor with two dimensional studies that extend beyond 14 days. This limitation may have a negative effect on data collection. For a material to be considered for tissue-engineering applications, it must be able to support viable cells throughout the bulk, both under *in vitro* conditions and eventually when implanted into a bone defect. Often bone graft material is packed around an implant or into a defect site where the fluid flow around it may be limited. The fluorescent images taken during this study of the cells growing on chronOS granules show that these three-dimensional

substrates were able to successfully support the cells for 17 days under static culture conditions with limited fluid exchange.

In our previous studies, cellular proliferation was estimated based on intracellular protein levels. While this method is a good indicator of cell proliferation, it is more of a qualitative assessment and can be influenced by factors other than cell number. The PicoGreen® assay gives a quantitative assessment of cell number based on the amount of double stranded DNA present in the samples and is insensitive to other cell growth indicators. Work done by Forsey and Chaudhuri has shown that PicoGreen® yields accurate results of cell number when cells are seeded within a three dimensional construct [2]. Their results have also indicated that using standards of known cell numbers to generate a standard curve, as in the present study, is more accurate than using the λDNA standard that is supplied in the kit [2]. Further supporting our conclusion that the three-dimensional ceramic granules were successful within this co-culture model, the results of this quantitative assay indicated that the cells in all five of the test groups significantly increased in number over the course of the 17 days ($p < 0.05$). Visual examination of the cells via the LIVE/DEAD assay confirmed this result (Figure 6.2); the ceramic granules were covered in live cells after 8 days in culture and remained covered through Day 17. The pattern of proliferation and metabolic activity observed for the groups that contained osteoclasts were consistent with our previous work that identified three phases of osteoclast differentiation (Chapter 3). Metabolic activity levels supported the results of the PicoGreen assay; however, the fluctuation in activity observed by the co-cultured cells was a good indication that metabolic activity is influenced by factors other than cell number.

Much of the work conducted involving RANKL and bone cells have focused on the expression of this molecule by osteoblasts and how it affects osteoclastogenesis and bone resorption [3, 14]. Little work has been conducted that isolates RANKL as an additive that may affect osteoblast differentiation. Lin and coworkers concluded that the addition of RANKL had no impact on the proliferation of primary osteoblasts isolated from rat calvariae [7]. Conversely, in a 2003 review, Teitelbaum and Ross first hypothesized that RANKL may activate osteoblast precursor cells and promote osteoblast formation [17]. The results of these past studies indicate that the effect of RANKL on osteoblasts is dependent on their level of maturation. Our data indicate this peptide had an effect on the proliferation of osteoblast precursor cells, but this effect was not immediate. An effect on the activation of BSP gene was also seen. This interesting finding will ensure that a control RANKL supplemented group will be incorporated into future studies to isolate how this peptide contributes to the changes seen in mesenchymal stem cells when osteoclast medium (containing RANKL) is incorporated into co-culture models.

The osteogenic medium had a significant impact on the synthesis of alkaline phosphatase by the MSCs when measure with the colorimetric assay, as illustrated by the OB test group. When RANKL was introduced as a variable, the cells did exhibit an increase in ALP levels over time, but at lower amounts than when it was not present in the medium. As discussed previously, exposure to osteoclast conditioned medium has been found to inhibit the expression of the ALP bone cell marker [6, 13]. Even with the change of exposure from conditioned medium to direct contact, the presence of osteoclasts inhibited the activity of ALP by the mesenchymal stem cells in this study. In both instances, there was an inverse relationship between cellular proliferation and ALP activity, which supports the theory that

MSCs will not transition along an osteogenic pathway if they are at a stage of active growth. Enzyme production occurs after active propagation of the cells has decreased.

In addition to the colorimetric assay used previously and within this study to determine the cellular expression of alkaline phosphatase by MSCs, RT-PCR was employed to better examine ALP activation at the molecular level. While it is hard to draw a direct correlation and firm conclusions without successful isolation of all of the RNA samples, both methods suggest that there was an increase in expression of this marker over time. It is important to note that gene expression data collected using PCR indicates that the target gene has been activated; however, it is not necessarily indicative of the amount of cellular synthesis of the protein. Liu and coworkers observed differences between real-time PCR results and results of other quantitative methods used to determine expression of Type I collagen at the biological level. These differences were attributed to the fact that the cell has the ability to move the protein to extracellular space from intracellular space without upregulating genetic expression of the protein at the RNA level [8].

The patterns of expression observed for the other two osteoblast specific markers were consistent with what would be expected for differentiation of osteoblasts, although further work must be conducted to improve RNA isolation techniques. Runx2 is a transcription factor specifically expressed by osteoblast progenitor cells. This factor has a major role in the regulation of other osteoblast-expressed genes, such as osteocalcin, which are initiated during the early stage of differentiation [5, 9]. The levels of Runx2 decreased between Day 9 and Day 17 in this study, suggesting that the gene was present early in the study. Successful isolation of RNA from samples within the first week of culture will be essential to better determine if the expression of this gene is affected by the presence of osteoclasts.

Expression of bone sialoprotein is expected to be highest in differentiating osteoblasts after two weeks in culture [8, 15], and levels were highest at Day 17 in the present study. Both co-culture groups had significantly greater levels of expression of BSP on Day 17 compared to the group seeded without osteoclasts (OBR), but there was no significant difference between the two groups that contained osteoclasts.

BSP is associated with the mineralization that occurs during the late stage of osteoblast differentiation and it has also been linked to the formation of mineral in cancer tumors [4, 19]. Osteoclast conditioned medium has been linked to increased levels of bone mineralization, and these data suggest this important mineralization gene may be influenced by the presence of osteoclasts. Curiously, osteoclasts may be able to influence factors from the initial and final stages of osteoblast differentiation without having a significant effect on markers linked to the middle stage. These findings warrant future investigations.

The maturation of osteoclasts was assessed by testing TRAP 5b activity levels in culture medium collected during the 17 day study. In the physiological environment it is likely that osteoclasts reach a point of terminal activity before bone formation occurs, and this study was used gain insight into the effect that the osteoclast terminal differentiation may have on MSCs that are integrated into the bone graft material. TRAP levels were negligent in the preOC group, but a steady increase in activity was observed for the osteoclasts seeded simultaneously with hMSCs. Gori and colleagues have previously documented the positive effect that undifferentiated MSCs in co-culture with mouse marrow cells have on the formation of osteoclasts. Their work was conducted on two-dimensional plates; these data show that the same positive effect, as measured by TRAP activity, was observed on three-dimensional substrates.

Even though other researchers have observed an increase in osteoblast differentiation on surfaces that have been pre-seeded with osteoclasts, pre-seeding the ceramics with osteoclasts did not have an impact on any of the osteoblast markers tested in this study when compared to the OBOC group. Spence and colleagues removed the resorbing osteoclasts from the ceramics before introducing osteoblast precursor cells. It was thought that the act of trypsinizing and removing the osteoclasts from the three-dimensional substrates would affect the ability of the MSC to attach, but removal of osteoclasts may be explored in the future.

6.5 Conclusions

This study was developed to gain further insight into the role that osteoclasts may play on the differentiation of mesenchymal stem cells when incorporated into a bone graft system. We have shown that the addition of RANKL and the presence of osteoclasts can positively influence the proliferation of mesenchymal stem cells and their expression of late-stage markers osteoblasts differentiation markers in an *in vitro* model. Findings from this study, coupled with the knowledge obtained from our previous work will aid in the development of a clinically viable mesenchymal stem cell based bone graft system.

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CHAPTER SEVEN

CONCLUSIONS

A successful tissue-engineered construct should offer a more desirable alternative to the autologous bone grafts that are currently the preferred choice of surgeons. Ideally, these constructs will possess the osteoinductive and osteogenic properties that are desired by surgeons for proper repair. The incorporation of cells and growth factors within a synthetic bone graft may improve its performance, but the optimal mix of cells, scaffold, and growth factors has yet to be determined. We hypothesized that osteoclasts may play an important role in the recruitment of mesenchymal stem cells (MSCs) to areas of bone resorption and the differentiation of osteoblasts and their presence would have an effect on the differentiation of osteoblasts.

To test this hypothesis, *in vitro* conditions were developed for culturing cells capable of expressing the osteoclast-specific molecule TRAP 5b. Osteoclast differentiation was achieved *in vitro* using cells from a mouse monocyte cell line cultured in medium and supplemented with RANKL. It was determined that the differentiation cycle of the bone resorbing cells could be divided into three phases. Medium from these successful osteoclast cultures was collected and used as 'conditioned medium'. MSCs from an established cell line were inhibited from differentiating along an osteogenic pathway when exposed to the osteoclast conditioned medium. Osteoclast conditioned medium did, however, have a positive effect on intracellular protein levels of MSCs. The phase of osteoclast differentiation from which condition medium was collected had no bearing on the overall effects of the conditioned medium on the development of the osteoblasts.

The effects of osteoclasts on the differentiation of MSCs were observed on both two-dimensional and three-dimensional surfaces. Osteoclasts grew successfully on 3-D ceramic substrates and expressed increased levels of TRAP 5b activity compared to osteoclasts grown on tissue culture plastic. When seeded directly with MSCs on the 3-D substrates, the osteoclasts had an increase in TRAP activity over time if seeded simultaneously. The co-culture setup also resulted in increased proliferation of MSCs. Late stage osteoblast differentiation markers (i.e. calcium, BSP) were also positively affected by both conditioned medium from, and direct co-culture with, osteoclasts. The addition of RANKL to the culture medium to induce osteoclastogenesis appears to be a factor in the observed responses by MSCS, but it is not the only factor influencing the mesenchymal stem cells. Osteoclasts were shown to have an influence on the development of mesenchymal stem cells into osteoblasts when cultured *in vitro*.

The aspirate material obtained from the femoral shaft using the Reamer/Irrigator/Aspirator device (Synthes, USA; Paoli, PA) was evaluated as a potential untapped source of mesenchymal stem cells that could aid in bone formation when incorporated into bone grafts. The fat layer of aspirate, typically discarded in the clinic, was the focus of this work. Cells were successfully and consistently isolated from the fatty layer of RIA aspirate using the same techniques with multiple patient samples. Cellular outgrowth was achieved from the fat tissue with and without the use of enzymatic digestion of the connective fatty tissue of the lipid-rich layer. Fat layer and bone layer aspirate cells had morphology comparable to those from an established mesenchymal stem cell line. Further characterization performed on the fat layer from multiple patient samples showed that the

fatty layer of aspirate was mainly composed of four main fatty acids: oleic, palmitic, linoleic, and stearic.

The isolated cells were successfully expanded using *in vitro* cell culture techniques and they were viable in culture following liquid nitrogen storage. Cells isolated from both the bone and fat layer of reamer aspirate grew successfully on tissue culture plastic and on 3-D ceramic substrates. When grown on ceramic granules, cells isolated from the fat layer of aspirate expressed higher levels of intracellular protein compared to those grown on tissue culture plastic but had lower levels of the osteogenic marker alkaline phosphatase, suggesting that the cells proliferate at higher levels on the ceramics, delaying differentiation. Aspirate cells were, however, able to express the osteoblast cell markers alkaline phosphatase and calcium when cultured in osteogenic differentiation medium on 2-D surfaces and, after extended time periods, on 3-D surfaces. The expression of these markers suggests that these isolated cells have the potential to be driven towards osteoblast phenotype when exposed to select conditions. The results of this work indicate that the fatty layer of RIA aspirate may be a new untapped source for autologous progenitor cells with bone forming capabilities.

CHAPTER EIGHT

RECOMMENDATIONS FOR FUTURE WORK

1. Additional *in vitro* osteoclast cell studies should be performed with human osteoclast precursor cells to determine if the cellular behavior observed in this work using murine cells is consistent across species.
2. Future co-culture studies should be performed within a bioreactor with dynamic fluid flow to allow continuous replenishment of supplements. A bioreactor environment will minimize rapid consumption of available nutrients that may occur win a static system and will facilitate the removal of cellular waste products.
3. Additional methods should be employed to more comprehensively assess osteoblast differentiation from mesenchymal stem cells. Recommendations include:
    a. Use immunofluorescence to detect osteoblast markers such as collagen Type I and alkaline phosphatase.
    b. Examine the expression of additional osteoblast makers, including osteocalcin, with RT-PCR techniques.
4. Improvements should be made to ensure successful isolation of RNA from cells growing within three-dimensional constructs. Recommendations include:
    a. Mechanically grind the substrates prior to performing the RNA isolation.
5. All aspirate studies should be repeated with more patient samples and an increased sample size should be used within each study. Studies should be performed that compare the activity of cells isolated from multiple patients.

6. Cells isolated from the reamer layers should be further characterized to determine if they are a pure population of mesenchymal stem cells. Additional characterization techniques could include:
    a. Identification of cell surface expression of MSC markers (i.e. CD34, CD105, CD44, etc.) by flow cytometry.
    b. Immunocytochemical staining, with antibodies against mesenchymal stem cells surface molecules, coupled with fluorescent microscopy to visualize fluorescently labeled cells.
7. Cellular studies to examine what effect the bulk fatty layer material has on MSCs when mixed with RIA bone fragments should be performed.
8. After additional *in vitro* studies are performed with both bulk aspirate material and cells isolated from the material, *in vivo* animal studies should be performed to determine if the incorporation of these components into a bone graft system will result in increased bone formation at the site of bone repair under physiological conditions.

APPENDICES

Appendix A

Protocol: Purification of Total RNA from Animal Cells Using Spin Technology*

*Adapted from the QIAGEN RNeasy® mini kit manual
For use with the QIAGEN RNeasy Mini Kit
Catalog no. 74106 (2009)

Use pipettes and tips designated for RNA work

Before starting:

- Mix 10 µl β-mercaptoethanol (β-ME) per 1 ml Buffer RLT in a fume hood, wearing appropriate protective clothing
- Add 4 volumes of 100% ethanol to the Buffer RPE supplied in the kit

Procedure for cells grown on three-dimensional substrates:

1. Disrupt the cells by adding Buffer RLT.
    a. Use a volume of Buffer that will cover the substrates: 350 µl or 600 µl
2. Mix samples on an orbital shaker for 2 hours
3. Pipette cell lysate directly into a QIAshredder spin column supplied within a 2 ml collection tube, and centrifuge for 2 min at full speed (14,000 rpm on Eppendorf centrifuge).
    a. Use one QIAshredder column per sample
    b. SAVE flow-through
4. Add 1 volume of 70% ethanol to the homoginized lysate (Step 3 flow-through), and mix well by pipetting. Mix any precipitate that forms.
5. Transfer up to 700 µl of the ethanol mixture to an RNeasy spin column supplied within a 2 ml collection tube. Centrifuge for 15s at ≥10,000 rpm.
    a. DISCARD flow-through.
    b. REUSE collection tube
6. Add 700 µl Buffer RW1 to the RNeasy spin column. Centrifuge for 15s at ≥10,000 rpm.
    a. Remove the RNeasy spin column from the collection tube so it does not contact the flow-through.
    b. DISCARD flow-through
    c. REUSE collection tube
7. Add 500 µl Buffer RPE to the RNeasy spin column. Centrifuge for 15s at ≥10,000 rpm.
    a. DISCARD flow-through
    b. REUSE collection tube
8. Add 500 µl Buffer RPE to the RNeasy spin column. Centrifuge for **2 min** at ≥10,000 rpm.

a. Remove the RNeasy spin column from the collection tube so it does not contact the flow-through.
b. DISCARD flow-through and collection tube

9. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 50 µl of RNase-free water into the spin column. Centrifuge for 1 minute at ≥10,000 rpm. **Flow-through contains the RNA.**
10. Repeat Step 9 if expected RNA yield is > 30 µg
11. Store RNA samples at -80°C

Appendix B

Protocol: RNA 6000 Nano Assay Protocol*
*Adapted from the Agilent RNA 6000 Nano Assay manual
For use with the Agilent RNA 6000 Nano Assay kit
Catalog no. 5065-4476 (10/2009)

Use pipettes and tips designated for RNA work
Always keep the samples and ladder on ice

Before starting:

- **Remove RNA 6000 Nano LabChip kit from the 4°C fridge equilibrate at room temperature for 30 minutes.**
- **Remove the ladder aliquot from -20°C freezer and thaw it on ice.**
- **Set water bath temperature to 70°C**

Procedure:
Preparing the Gel

1. Place 550 µl of RNA 6000 Nano gel matrix (red top vial) into the top receptacle of a spin filter (supplied).
2. Centrifuge the spin filter for 10 minutes at 4000 rpm (Eppendorf microcentrifuge).
3. Aliquot 65 µl filtered gel into 0.5 ml RNase-free microcentrifuge tubes (supplied). Store the aliquots at 4 °C and date. Use gel aliquots within one month of preparation.

Preparing the Gel-Dye Mix

1. Protect the RNA 6000 Nano dye (blue top vial) from light at all times.
2. Vortex the dye for 10 seconds; spin down.
3. Add 1 µl of dye to a 65 µl aliquot of filtered gel.
4. Vortex to ensure proper mixing (visually inspect).
5. Centrifuge the gel-dye mix for 10 minutes at room temperature at 14,000 rpm (Eppendorf microcentrifuge).
6. Use the centrifuged mixture within 24 hours.

Loading the Gel-Dye Mix

1. Always use a new RNA Nano chip, removed from a sealed bag.
2. Place the chip on the Priming Station. Priming station base plate should be in C position.
3. Pipette 9 µl of the gel-dye mix into the bottom of well marked G. to prevent air bubbles from forming in the chip, do not 'blow' the last bit of mix from the pipette.

4. Close and latch the Chip Priming Station. With plunger set at 1ml, press plunger until it is held by the clip and leave for 30 seconds.
5. After 30 seconds, release the plunger from the clip. Wait 5 seconds and return plunger to 1 ml position.
6. Open Priming Station and pipette 9 µl of the gel-dye mix into the bottom of wells marked G.

Loading the RNA 6000 Nano Marker

1. Pipette 5 µl of the RNA 6000 Nano Marker (green top vial) into the well marked with the ladder icon and into each of the 12 sample wells. Add 6 µl of Marker to and wells that will not be used with RNA samples.

Loading the RNA 6000 Nano Marker

1. Pipette 1.3 µl of thawed RNA 6000 ladder into an RNase-free tube and denature in the 70°C water bath for 2 minutes.
2. Pipette 1 µl of the denatured ladder into the well marked with the ladder icon.
3. Pipette 1 µl of each RNA sample into the sample wells on the chip (#1-12).
4. Vortex the chip for 1 minute at 2000 rpm on the IKA vortexer with chip adapter.
5. Analyze within 5 minutes

Chip Analysis in Agilent 2100 Bioanalyzer

1. Place chip into receptacle within the bioanalyzer, it only fits one way. Close the lid.
2. Select Instrument.
3. Select Assay → Electrophoresis → RNA → Eukaryote Total RNA Nano
4. Set 'Destination' for files to be saved.
5. Click START.
6. Data file will be saved in 'Destination' folder when run is complete.