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In Vitro Simulation of Pathological Bone Conditions to Predict Clinical Outcome of Bone Tissue Engineered Materials

Duong Nguyen
Clemson University, duongn@g.clemson.edu

IN VITRO SIMULATION OF PATHOLOGICAL BONE CONDITIONS TO PREDICT CLINICAL OUTCOME OF BONE TISSUE ENGINEERED MATERIALS

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Duong Thuy Thi Nguyen
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Accepted by:
Dr. Karen J. L. Burg, Committee Chair
Dr. Martine LaBerge
Dr. Jeoungsoo Lee
Dr. Melinda Harman

ABSTRACT

According to the Centers for Disease Control, the geriatric population of ≥65 years of age will increase to 51.5 million in 2020; 40% of white women and 13% of white men will be at risk for fragility fractures or fractures sustained under normal stress and loading conditions due to bone disease, leading to hospitalization and surgical treatment. Fracture management strategies can be divided into pharmaceutical therapy, surgical intervention, and tissue regeneration for fracture prevention, fracture stabilization, and fracture site regeneration, respectively. However, these strategies fail to accommodate the pathological nature of fragility fractures leading to unwanted side effects, implant failures, and non-unions.

Compromised innate bone healing reactions of patients with bone diseases is exacerbated with protective bone therapy. Once these patients sustain a fracture, bone healing is a challenge especially when fracture stabilization is unsuccessful. Traditional stabilizing screw and plate systems were designed with emphasis on bone mechanics rather than biology. Bone grafts are often used with fixation devices to provide skeletal continuity at the fracture gap. Current bone grafts include autologous bone tissue and donor bone tissue; however, there is insufficient quality and quantity demanded by fragility fractures sustained by high-risk geriatric patients and patients with bone diseases. Consequently, bone tissue engineering strategies are advancing towards functionalized bone substitutes to resolve shortages in fracture reconstruction while effectively mediating bone healing in normal and diseased fracture environments.

In order to target fragility fractures, fracture management strategies should be integrated for a synchronized treatment of prevention without hindrance to bone regeneration and fracture stabilization with bioactive bone substitutes designed for the pathological environment. However, the clinical outcome of these materials must be predictable within various disease environments. Initial development of a targeted treatment strategy should focus on simulating physiological in vitro bone environment to predict clinical effectiveness of engineered bone while understanding cellular responses due to the alternative agents and bioactive scaffolds. An in vitro testing system can be the predicate to reducing implant failures and non-unions in fragility fractures.

DEDICATION

I dedicate this work to my parents, Xuan and Loc Nguyen, and my siblings, Long and Lisa Nguyen. They have encouraged me to beyond the bonds of society and achieve high goals.

I am also dedicating this to my future husband, Sebastian Christensson, for his endless love and silliness to keep me happy and smiling.

When times are tough and stress is high, I can always depend on my friends help calm me down and bring the smile back to my face. The friends I have made at Clemson are my life friends because of the challenges and fun times we have shared. Getting to know them has made me into a wiser and more mature person. The special people are as follows: Hannah Murphy, Rachel Riti, Katie Dunn, Egleide Elenes, Brittany MacGowan, Lindsey Sanders, Aditi Sinha, Kayode Karunwi, and Suzanne Tabbaa. One person that has made my journey here memorable was my very first neighbor, Hannah Murphy, who will always be my neighbor. A special recognition to my first classmate friend, Rachel Riti, movie partner, pickiest eater I know, and the person that almost burnt down the apartment boiling water; those were the fun times. A special thanks to my longest friend, Egleide Elenes, who is still close to me even though we are states apart. Many thanks to my ladies, Brittany, Lindsey, and Aditi for the laughter, fitness encouragement, and listening to my rants.

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TABLE OF CONTENTS

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

PREFACE

The number of bone grafting procedures performed annually was estimated, in 2001, to be 500,000 in the US and over 2.2 million worldwide, with an expected increase of 13% per year exceeding the procurement of donor tissue [5-8]. The bone graft of choice for surgeons is autologous bone tissue, harvested from the patient's own bone, commonly from the iliac crest. However, for patients with bone diseases, the natural regenerative capability of bone is greatly hindered and further exaggerated with bone protective drug therapies. The preferred alternative to an autograft is an allograft obtained from donor tissue; however, limited donor supply has driven the development of substitute bone biomaterials composed of polymers, ceramics, and their composites.

Bone tissue engineers have innovated and investigated a vast array of biomaterials to mimic the mechanical, physiochemical, and biological properties of bone. Additionally, chemical, molecular, and cellular mediators have been incorporated into biomaterials to stimulate and enhance the bone healing cascade [11-14]. Early testing of enhanced biomaterials are limited to *in vitro* characterizations of monocultures and co-cultures that are inconsistent in design and mimicry to the natural bone environment. With advance in understanding cellular and molecular biology of bone, a better *in vitro* culture can be developed to be able to predict clinical outcome of the biomaterial for an expedited journey to clinical applications.

An established standard in vitro multicellular culture system simulating aspects of bone cell pathology will emphasize and overcome the pathological limitations of fracture healing. A greater understanding of bone healing under pathological conditions have

allowed researchers to design cellular bone replacements that significantly reduce non-healing fractures. Hence, a co-culture test system with relevant cell ratio to simulate pathological conditions can account for specific environments or exaggerate the anomalies. The proposed test system will utilized precursor osteoclasts and osteoblasts to understand bone cell differentiation on commercially available bone substitute ChronOS granules. The precursor cells are cultured at varying cell ratios of 1:1, 1:10, 1:100 (precursor osteoclast:osteoblast) to simulate pathological bone cell conditions in order to predict clinical outcome of ChronOS as a treatment option for patients with abnormal bone cell activity in bone formation and resorption of the material. The overall research objective is to establish a standard co-culture condition using murine RAW monocytes and D1 stromal cells at the specified cell ratios to elicit characteristic metabolic activity, gene expression, and protein production native to bone formation. To translate research concepts to the K-12 community, a teaching module was designed to introduce middle and high school students to bone tissue engineering as a possible career aspiration within biomedical engineering. To accomplish the overall objectives, four specific aims were recognized as follows:

Aim 1: Determine co-culture cell ratio for RAW:D1 to differentiate into osteoclast:osteoblast

Aim 2: Determine necessity for RANK ligand for osteoclastogenesis in presence of osteoblasts

Aim 3: Determine multicellular culture with co-culture of RAW:D1 for osteoclast:osteoblast differentiation in presence of indirect culture with adipocytes

Aim 4: Develop a workshop lesson plan and hands-on activity to effectively introduce bone tissue engineering to young students by demonstrating bone biology and implant development

As detailed in Chapter 2, Aim 1 study was designed to simulate the natural bone environment, considerations for cell ratio were taken into account to demonstrate the differences in cellular interaction, communication, and activity. The culture system used 3D ChronOS bone granules (Synthes) to simulate the complex architecture of bone extracellular matrix. Precursor bone cells were seeded at three RAW:D1 ratios (1:1, 1:10, 1:100) to culture for 35 days under osteogenic condition to monitor cellular differentiation and activity. Analysis of relative gene expression and protein levels were quantified to determine which cell ratio follows physiological behavior at key stages of maturation. Visual observations via fluorescent microscopy confirmed cell attachment, proliferation, and morphology.

Experimental details for Aim 2, as detailed in chapter 2, was conducted simultaneously with Aim 1 by including an additional culture condition of osteogenic medium supplemented with RANK ligand. Osteoblast and osteoclast maturation will be monitored for relative gene expression and protein production.

Aim 3, detailed in Chapter 3, focused on the multi-cellular culture of osteoclasts and osteoblasts in the presence of differentiated adipocytes to determine the influence of

adipocytes on bone cell activity. To simulate direct communication of differentiating precursor osteoclasts with osteoblasts and indirect communication with adipocytes, Netwell inserts were used to suspend the co-culture within a well compartment with adipocytes. Adipogenic differentiation of D1 cells on well-plates began 7 days prior to tri-culture simulation. Seeding of co-culture of RAW:D1 occurred 3 days prior to tri-culture for 14 days. Information on cell ratio gleaned from Aim 1 and Aim 2 were implemented in the experimental setup for Aim 3 in simulating normal bone cell interactions. By applying the co-culture to the tri-culture system with adipocytes, effects of adipogenic factors (lipids, hormones) on osteoclast and osteoblast activity were examined with gene expression and protein production. Behavior characterization methods for osteoclasts and osteoblasts were duplicated from Aims 1 and 2 with the addition of adipogenic markers.

The educational outreach workshop from Aim 4, as detailed in Chapter 4, introduced bone tissue engineering technologies to a group of girl scouts and high school students through a presentation, a hands-on activity, and an interactive communication. The teaching module incorporated biomedical engineering, bone biology, and medical devices for fracture management. The teaching module was implemented during two separate events with other teaching modules that also focused on introducing engineering and science to the students. Hence, the biomedical engineering teaching module was limited to 1-hour. The presentation provided an overview of bone physiology, various bone diseases, and orthopedic implants ending with a problem statement for the students to address in their hands-on activity. With the information given to them, the students

were challenged to strategize, implement, and test a plan to stabilize a simulated normal fracture and an osteoporotic fracture. Instructions required the girls to follow the development scheme of brainstorming, designing, prototyping, and testing. The hands-on activity will be made into a kit consisting of the tools, simulated fractures (cardboard tubes filled with styrofoam and insulation foam), screws, and a metal or plastic mending plate as internal fixation screw and plate system. Effectiveness and influence of the demonstration were measured by social cognitive theory pre- and post- survey questions, including rank based and open-ended questions. Due to the time constraint for each group of students, measurements of career interest incorporated engineering, science, and math in general rather than a focus on biomedical engineering.

CHAPTER ONE

INTRODUCTION

1.1 Clinical Significance

Tissue engineering and regenerative medicine emerged to resolve shortages in tissue transplantation for treatment of damaged tissues and organs. The theory is that engineered biotechnologies incorporating biomaterials, chemical mediators, and stem cells can produce functional tissues that repair and prevent the loss of damaged tissues. Many advances have been possible due to increased understanding and discoveries of human pathology at the tissue to molecular level. Innovations in medicine are driven by the objective to decrease patient suffering and increase longevity, especially as the population and life expectancy grow. However, as the past generations age and the future generations become more active, the frequency of injuries and diseases will increase dramatically. The Centers for Disease Control has predicted that, in 2020, over 51.5 million people in the United States will be ≥65 years old, while the United States Census Bureau estimates the world population will reach 8 billion people. The rise in population will amplify the strain on the medical industry to maintain a healthy population. Since traumatic injuries can result in tissue or organ failure, tissue transplantation will become a necessity. There are many complications associated with tissue transplantation and a major challenge is obtaining viable donor tissue. Consequently, tissue engineering and regenerative medicine is evolving to develop patient-specific and biologically functional tissues.

Bone grafts are the second most transplanted tissues, exceeded only by blood [4]. Currently, over 500,000 bone graft implantations occur annually in the United States, with surgeons preferentially using gold standard autografts, opting for allografts as a second choice, to treat large bone fractures and defects. Further considerations of fracture severity, fracture location (long or flat bone), and bone type (cancellous or cortical) are required to choose the optimal graft to induce an effective bone healing response [5]. However, autografts and allografts are also the top choices for patients with bone diseases and impaired healing reactions. Normal healing time for cortical (compact) bones are much longer than cancellous (spongy) bones due to the differences in bone density, but bones with impaired healing will, at best, heal at the slowest rate or, at worst, have incomplete healing. Even though there are limitations to bone grafting, especially for patients with degenerative bone diseases, both cortical and cancellous autografts and allografts are used.

Autografts and allografts bridge the gaps at fracture sites to provide skeletal continuity and encourage the innate bone healing cascade. The transplanted grafts are considered necrotic tissues that serve as the template for bone regeneration. The bone healing and repair reactions start with the formation of a hematoma to induce revascularization and recruit progenitor bone cells to the site of injury within 2 weeks. Bone cells then form new woven bone to stabilize and establish skeletal continuity at the fracture, which can take 6 weeks to 6 months. The woven bone is eventually remodeled into mature lamellar bone, years following the implantation [5, 6]. The rate and success of bone repair and regeneration depends on the quality and type of grafts transplanted.

Autologous and donor bone tissue can be cortical (compact) or cancellous (spongy), the two natural organizations and architectures of bone. Cortical and cancellous graft transplantations will each induce a different healing response and graft integration with host bone tissue (osteointegration) [6]. Cortical grafts have low porosity that results in minimal neo-vascular formation, resorption of the graft before woven bone regeneration, and remodeling of the graft. Due to the lack of vascular infiltration and density of the graft, osteointegration is limited to the exterior where surface bone resorption provides space for woven bone formation. Consequently, 50-90% of residual necrotic graft tissues remains and can diminish the mechanical integrity of bone at the fracture gap. Cancellous grafts, on the other hand, have high porosity to induce ingrowth of new blood vessels, new woven bone, and complete remodeling of the graft in which lamellar bone replaces both the woven bone and graft material [6]. Clearly, if not for the limited quantity of autografts and low quality of allografts, the graft of choice is a cancellous graft.

Autografts are bone tissue retrieved from the patient's own bone through a surgical extraction procedure, most commonly at the iliac crest. It is thought that transplantation of the bone will provide viable tissue with biological function; however, the removal will damage the cellular components, tissue continuity, and the tissue's regenerative ability. Compared to allografts and donor bone tissue, autografts increase patient risk during extraction procedures but have enhanced graft-to-tissue integration (osteointegration) and compressive strength. The autograft harvesting causes complications in 8-20% of all patients, including blood loss, nerve damage, artery damage, chronic pain, tissue necrosis, and infection [4]. For patients with bone disease,

autograft extraction and implantation significantly increases healing complications due to decreased bone quality and regeneration ability; hence, allografts are the alternative of choice.

Allograft is donor bone tissue that has been processed to remove all cellular, bacterial, and viral components to eliminate immune response and disease transmission [7]. Even though processing significantly compromises osteogenic and mechanical properties of the tissue, it is the material of choice for 35% of all grafting procedures because of its availability, shelf-life, and customizable type and size [8]. The physiochemical properties of allografts are different for fresh, frozen, or freeze-dried allografts. Fresh allografts are rarely used because of the extended time required for screening to prevent disease transmission. Processed allografts can be frozen at -60°C or freeze-dried to decrease enzymatic activity and immune response or destroy all cellular components and completely eliminate immune responses. These processing methods decrease the tissue's ability to recruit progenitor bone cells (osteoinduction) and to mediate differentiation of bone cells. However, with the introduction of bone morphogenic protein (BMP) into allografts, a 15-fold increase in allograft implantation occurred over the past decade [4, 9].

With the increased frequency of bone fractures and with the low and costly allograft supply, surgeons are more frequently opting for bone substitute materials. The development of substitute biomaterials for bone constructs will allow customizable mechanical and biological properties native to bone. Eventually, enhancements will incorporate osteoinductive and osteoconductive properties for constructs, specifically for

mediating bone healing in diseased bone. Synthetic and natural biomaterials with physicochemical properties similar to the inorganic and organic components of bone are a focus of ongoing investigation. The design and construction of a temporary 3D template that mimics the inherent architecture and compressive strength are ongoing challenges within the evolving field of bone tissue engineering. Furthermore, the future direction of substitute bone construct design and development should target fragility fractures in diseased bone.

1.2 Background in Bone Tissue Engineering

Before regenerative medicine and bone grafts were developed in widespread form, large segmental bone defects lead to amputations. However, recent advances in fixation devices, bone tissue engineering, and surgical procedures have lead to restoration options for bone and limb tissue [9]. The first documented bone tissue engineering attempt was in 1668 when bone grafting was first attempted and evolved into a multidisciplinary science that has facilitated the development of biotechnologies and management procedures for treating various bone defects and diseases [6]. By investigating mechanisms of bone pathology, researchers are able to map physiological repair and remodeling reactions and pathways in bone metabolism. A thorough understanding of bone tissue and bone remodeling is essential to designing regenerative solutions that maintain bone integrity and target degenerative bone diseases.

1.1.1 Bone Composition and Structure

At the surface, bone looks simple and non-viable but at the microscopic level the complexity and dynamic nature of bone matrix and bone cells are evident. Bone tissue

harnesses an innate ability to self heal from micro- and macro-fractures throughout a person's lifetime, while providing structural mechanics, movement, and protection. The 206 bones in the body assemble into an upright skeleton to support and protect all other soft tissues. The irregular shapes of bones have been optimized for ease of movement at each joint, transmission of external loads, and protection for each vital organ. Bone is composed of inorganic (hydroxyapatite) and organic matrix (collagen and proteoglycans) organized into a 3D structure of Haversian and Volkmann canals.

The architectural organization of bone can be classified into cancellous and cortical, according to structural density and porosity. Bone matrix is a combination of highly compressive hydroxyapatite, crystalline and highly ductile collagen, and proteoglycans; this combination allows the tissue to withstand varying loads of tension, compression, and shear encountered by the body. To further reinforce structural integrity, the matrix is arranged into parallel or circumferential lamellae to form cancellous or cortical bone, respectively. Due to the longitudinal organization, bone is an anisotropic material with higher resistance to longitudinal forces than latitudinal.

Cortical bone serves as the outer lining for most bones because it is stronger and heavier than cancellous bone. This highly compact bone with <10% porosity is made of longitudinal concentric lamellae (layers), with interstitial and circumferential lamellae for compressive strength and load transmission. Within compact matrix, there is a network of Haversian and Volkmann canals through which vascular structures pass, as well as lacunae-containing osteocytes (mature bone cells). The compressive modulus of cortical bone is ~17.0 GPa in the longitudinal direction, ~11.5 GPa in the transverse direction,

and ~3.3GPa in shear [10]. Cancellous bone, on the other hand, has a parallel lamellar organization that forms interconnected struts called trabeculae. Enclosed by cortical bone, the trabecular organization of cancellous bone has a density range of 5-90% depending on location, resulting in much lower weight and compressive moduli of 291-445 MPa. However, these lightweight struts serve to redistribute load more effectively along the bone while the interconnected pores store bone marrow and a vascular network [10]. These two types of bone are then arranged in various configurations to form long segmental, flat, or irregular bones and serve their specific function of locomotion or protection. Since bone is a living tissue, it is composed of specialized cells with innate capacities to maintain bone integrity by continually remodeling old bone and repairing damaged bone.

These structural organization and mechanical properties are challenging to integrate in bone substitute constructs due to material and fabrication limitations of current technologies. Innovative bone construct designs have included hardened sponges, sintered microspheres, fibrous matrices, and rapid prototyped woven matrices [11-16].

1.1.2 Bone Cellular Components

At the core of hollow long bones lies bone marrow, a source for skeletal progenitor cells that have been shown to differentiate along osteoblastic, adipogenic, and chondrogenic lineages [17]. Friedenstein and coworkers discovered bone marrow during their investigation of bone's innate healing capacity as related to stem/progenitor cell involvement and availability [17-19]. Further characterization of heterogeneous mixtures of bone marrow stem and progenitor cells has lead to understanding the differentiation

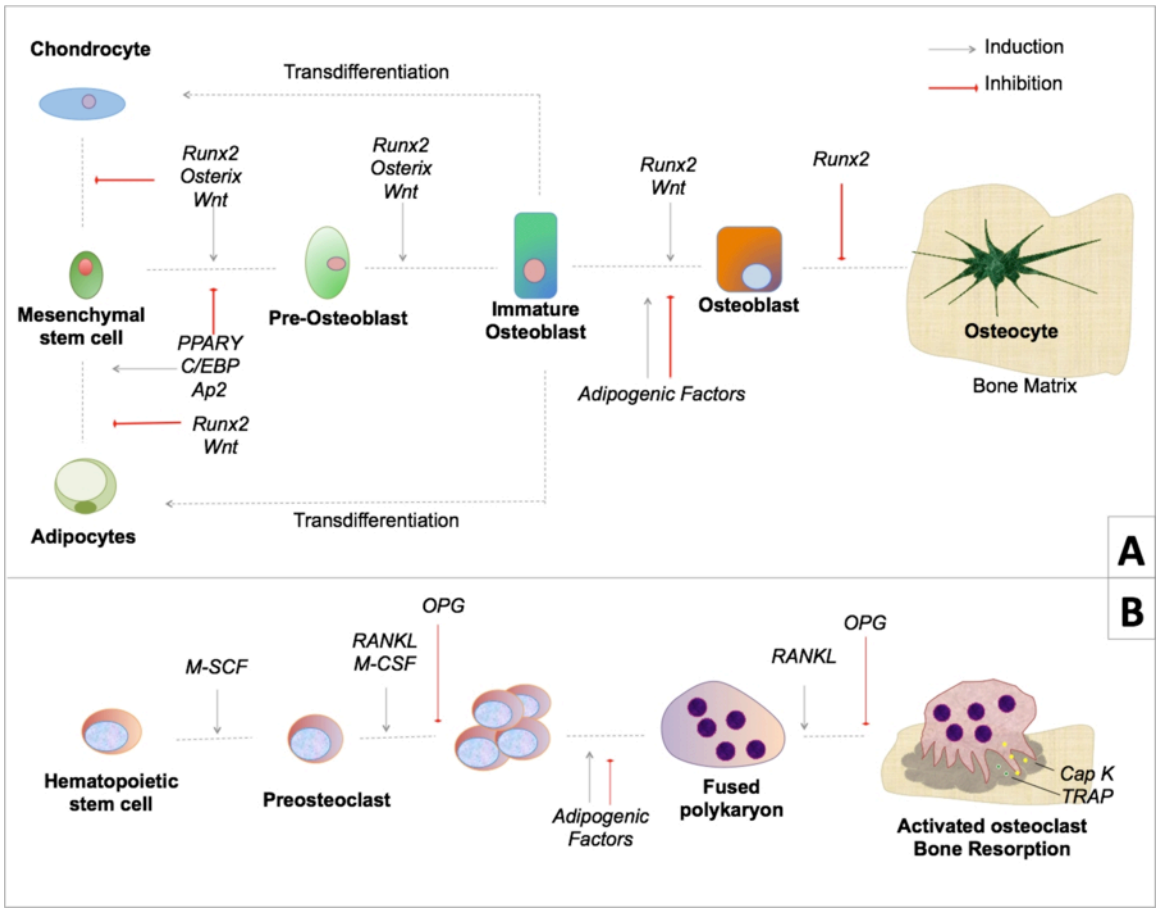

Figure 1.1. Differentiation of osteoblasts (A) and osteoclasts (B) in response to internal cytokines and molecular mediators.

potential of the multipotent adult progenitor cells, mesenchymal stem cells, bone marrow stromal cells, and hematopoietic stem cells [17].

The cellular components of bone are under highly regulated coordination in response to internal signaling and external mechanical loading. The resorption and production of bone matrix are the results of activated osteogenic cells, differentiated from mesenchymal stem cells (MSC) and hematopoietic stem cells in response to cytokines and growth factors (Figure 1.1). Recruited hematopoietic stem cells, specifically monocytes, are directed by macrophage colony stimulating factor (M-CSF), receptor activator of NFκ (RANK), and RANK ligand (RANKL) to fuse into inactive

multinucleated osteoclasts. Further interactions of RANK, a surface receptor of osteoclastic cells, and RANKL, a surface marker of osteoblasts, will polarize osteoclasts to develop resorptive ruffled borders that attach to the bone matrix. The ruffled borders enclose an area marked for resorption and the secretion of protolytic enzyme cathepsin K and tartrate-resistant acid phosphatase (TRAP) for the degradation of the unwanted bone matrix. However, osteoprotegerin (OPG) serves as the negative feedback that interferes with RANKL and RANK signaling to inhibit and regulate osteoclast polarization and activation. The enzymatic resorption process creates pits on the bone surface called "Howship's lacuna", which are coated with cytokines and factors to recruit osteoblastic cells to the excavation site to deposit new bone at the eroded surface [1]. Osteoblasts differentiate from mesenchymal stem cells, with transcription factor signaling via Runx2, osterix, and β-catenin. The progenitor cells differentiate into preosteoblasts, then immature osteoblasts, expressing high levels of osteopontin. Osteoblast maturation continues under the control of Runx2. During this time the mature osteoblasts release high levels of alkaline phosphatase (ALP) and express osteocalcin, as mineralization occurs and new bone matrix is deposited [20]. As new extracellular matrix composed of the inorganic and organic phase of bone accumulates, osteoblasts become embedded within the matrix, leading to their differentiation into osteocytes; subsequently, these cells remain latent in the lacunae to monitor the health of the bone. Osteocytes are able to communicate and interact directly with vasculature and other osteocytes because of their numerous cytoskeletal extensions that travel along microscopic channels called canaliculi

[1]. Consequently, when there is a disturbance in bone integrity, osteocytes initiate bone healing reactions.

A recent discovery of fatty acid secretions mediating bone mineral density has led investigators to study the influence of adipocytes, or fat cells, on bone formation [21, 22]. Adipocyte secretions have been shown to regulate the activity of both osteoclasts and osteoblasts. Adipocytes are found in close proximity to bone cells in brown and white marrow fat and their fatty acid secretions can diffuse into active bone cells undergoing bone remodeling. Studies have shown that stearic and palmitic acids decrease the expression of alkaline phosphatase (ALP), which is an indicator of osteoblast mineralization, while linoleic acids increase the expression of ALP [21, 23]. However, fatty acids such as dexamethasone and prostaglandin E_2 promote osteoclast resorption. This regulation by fatty acids establishes a direct relationship of aging bone with increased fatty marrow to low bone mineral density in geriatric patients [24].

As bone cells differentiate under high regulation, favorable conditions, and mediators, bone remodeling and healing will proceed with a balance in bone degradation and deposition for optimal bone integrity and health. Unfortunately, traumatic injuries and cellular imbalances occur which challenge the innate self-healing capacity and the regulated feedback mechanisms, respectively.

1.1.3 The Bone Healing Process and Bone Cell Communication

Bone is the infrastructure of the body, possessing mechanical and biological properties vital for support, protection, growth, and immunity. However, like most tissues in the body, bone has a physiological carrying capacity, namely its mechanical strength.

When skeletal continuity is disrupted due to excessive stress and loading, bone can repair and heal itself without producing a scar. The physiological healing process is extensive, involving a vast network of cellular signals to recruit and differentiate progenitor stem cells to osteoclasts and osteoblasts in order to resorb and deposit new bone [25]. Since bone serves as the structural support for the body, healing and repairing reactions are impacted by the mechanical stability and the biological environment of the damage or fracture site, along with the severity of injury of the surrounding soft tissues. After an assessment of the damage, primary or secondary healing reactions will occur.

Primary fracture healing via a "cutting cone" occurs with absolute stability, requiring no external callus bridging. However, if the fracture gap exceeds 200μm, osteoclasts are hindered from constructing the "cutting cone", which can delay bone union. The "cutting cone" is the organization of osteoclasts that tunnel across the fracture line to resorb bone while osteoblasts are recruited to deposit new bone and reconstruct the bone union [5]. For secondary bone healing, there is a strain between the fracture surfaces that necessitates the formation of a callus bridge to stabilize the fracture for ossification. This type of healing is typical for patients with bone graft implantations and/or internal fixation devices. There are four phases to secondary healing, all of which are regulated at the cellular and molecular level for neo-vascularization and bone regeneration. The process starts with non-specific signaling to respond to the trauma-related inflammation and hematoma formation, proceeds to fracture bridging via soft callus, then to hard callus formation, and finally to specific regulation of bone remodeling [1, 5, 25-29]. Even

though the repair process is highly regulated, participating cellular and molecular components contribute at each phase, overlapping as seen in Figure 1.2.

Phase 1: Inflammation

Fractures due to trauma also result in disruption of the surrounding tissues, vasculature, and bone integrity. This trauma causes an immediate, nonspecific response in which the

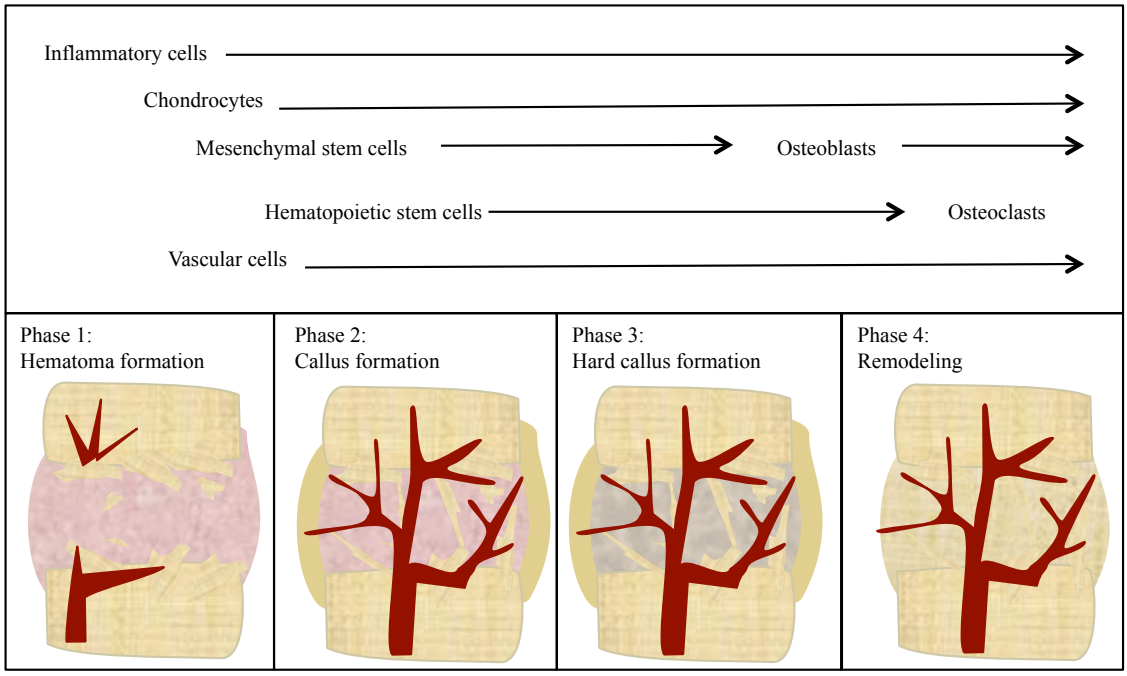

Figure 1.2. Timeline of cellular contributors during the four phases of bone remodeling. (Adapted from Schindeler et al., 2008, and Carano and Filvaroff [1, 2]).

pooling of blood and accumulation of inflammatory cells at the injury site form a hematoma encased by surrounding tissues. The inflammatory cells, such as degranulated platelets, macrophages, monocytes, and lymphocytes, are the first responders to form a blot clot and remodel the hematoma into granulation tissue as macrophages and giant cells remove necrotic cells. Cellular coordination is conducted through the secretion of cytokines and growth factors, such as vascular endothelial growth factors (VEGF),

platelet-derived growth factor (PDGF), and others seen in Table 1.1, to mediate recruitment of more inflammatory cells as well as chondrogenic and osteoprogenitor cells [1, 29].

Table 1.1. Molecular Contributors to Bone Healing

Pro-inflammatory cytokines and growth factors	Pro-osteogenic factors	Angiogenic factors
Transforming growth factor-β (TGF-β)	Runt-mediated transcription factor (Runx2)	VEGF
Platelet-derived growth factor (PDGF)	Receptor activator of NFκB (RANK)	BMPs
Fibroblast growth factor-2 (FGF-2)	RANK ligand (RANKL)	FGF-1
Vascular endothelial growth factor (VEGF)	BMPs	TGF-β
Macrophage colony stimulating factor (M-CSF)	M-CSF	Angiopoietin I and II
Interleukin-1 and -6 (IL-1, IL-6)	Osteoprotegrin (OPG)	
Bone morphogenetic proteins (BMPs)		
Tumor necrosis factor-α (TNF-α)		

Phase 2: Soft callus (fibrocartilage) formation

Once chondrogenic and fibroblastic cells reach the bone fracture, endochondral ossification occurs as a fibrocartilage soft callus is formed. Since the fracture site is mechanically unstable, a soft callus provides stability and a template for primary bone formation. Recruited mesenchymal progenitor cells differentiate into chondrocytes to produce the cartilaginous matrix that merges with fibrous tissue produced by fibroblasts to establish a continuous bone bridge [1]. Bone morphogenetic protein and fibroblast

growth factor (FGF) are the major signaling molecules for ossification [30]. Angiogenesis, or the formation of new blood vessels, occurs concurrently with ossification under the control of VEGF and angiopoietin I and II [26]. However, VEGF expression is dependent on the expression of Runx2 (early osteoblastic marker) by osteogenic cells activated for hard callus formation [1].

Phase 3: Hard callus formation

This phase is the major component of osteogenesis and includes a high level of osteoblast matrix deposition onto the soft callus template, following which the mineralized bone matrix converts the soft callus into a hard callus or woven bone. Activated osteoblasts are recruited with osteogenic factors from the super family of transforming growth factor-β (TGF-β), such as BMP-differentiating osteoprogenitor cells recruited during hematoma formation. With the help of vascular networks, osteoprogenitor cells and their differentiation signals are able to infiltrate the soft callus to efficiently mineralize the woven bone [1, 30]. However, innate bone repair continues to reestablish structural organization of native bone and converts woven bone into lamellar bone.

Phase 4: Bone remodeling

The conversion to lamellar bone requires resorption of woven bone by osteoclasts. Osteoclasts differentiated from monocytes remodel the woven bone hard callus into lamellar bone in the appropriate cortical or cancellous configuration. Monocytes are first recruited to the site for remodeling, then the cells mature into polarized osteoclasts and adhere to the mineralized surface. The attached ruffled borders of osteoclasts secrete

proteases such as cathepsin K and tartrate-resistant acid phosphatase (TRAP) to degrade the woven bone. The resorption process creates pits on the bone surface called "Howship's lacuna" that recruit and activate osteoblasts to regenerate new bone at the eroded surface. Cytokines such as macrophage colony stimulating factor (M-CSF) regulate osteoblast bone mineralization, while receptor activator of NFκ (RANK) and RANK ligand (RANKL) activate osteoclast resorption and osteoprotegerin (OPG) to inactivate resorption as needed [1, 25, 29, 31-33]. The bone remodeling phase is a juxtaposition of osteoblast anabolism and osteoclast catabolism of bone matrix [1].

The four phases of bone healing occur in conjunction with each other with no real separation between the end of one phase and the start of another. The balanced reaction is highly regulated and coordinated at the cellular and molecular level to produce effective healing responses. Healing rates are dependent on the implanted grafts (cortical or cancellous), the health of the patient, and location and severity of the fracture.

1.3 Bone Pathology

Bone healing reactions of adults and children suffering with bone diseases and defects are compromised, leading to complications with graft implantation and internal fixation treatments. When imbalances occur within the highly regulated bone repair and remodeling processes, bone diseases arise from abnormal bone cell activity and metabolism. The majority of bone diseases are due to overactive or inactive osteoclasts, resulting in decreased bone density or increased bone mass, respectively. The most diagnosed bone disease is osteoporosis, or low bone density, a degenerative disease affecting the aged population. The onset of osteoporosis is osteopenia, or the gradual loss

of bone mass associated with high osteoclast resorptive activity exceeding osteoblastic regenerative activity [34]. Risk factors for developing osteopenia and osteoporosis include low physical activity and vitamin D deficiency, which are physical and chemical stimulators for bone remodeling. The combination of aging and low physical activity will increase osteoporosis risk, since factors will increase marrow fat concentration which can interfere with osteoblast activity while promoting osteoclast metabolism [35-37]. Johnell and Kanis estimated the world burden of osteoporotic fractures to be 9 million occurrences in the year 2000, with 61% affiliated with women over the age of 50. The most common fractures incurred by osteoporotic patients are vertebral fractures which can be fatal if not debilitating [38]. Current pharmaceutical agents for osteoporosis target and interfere with RANK/RANKL and/or induce the OPG signaling pathway to prevent osteoclastogenesis (osteoclast differentiation and activation) [3].

At the other extreme, impairment of osteoclast resorption or osteoclastogenesis will result in osteopetrosis, sclerosteosis, or Paget's disease conditions, i.e. high bone mass due to osteoblastic bone matrix construction in the absence of osteoclastic bone matrix destruction [3]. Osteopetrosis is a rare hereditary genetic disease, involving osteoclastogenesis inhibition and associated low supply of bone marrow, osteosclerosis, short stature, brittle bones, and even cranial nerve compression due to the closure of the cancellous bone cavities [39, 40]. Sclerosteosis is caused by interference of osteoclastogenesis via the Wnt signaling pathway that regulates production of RANKL and OPG in osteoblasts [3]. The dense bone mass in Paget's disease is due to accelerated bone remodeling in which bone formation compensates for increased resorption from

hypersensitive osteoclasts [40]. Even though bone mineral and matrix are dense in these disease states, bone fragility increases since the disorganized accumulation of bone will lead to decreased mechanical strength and structural integrity.

Osteogenesis imperfecta is another genetic bone disease affecting the differentiation of mesenchymal stem cells into osteoblasts to produce collagen Type I, an important matrix component of bone that provides tensile strength. Patients with osteogenesis imperfecta have bone fragility leading to multiple fractures, skeletal development retardation, and skeletal deformities. This disease can affect all age and gender groups and currently no cure is available. Bisphosphonates are the only known broad-spectrum treatment for mild cases; however, in severe cases, especially in children, bone marrow transplants are common [41]. Adults with osteogenesis imperfecta can also have osteoporosis and threefold higher risk of fractures [42].

Metastatic bone disease occurs in cancer patients; breast and prostate cancer patients have the highest risk, due to radiation chemotherapy and hormonal therapy [43]. For example, breast cancer cells express runt-mediated transcription factor 2 (Runx2), which is also a master transcription factor for osteoblast differentiation. Runx2 promotes the osteoblast lineage in the differentiation of mesenchymal stem cells into immature, then mature, osteoblasts. Consequently, mutations of Runx2 are associated with bone cancer (osteosarcoma), with undefined pathological mechanisms afflicting children and young adults, especially during growth spurts [44, 45]. Management of metastatic bone disease and osteosarcoma involves chemotherapy and bisphosphonate therapy to inhibit the growth of cancerous bone cells [45, 46].

The pathological environment of bone diseases alters not only bone cell activity but also morphology and concentration of osteoblastic and osteoclastic cells. In examining the limited histomorphometric studies, the range of precursor bone cell ratios for physiological and bone diseases was determined (Table 1.2)[47-50]. The table lists number of osteoclasts and osteoblasts per millimeter of bone perimeter, following the standard of histomorphometry from the American Society for Bone and Mineral

Table 1.2. Bone Cell Number via Histomorphometric Analysis of Bone Biopsy

	Osteoclast (OC)	Osteoblast (OB)	OC:OB	Location	Reference
Control	$0.26\pm0.15 mm^{-1}$	$4.03 \pm 1.30\ mm^{-1}$	1:15.5	Vertebra	Pestka. Eur Spine, 2012.
Control	$0.10\pm0.1 mm^{-1}$	$1.5 \pm 0.30\ mm^{-1}$	1:15	Femor	Vukmirovic-popovic. Bone, 2002.
Control	$0.35\pm0.18 mm^{-1}$	-	-	Iliac crest	Rauch. Bone, 2000.
Control	$0.30\pm1.68 mm^{-1}$	-	-	Iliac crest	Rauch. J of Bone and Mineral Res, 2000.
Paget's Disease	$0.92\pm0.33 mm^{-1}$	$21.27\pm10.51 mm^{-1}$	1:23.1	Vertebra	Pestka. Eur Spine, 2012.
Bone Metastasis Breast Carcinoma	$1.7\pm0.5 mm^{-1}$	$2.7\pm0.5 mm^{-1}$	1:1.6	Femur	Vukmirovic-popovic. Bone, 2002.
Osteogenesis Imperfecta I	$0.47\pm0.29 mm^{-1}$	-	-	Iliac crest	Rauch. Bone, 2000.
Osteoporosis	$0.20\pm2.04 mm^{-1}$	-	-	Iliac crest	Rauch. J of Bone and Mineral Res, 2000.

Research. From the number of osteoclasts and osteoblasts per bone surface, the cell ratio was calculated. The ratios indicate a range of variability in the disease state, a key finding for simulating pathological bone environment in co-culture. Furthermore, histology shows morphology differences, as compared with normal cells, in osteoclasts and osteoblasts involved in Paget's disease. Advances in understanding the cellular etiology

of common and rare bone diseases will demonstrate the morphological abnormalities and concentration differences of osteoblastic and osteoclastic cells.

For patients with bone diseases, the inherent increased risk of bone fractures is dramatic, including increased complications in fracture healing and management. These patients must take a high number of precautions during everyday activities and avoid the fundamental causes of fracture.

1.1.4 Causes of Fractures

Patients suffering from bone diseases are more likely to incur a bone fracture due to the compromised mechanical properties of their bones. When bone experiences tensile or compressive stresses (cyclic or direct) that exceed the limits of normal bone strength, it will fracture. Bones that fracture under normal physiological stress and loading are diseased bones with reduced mechanical and physicochemical properties. The anisotropic mechanical strength of bone (high longitudinal strength, low latitudinal strength) means that bone will most likely fracture into multiple fragments under extreme perpendicular and rotational stresses. Fractures can have different levels of severity, from a minor micro-crack that goes unnoticed to a major open fracture with a break in the skin and damage to the surrounding tissues or organs. Fracture types or patterns are classified as [5]:

- Complete or incomplete
- Displaced or undisplaced
- Simple or comminuted
- Open or closed

Each classification has subcategories so that all details of the fracture are accounted in order to choose the most effective treatment and management solutions. However, an important indicator for intervention options is the patient. Physicians must consider the patient age, health condition, type of trauma, site of fracture, bone type, and fracture type.

In the case of osteoporotic fractures, prevalence of fractures in the spine and proximal femur are higher in older patients as compared to younger patients, with high risk for distal radial fractures. Vertebral fractures in osteoporotic patients occur under normal body weight and will cause deformities of the spine and chronic back pain. Management of spinal fractures includes use of bone grafts and fixation devices for spinal fusion to redistribute load, while femoral fractures are immobilized by intramedullary nails or plates to induce bone healing.

Since bone fractures are more prevalent in patients with bone diseases, pharmacological treatments targeting specific remodeling processes will suppress healing reactions at the fracture site. This suppression will lead to high rates of non-unions for this category of patients [30, 51]. The juxtaposition of inhibitory protective agents and fracture healing has little consideration in the management of bone fractures and the development of substitute bone. Hence it is important to review the advances and gaps with respect to implementation of pharmaceutical agents and bone substitute materials for fracture healing in diseased bone.

1.4 Review of Advances in Bone Tissue Engineering and Regenerative Medicine

1.1.5 Clinical Approaches to Bone Diseases and Defects

Expanding knowledge of bone abnormalities has enabled innovative diagnosis capabilities, surgical procedures, and therapeutic solutions. Clinical approaches focus on bone protective therapy for the aging population and cancer patients with osteoporosis and bone metastases, respectively. The majority of therapeutic medicines for bone diseases disrupt the osteoclastic (anti-resorptive/anti-catabolic) pathway and promote the osteoblastic (pro-anabolic) pathway [3]. Fracture management surgical interventions for diseased bones are limited to solutions designed for healthy bone with normal bone cell activity.

Pharmacological therapies targeting abnormal cell signaling in benign and malignant bone diseases can provide effective solutions but have complicated side effects [52, 53]. For example, bisphosphonates are a broad spectrum class of drugs used for many osteoporotic types to inhibit resorptive activities; however, the side effects can include renal dysfunction, gastrointestinal complications, or even osteonecrosis of the jaw [3, 46, 54]. Other studies have revealed the benefits of bisphosphonates, which have apparent anti-tumor effects when administered to cancer patients suffering from osteolysis or which, in combination with chemotherapy, inhibit the growth of osteosarcoma cells [45, 53]. Bisphosphonates are also administered to osteoporotic patients with total hip replacements to prevent aseptic loosening and peri-implant osteolysis [55]. Denosumad is an anti-resorptive pharmaceutical agent targeting RANKL signaling in osteoclast activation; a clinical study showed the agent's anti-fracture efficacy for women with postmenopausal osteoporosis, i.e. reduced fracture incidence in

vertebrae and increased bone mineral density in the hip [3]. Other bone-modulating agents target the protolytic enzyme cathepsin K, the Wnt pathway (indirect RANKL and OPG regulation), and calcium-sensing receptors to downregulate hormonal stimulators (parathyroid hormone) in bone remodeling [3, 25]. These agents are only medicinal therapies that prevent disease exacerbation; they do not prevent bone fragility and risk for painful fractures.

Surgical intervention is used to stabilize fractures in healthy bone with internal fixation devices and bone substitutes, providing a mechanically favorable environment and template for bone healing. For fractures in long bones, like the femur and humorous, implanted fixation devices provide rigid stability using screws and plates; bone substitutes are sometimes used to bridge the fracture gap. The traditional screws and plates are also used for the fixation of osteoporotic fractures in which the stability of the device depends on the integration of the screw to the bone. However, the stability of the traditional fixation system is compromised by the low bone mass in osteoporotic bone. Consequently, implant loosening and progressive instability will cause nonunions at the fracture gap [56]. Proximal femoral fractures in older patients are treated with urgent attention to control of bleeding and to achieve successful fixation and minimize future complications. In bone metastatic diseases, surgical intervention is intended to control tumor growth and provide load-bearing capabilities to the defective area [57]. Insufficient recognition of compromised bone mechanics in diseased bone has lead to implant failures and increasing patient suffering [58]. However, efforts towards redesigning the screw and

plate systems for low quality bone have been ongoing with products such as angular stability screws and plates, and bicortical screws [56, 58, 59].

Fracture management also depends on the enhancement of fracture healing with autografts and allografts. The practice of graft implantation has grown in the past two decades, as indicated by the $300 million market in 1999, to an astounding $1.6 billion in 2008 as estimated by the Orthopedic Network News [60]. Bone grafting procedures are predominantly performed in the spine (80%), with combinations of bone morphogenic protein to encourage regenerative bone fusion. The incorporation of BMP was one of the first approaches to bone tissue engineering; many advances have been achieved to enhance bone healing and formation, with some focus in bone diseases.

1.1.6 Bioactive and Regenerative Advances

The transition into regenerative practices is evident in the rise of bone substitute purchases of 28.6% in 2006 to 51.6% in 2007, in conjunction with the ~12% increase in BMP purchases [60]. The shift in focus is an effort to explore promising pathways of a functional bone substitute via cellular factors, chemical factors, and molecular factors to stimulate bone regeneration, even in abnormal bone conditions. The use of autologous bone marrow and its cellular components with bone substitutes is an emerging alternative to enhance osteogenicity and osteoconductivity [61]. Bone substitutes can also be carriers for disease-targeting macromolecules in the anti-catabolic and pro-anabolic pathways for local delivery [62]. Other strategies combine the effects of systemic bone protective agents with local delivery of molecular osteogenic factors [63]. Innovative development

of functionalized substitute bone has the capacity to resolve the rising demand for bone tissue transplantation in the aging population due to the rise in bone diseases.

The bone substitute construct or scaffold serves as a temporary template for cellular recruitment, differentiation, and matrix deposition in bone regeneration and provides continuity in mechanical stability at the defective area. The strategy of using functionalized bone scaffolds focuses on stimulating and directing all four phases in the bone healing reaction. The functionality depends mainly on the biomaterial of choice and the retention of potency and efficacy of the supplements within the defect bone.

Fabricating a sophisticated biomaterial that can mimic the innate regenerative capacity of bone tissue is a challenge. Strategies incorporating biomimetic bone scaffolds have focused on the use of bone marrow and platelet rich plasma to enhance osteoinductive properties of substitute bone grafts in an intraoperative procedure [61]. Researchers have infused anti-anabolic and pro-catabolic agents and molecular factors to stimulate bone cell activity in various bone scaffolds [64].

Cellular Factors: Bone Marrow

Bone marrow is a source for osteoprogenitor and hematopoietic stems cells; therefore, cellular grafting of autologous marrow aspirate is of high interest for enhancing fracture unions [63, 65]. Bone marrow has been used in its entirety and in fractions, depending on the bone defect. Various methods of bone marrow extraction have been evaluated to isolate marrow stromal cells, mesenchymal cells, hematopoietic stem cells, and even marrow fat cells [61, 66]. Whole bone marrow transplants are commonly performed for genetic bone diseases such as osteopetrosis and osteogenesis imperfecta in

order to replace abnormal bone cells with normal cells. As for alveolar bone defects in the maxilla, allografts are pre-soaked in bone marrow before implantation to mediate enhanced bone healing [67, 68]. In long bone fractures, intramedullary nails are implanted into the canal of the long bone to provide rigid stability for enhanced union. The implantation of the intramedullary nail requires the surgeon to ream the bone canal, which generates reamed aspirate that is filtered and divided into intraoperative autologous osseous particle and filtrate waste. The osseous aspirate material containing bone fragments is placed onto the defect to enhance osteoconductivity of the allograft or bone substitute bridging the fracture gap [69, 70].

However, the waste filtrate or the liquid flow-through from the reaming process is of high interest for its osteogenic potential with cellular components and various growth factors involved in bone metabolism [65]. Porter and coworkers confirmed that the filtrate had growth factors such as PDGF, VEGF, and TGF, along with multipotent cells expressing an MSC phenotype [65, 71]. This conservative approach to re-incorporate the filtrate will further enhance the osteoinductive environment of the allograft and bone scaffold.

Current intraoperative enhancements of allografts and commercially available bone substitutes using whole bone marrow can have complications. The recent discovery that adipocytes found in bone marrow regulate osteoblast bone formation and osteoclast bone resorption through fatty acid and hormonal secretions may explain the prevalence of osteoporosis in geriatric patients since marrow fat increases as bone ages [35]. The use of bone marrow cellular components refined for optimal bone formation and resorption can

be of benefit. However, bone marrow and its components are limited in quality, and even quantity, depending on the patient and the marrow extraction procedure [70].

Chemical Factors: Protective Biomolecules

An alternate treatment should be chosen for patients with bone diseases, who are incompatible for marrow transplants since the abnormal potential of autologous marrow stem cells could result in non-union healing. In these instances, strategies of

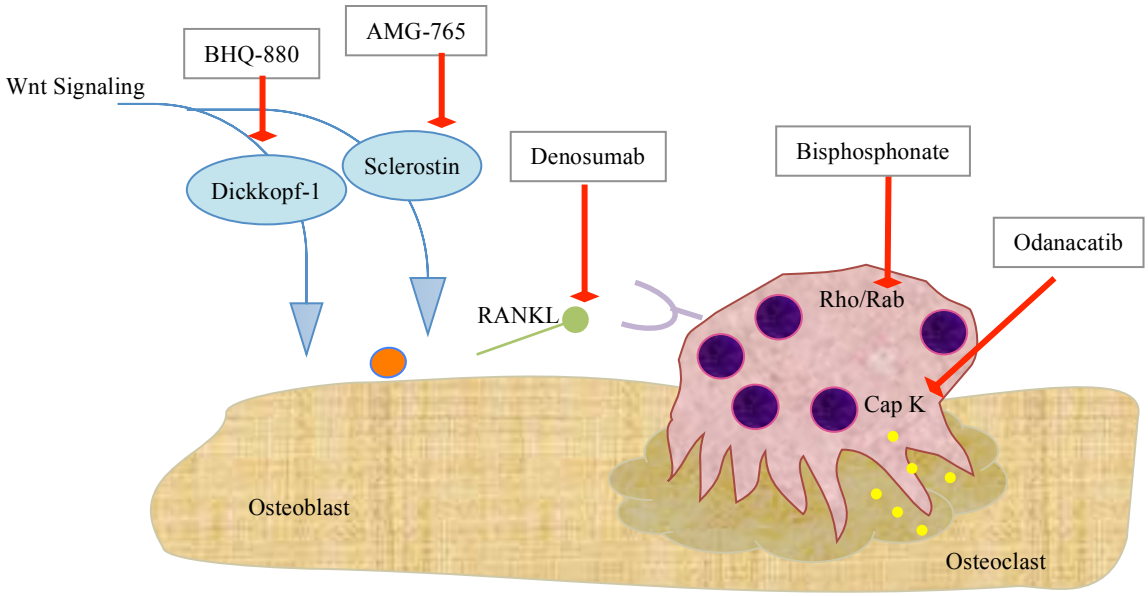

Figure 1.3. Bone protective agents targeting specific osteoblast anabolic and osteoclast catabolic activities to improve bone quality and prevent disease acceleration. Adapted from [3].

incorporating and loading bone-protective agents within the bone substitute for local delivery are more advantageous [62, 72, 73]. Current understanding of the exact mechanism, release rate, degradation, and dosage of the biomolecule in the scaffold is limited, and future work should include *in vitro* or *in vivo* simulations to understand the interactions at the cellular and system level [74].

Table 1.3. Bone Protective Agents	
Name	Activity
BHQ-880	Inhibits dickkopf-1 from interfering with Wnt signaling to promote bone formation
AMG-765	Inhibits sclerostin from interfering with Wnt signaling to promote bone formation
Denosumab	Inhibits RANKL and RANK signaling to prevent osteoclast activation
Bisphosphonate	Inhibits Rho and Rab signaling for osteoclast survival and activity
Odanacatib	Inhibits cathepsin K (Cap K) production of degradation lysozyme

Pharmacological agents used to treat bone diseases target specific signaling pathways within bone cell differentiation and activity (Figure 1.3 and Table 1.3). These biomolecules act to neutralize the inactivity of osteoblasts and hyperactivity of osteoclasts commonly found in osteoporosis for a more effective and accurate induction or inhibition, with limited side effects. Bisphosphonates are currently the most widely used protective agents, due to their approved status for treating osteoporosis, the most common bone disease. Consequently, strategies for loading protective biomolecules concentrate on incorporation of bisphosphonates, mainly in soluble calcium phosphate-based scaffolds and bone cement [73, 75, 76]. Faucheux and coworkers demonstrated that zoledronate, a potent bisphosphonate, loaded on calcium phosphate inhibited osteoclast activity without affecting osteoblast activity [77]. With local inhibition of osteoclast activity, investigators speculate that the initial application of the drug encourages bone formation within the bone scaffold, then allows osteoclast resorption to remodel the bone scaffold and woven bone. Further development is still needed to determine the precise release kinetics, loading efficacy, and distribution zone, while conserving the integrity of the bisphosphonates for an optimal bioactive scaffold. As more anti-catabolic and pro-

anabolic pharmaceutical treatments become available, strategies for different local drug delivery systems will evolve to allow the development of functional bone scaffolds specific to various bone diseases.

Molecular Factors: Cytokines, Steroids, and Growth Factors

A more direct approach is to deliver the molecules orchestrating bone resorption and formation reactions with the bone scaffolds. Some investigators have loaded cytokines, steroids, and growth factors to emphasize anabolic or catabolic activity during the phases of bone healing. To initiate phase 1 of the bone healing reactions, platelet-rich plasma, a source for platelet-derived growth factor and transforming growth factor, has demonstrated its ability to activate inflammation and coagulation for recruitment of progenitor and stem cells [63, 78, 79]. Since the discovery of recombinant human BMP-2 and BMP-7 to promote osteoblast differentiation, the use of allografts loaded with BMP has increased for spinal fusion and tibial fracture repair. Li and coworkers developed a method to load BMP-2, using gelatin microspheres, into macroporous calcium phosphate cement for controlled release with enhanced osteoinductivity [80]. The efficacy of BMP in long bone fractures is still under investigation; hence, there is an ongoing search for alternate proteins to stimulate good bone healing reactions. Miller and coworkers have investigated an intraoperative approach to incorporate dexamethasone, a synthetic steroid stimulating differentiation of MSCs into osteoblasts, with bone aspirate to mediate reclaimed progenitor cells towards osteoblastic commitment [81]. Another approach by Arrighi and coworkers was to immobilize active fragments of parathyroid hormone (PTH) on fibrin matrices for local delivery, to increase bone turnover without systemic

side effects [82]. Parathyroid hormone treatments have been used for osteoporosis treatment to maintain calcium homeostasis in bone for indirect regulation of bone turnover; however, PTH is a broad-spectrum mediator of other metabolic activities in which a systematic exposure would produce additional complications [63].

With increased sophistication of bioactive scaffolds, design challenges and constraints will increase. Variability of the bone substitute will increase due to the incorporation of cellular, chemical, and molecular factors. Preservation and retention of bioactive structure and potency can be insufficient due to material processing and scaffold construction. Consequently, with limited pharmaceutical agents approved for clinical use, a collaborative effort from bone tissue engineering, regenerative medicine, and pharmaceutical sciences is necessary to investigate synergistic approaches for a bioactive bone substitute scaffold for treatment of fragility fractures in patients with bone diseases.

1.1.7 Bone Substitute Biomaterials and Constructs

The basis for a sophisticated bone scaffold is the biomaterial that embodies the mechanical and physiological properties of the scaffold. Substitute bone constructs must provide skeletal continuity with mechanical integrity to transmit load, biological compatibility to avoid immune response, nontoxic degradation to allow new bone replacement, and mediators to encourage bone healing, even in unfavorable environments of diseased bone. Materials of choice include ceramics, natural polymers, synthetic polymers, and their composites. Bone tissue engineers will use biomaterials to engineer

bone substitute constructs that can mimic aspects of native bone to bridge the fracture gap as a temporary 3D template with regeneration potential.

Ceramics are the most commonly used materials in bone fracture repair because of their compressive strength and abundant supply; additionally, the solubility of calcium phosphate and hydroxyapatite compliments that of common bone protective agents. Commercially-available bone substitutes, listed in Table 1.4, are mainly based on

Table 1.4. Commercially-Available Bone Substitutes

Company	Product	Material
Biomet Spine	Pro-Osteon	Hydroxyapatite over a calcium carbonate core
Orthovita	Vitoss	β-Tri-calcium phosphate (β-TCP)
Smith & Nephew	Jax Bone Void	Calcium sulfate
Sofamor Danek	MasterGraft Mix	β-TCP and hydroxyapatite
Stryker	HydroSet HA Bone Calstrux	Calcium phosphate cement β-TCP granules and carboxymethylcellulose
Synthes	chronOS	β-TCP granules
Wright Medical Group	Osteoset 3.0 Pellets MIIG Pro-Dense	Calcium sulfate Proprietary alpha crystal technology Triphasic calcium salt
Zimmer	CopiOs	Calcium phosphate

ceramics, leveraging their osteoconductive nature, nontoxic degradability, compressive strength and long shelf-life. While variability for clinically-approved materials is limited, calcium phosphates, calcium sulfates, and hydroxyapatite materials are versatile, soluble materials that can be combined with ductile polymers and carry mediators. Currently, the clinically available products are essentially serving as bone fillers; however, transition of the clinically-approved bone substitutes into bioactive or functional substitute bone would be easier and quicker than developing novel biomaterials for treating fragility fractures in diseased bone.

Table 1.5. Examples of 3D Bone Substitute Construct Designs

Architecture	Ref.	Material Composition	Fabrication	Characteristics	Construct Variables	Disadvantages
Macroporous sponge	Pol et al. 2010.	βTCP and PL	Supercritical gas foaming	Macroporous	Pore size Mechanical strength	Undetermined degradation Undetermined drug encapsulation capacity
Sintered microspheres	Shi et al. 2010	PLG and HA	Heat sintering	High mechanical strength Controlled release function Macroporous	Porosity Mechanical Strength Drug encapsulation	Undetermined mechanical strength
Woven fiber	Lee et al. 2011	αTCP and sodium alginate	Solution hardening	Microporous	Drug encapsulation	Limited scaffold volume Low mechanical strength
Rapid prototyped matrix	Wilson et al. 2011	βTCP, BCP, HA	Rapid prototype casting	Macroporous	Pore size Organization Scaffold volume Composite blends	Preliminary stage of evaluating fabrication technique
Electrospun fibers	Li 2006	Silk fibroin and HA	Electrospinning	Microporous (interconnected) Nanofibrous	Material blends Drug encapsulation	Low mechanical strength Limited scaffold volume

βTCP = β-tricalcium phosphate, αTCP = α -tricalcium phosphate PL = polylactide, PLG = poly(lactide-co-glycolide), HA = hydroxyapatite, BCP = biphasic calcium phosphate

Polymers, on the other hand, are comparatively more versatile than ceramics in processing, manufacturing, and manipulation, including rapid prototyping, electrospinning, and *in situ* hardening of nanofibers, macroporous sponges, and microspheres (Table 1.5). Natural polymers like chitosan, collagen, and hyaluronic acid have inherent physicochemical and mechanical properties that can be adjusted through processing for an intended use [83]. For example, chitosan derived from the exoskeleton

of crustaceans has intrinsic antibacterial properties, biologically reactive functional groups, nontoxic degradation, and ductility [83, 84]. There also exist synthetic polymers such as polylactide (PL), polyglycolide (PG), polyanhydride, and their copolymers that can be fabricated into varying 3D matrix shapes with sufficient mechanical strength and controlled degradation [83, 85]. However, the foreign body response to degradation by-products of orthopedic implants made from synthetic polymers such as polyglycolide and PL orthopedic implants is non-ideal [86]. To minimize PL or PG bulk mass loss, scaffolds are fabricated with composite materials incorporating ceramics and polymers. Composites such as chitosan/calcium phosphate and poly(lactide-co-glycolide) (PLG)/hydroxyapatite (HA) have been manipulated to mimic bone architecture and increase biocompatibility [87].

Once a biomaterial or a composite of biomaterials is chosen, the challenge is to engineer substitute constructs conforming to an array of criteria – architectural organization, biocompatibility, osteoinductivity, osteoconductivity, biomechanics, biodegradability, and, most recently, bioactivity – to become biomimetic and functional as autologous bone. The 3D architectural organization of the material may have interconnected pores to allow host cell migration, nutrient and waste diffusion, and blood capillary formation. However, finding a synthetic material with high porosity and mechanical integrity proves to be a challenging proposition. In addition to overall architecture, surface micro-topography or roughness can enhance cell attachment and protein adsorption in bone regeneration. A biomaterial scaffold should be relatively biocompatible, eliciting no immune response while encouraging cell attachment and

proliferation. The scaffold matrix should provide an osteoconductive and osteoinductive environment to recruit progenitor cell attachment, then support and stimulate differentiation of active bone cells for fracture healing. The scaffold should be absorbed or remodeled by bone cells and absorbed, remodeling/absorbing at a rate at which biomechanical stability is sustained and cellular responses stimulated as load is transmitted.

To advance regenerative medicine, bone substitute constructs have become biomimetic, bioactive, and functional, serving as drug delivery systems [88]. Current investigations are focused on mechanisms of loading and solubilizing a biomolecule into the material itself before scaffold construction, or adsorbing and absorbing the biomolecule into the scaffold after production. Novel approaches use calcium phosphate as a drug carrier in electrospun polymers or injectable bone substitutes [55, 74, 76].

In vitro and *in vivo* assessments are essential to evaluate scaffold properties and potentials in encapsulating and eluting drugs in a controlled manner to target the pathology of the particular bone disease. As a review by Baroli suggested, pharmaceutical scientists focused on bone regeneration will face the challenge of choosing a specified agent for one particular mechanism in spite of the reality of a multitude of pathways that orchestrate bone healing reactions [62]. An *in vitro* test system can simulate a specific abnormal pathway in the bone disease and allow consistent characterization of the molecular agent of interest along with fundamental understanding of the bone substitutes in the pathological conditions. Each molecular factor, cytokine, and hormone supplement in cell culture medium has been extensively

studied to obtain the optimal concentration and ratio; however, the effects of cell concentration and ratio with respect to each other have been of little interest. Furthermore, the determination of the influence of adipocytes in bone cell regulation has received inadequate consideration. Some bone diseases are due to the imbalance of cell ratios and cellular signaling, which can lead to hyperactivity of one and hypoactivity of the other, resulting in bone malignancies. To simulate *in vivo* conditions effectively, a standardized multicellular system should be established to incorporate key cell types, comparable cell ratios, and molecular controls to allow a coordinated stimulatory environment for all cell types within an *in vitro* culture. A multicellular system will allow efficient characterization and evaluation of the bone scaffold before advancing into *in vivo* and clinical testing.

For example, in a case study presented by Eder and coworkers chronOS produced by Synthes was explanted 28 months after a spinal fusion of a 41 year old female patient with scoliosis [89]. The explant showed no sign of material resorption and no sign of bone cell attachment or proliferation. The manufacturer claimed complete resorption of the β-tri-calcium phosphate within 6-18 months, which was most likely estimated with no consideration of pathological bone metabolism [89]. With an *in vitro* system that can characterize the synthetic material under pathological conditions, an expedited preliminary screening can estimate potential disadvantages of the material.

The evolution of bone tissue engineering must include high consideration for overall patient physiology as well as the objective of fracture management. The process

of translating scaffold innovations into clinical applications will involve extensive *in vitro* and *in vivo* evaluations to ensure all essential criteria are incorporated – biodegradability, compressive strength, bioactivity, 3D architecture, manufacturing, handling, etc. By implementing more requirements for developing functional scaffolds, investigators will face more constraints in their challenging endeavor for an ideal regenerative bone substitute.

1.1.8 Constraints in Bone Tissue Engineering

The design of a drug-delivering scaffold to serve as a template for bone formation and structural support, with the ability to elute biomolecules to mediate normal bone healing reactions in an abnormal environment, is a challenging endeavor. Systemic delivery of protective bone agents inhibits bone cell malfunctions and interferes with the bone's capacity to repair, while local delivery of growth factors and cytokines stimulates bone cell remodeling activities at the fracture site. An ideal bone substitute scaffold would have controlled and sustained drug release, would conserve drug potency and efficacy, would be low cost, and have a long shelf-life. The encapsulation techniques should allow predictable control of a biomolecule concentration and of the elution profile; however, potency and efficacy can be limited by the degradation profile of the material and the stability of the molecule immobilized in the scaffold. During the incorporation of the drug into the scaffold, minimal or no conformational changes to the molecular structure should occur to avoid alterations to drug activity and potency [89].

The production cost of a scaffold should be minimal, allowing ample supply with no loss in scaffold functionality. The addition of the biomolecule is the limiting factor

constraining the advances in bioactive scaffolds, especially when dealing with molecular factors of purified proteins, cytokines, and growth factors. Constraints in potency and efficacy can be a comparable tradeoff for quantity and quality limitations of autografts and allografts. Tunability in construct organization and physiochemical properties will allow specialized substitute scaffolds for different abnormal physiological environments.

1.5 Summary

As commonly taught in biology classes, function follows form; however, in the case of the body, function follows physiology. Therefore, the fracture management strategies in pharmaceutical therapy, surgical intervention, and tissue regeneration should follow the patient's bone physiology. Patients with bone diseases undergo protective bone treatments to prevent fragility fractures by inhibiting abnormal cell activity, not by restoring normal activity. However, when a pathological fracture is sustained, surgical interventions tend to implement traditional methods of stabilization and fail to account for compromised bone mechanics. Regenerative bone grafts are commonly used to provide skeletal continuity for critical size fractures; however, the regenerative capacity of the graft can be hindered by the protective bone agent. Consequently, it is essential to integrate all three management strategies to establish a well-rounded treatment that can mediate bone healing in a diseased bone environment.

Engineering approaches are evolving toward treatment of high risk fragility fractures in pathological bone with low quality bone fixations and functionalized bone scaffolds. Hence, function will follow pathology as increased understanding of bone diseases is applied towards designing disease-specific systemic and local bone

treatments. Incorporation of bioactive factors should be the norm in the advancement of engineered fixation devices and bone scaffolds. However, much research is needed to determine optimal methods and procedures to load and encapsulate the factors without compromising efficacy. By focusing on regenerative responses in the pathological bone environments, bone tissue engineering will be able to target the specific abnormality of the bone disease for the most beneficial and integrated fracture treatment and management.

References

[1] Schindeler A, McDonald MM, Bokko P, Little DG. Bone remodeling during fracture repair: The cellular picture. *Seminar in Cellular Development and Biology*. 2008 Oct; 19 (5): 459-466.
[2] Carano RA, Filvaroff EH. Angiogenesis and bone repair. *Drug Discovery Today*. 2003 Nov 1; 8 (21): 980-989.
[3] Rachner TD, Hadji P, Hofbauer LC. Novel therapies in benign and malignant bone diseases. *Pharmacology and Therapeutics*. 2012 Feb 28; 134 338-344.
[4] Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. *Injury*. 2005 Nov; 36 Suppl 3 S20-27.
[5] Wraighte PJ, Scammell BE. Principles of Fracture Healing. *General Principles of Orthopedic Surgery*. 2006 24 (6): 198-207.
[6] Yaszemski MJ, Payne RG, Hayes WC, Langer R, Mikos AG. Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. *Biomaterials*. 1996 Jan; 17 (2): 175-185.
[7] Khan SN, Cammisa FP, Jr., Sandhu HS, Diwan AD, Girardi FP, Lane JM. The biology of bone grafting. *Journal of the American Academy of Orthopaedic Surgeons*. 2005 Jan-Feb; 13 (1): 77-86.
[8] Blokhuis TJ, Lindner T. Allograft and bone morphogenetic proteins: an overview. *Injury*. 2008 Sep; 39 Suppl 2 S33-36.
[9] McCall TA, Brokaw DS, Jelen BA, Scheid DK, Scharfenberger AV, Maar DC, Green JM, Shipps MR, Stone MB, Musapatika D, Weber TG. Treatment of large segmental bone defects with reamer-irrigator-aspirator bone graft: technique and case series. *Orthopedic Clinics of North America*. 2010 Jan; 41 (1): 63-73.
[10] Ethier CRS, Craig A (2007) Introductory BiomechanicsCambridge University Press, Cambridge
[11] Schmidlin PR, Nicholls F, Kruse A, Zwahlen RA, Weber FE. Evaluation of moldable, in situ hardening calcium phosphate bone graft substitutes. *Clinical Oral Implants Research*. 2011 Nov 1.
[12] Lee GS, Park JH, Shin US, Kim HW. Direct deposited porous scaffolds of calcium phosphate cement with alginate for drug delivery and bone tissue engineering. *Acta Biomaterialia*. 2011 Aug; 7 (8): 3178-3186.
[13] Butscher A, Bohner M, Hofmann S, Gauckler L, Muller R. Structural and material approaches to bone tissue engineering in powder-based three-dimensional printing. *Acta Biomaterialia*. 2011 Mar; 7 (3): 907-920.
[14] Jiang T, Khan Y, Nair LS, Abdel-Fattah WI, Laurencin CT. Functionalization of chitosan/poly(lactic acid-glycolic acid) sintered microsphere scaffolds via surface heparinization for bone tissue engineering. *Journal of Biomedical Materials Research. Part A*. 2009 Sep 23.
[15] Shi X, Su K, Varshney RR, Wang Y, Wang DA. Sintered microsphere scaffolds for controlled release and tissue engineering. *Pharmaceutical Research*. 2011 May; 28 (5): 1224-1228.

[16] Wilson CE, van Blitterswijk CA, Verbout AJ, Dhert WJ, de Bruijn JD. Scaffolds with a standardized macro-architecture fabricated from several calcium phosphate ceramics using an indirect rapid prototyping technique. *Journal of Materials Science. Materials in Medicine*. 2011 Jan; 22 (1): 97-105.
[17] Derubeis AR, Cancedda R. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. *Annals of Biomedical Engineering*. 2004 Jan; 32 (1): 160-165.
[18] Friedenstein AJ. Precursor cells of mechanocytes. *International Review of Cytology*. 1976 47 327-359.
[19] Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*. 1968 Mar; 6 (2): 230-247.
[20] Komori T. Regulation of osteoblast differentiation by transcription factors. *Journal of Cellular Biochemistry*. 2006 Dec 1; 99 (5): 1233-1239.
[21] Kruger MC, Coetzee M, Haag M, Weiler H. Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone. *Progress in Lipid Research*. 2010 Oct; 49 (4): 438-449.
[22] Schilling T, Noth U, Klein-Hitpass L, Jakob F, Schutze N. Plasticity in adipogenesis and osteogenesis of human mesenchymal stem cells. *Molecular and Cellular Endocrinology*. 2007 Jun 15; 271 (1-2): 1-17.
[23] Elbaz A, Wu X, Rivas D, Gimble JM, Duque G. Inhibition of fatty acid biosynthesis prevents adipocyte lipotoxicity on human osteoblasts in vitro. *Journal of Cellular and Molecular Medicine*. 2010 Apr; 14 (4): 982-991.
[24] Hozumi A, Osaki M, Goto H, Sakamoto K, Inokuchi S, Shindo H. Bone marrow adipocytes support dexamethasone-induced osteoclast differentiation. *Biochemical and Biophysical Research Communications*. 2009 May 15; 382 (4): 780-784.
[25] Deschaseaux F, Sensebe L, Heymann D. Mechanisms of bone repair and regeneration. *Trends Mol Med*. 2009 Sep; 15 (9): 417-429.
[26] Carano RA, Filvaroff EH. Angiogenesis and bone repair. *Drug Discov Today*. 2003 Nov 1; 8 (21): 980-989.
[27] Giannoudis PV, Einhorn TA, Marsh D. Fracture healing: the diamond concept. *Injury*. 2007 Sep; 38 Suppl 4 S3-6.
[28] Keramaris NC, Calori GM, Nikolaou VS, Schemitsch EH, Giannoudis PV. Fracture vascularity and bone healing: a systematic review of the role of VEGF. *Injury*. 2008 Sep; 39 Suppl 2 S45-57.
[29] Soucacos PN, Johnson EO, Babis G. An update on recent advances in bone regeneration. *Injury*. 2008 Sep; 39 Suppl 2 S1-4.
[30] Tsiridis E, Upadhyay N, Giannoudis P. Molecular aspects of fracture healing: which are the important molecules? *Injury*. 2007 Mar; 38 Suppl 1 S11-25.
[31] Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Research and Therapy*. 2007 9 Suppl 1 S1.
[32] Egusa H, Doi M, Saeki M, Fukuyasu S, Akashi Y, Yokota Y, Yatani H, Kamisaki Y. The small molecule harmine regulates NFATc1 and Id2 expression in osteoclast progenitor cells. *Bone*. 2011 Aug; 49 (2): 264-274.

[33] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature*. 2003 May 15; 423 (6937): 337-342.
[34] Hofbauer LC, Kuhne CA, Viereck V. The OPG/RANKL/RANK system in metabolic bone diseases. *J Musculoskelet Neuronal Interact*. 2004 Sep; 4 (3): 268-275.
[35] Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? *Nature Clinical Practice. Rheumatology*. 2006 Jan; 2 (1): 35-43.
[36] Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. *Progress in Lipid Research*. 2001 Jan-Mar; 40 (1-2): 125-148.
[37] Justesen J, Pedersen SB, Stenderup K, Kassem M. Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo. *Tissue Engineering*. 2004 Mar-Apr; 10 (3-4): 381-391.
[38] Johnell O, Kanis JA. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporosis International*. 2006 Dec; 17 (12): 1726-1733.
[39] Del Fattore A, Cappariello A, Teti A. Genetics, pathogenesis and complications of osteopetrosis. *Bone*. 2008 Jan; 42 (1): 19-29.
[40] Cundy T, Bolland M. Paget disease of bone. *Trends Endocrinology Metabolism*. 2008 Sep; 19 (7): 246-253.
[41] Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proceedings of the National Academy of Sciences of the United States of America*. 2002 Jun 25; 99 (13): 8932-8937.
[42] Wekre LL, Eriksen EF, Falch JA. Bone mass, bone markers and prevalence of fractures in adults with osteogenesis imperfecta. *Archives of Osteoporosis*. 2011 Dec; 6 (1-2): 31-38.
[43] Pratap J, Lian JB, Stein GS. Metastatic bone disease: role of transcription factors and future targets. *Bone*. 2011 Jan; 48 (1): 30-36.
[44] Nathan SS, Pereira BP, Zhou YF, Gupta A, Dombrowski C, Soong R, Pho RW, Stein GS, Salto-Tellez M, Cool SM, van Wijnen AJ. Elevated expression of Runx2 as a key parameter in the etiology of osteosarcoma. *Molecular Biology Reports*. 2009 Jan; 36 (1): 153-158.
[45] Ta HT, Dass CR, Choong PF, Dunstan DE. Osteosarcoma treatment: state of the art. *Cancer and Metastasis Reviews*. 2009 Jun; 28 (1-2): 247-263.
[46] Neville-Webbe HL, Coleman RE. Bisphosphonates and RANK ligand inhibitors for the treatment and prevention of metastatic bone disease. *European Journal of Cancer*. 2010 May; 46 (7): 1211-1222.
[47] Pestka JM, Seitz S, Zustin J, Puschel K, Amling M, Barvencik F. Paget disease of the spine: an evaluation of 101 patients with a histomorphometric analysis of 29 cases. *Eur Spine J*. 2012 May; 21 (5): 999-1006.
[48] Vukmirovic-Popovic S, Colterjohn N, Lhotak S, Duivenvoorden WC, Orr FW, Singh G. Morphological, histomorphometric, and microstructural alterations in human bone metastasis from breast carcinoma. *Bone*. 2002 Oct; 31 (4): 529-535.

[49] Rauch F, Travers R, Parfitt AM, Glorieux FH. Static and dynamic bone histomorphometry in children with osteogenesis imperfecta. *Bone*. 2000 Jun; 26 (6): 581-589.
[50] Rauch F, Travers R, Norman ME, Taylor A, Parfitt AM, Glorieux FH. Deficient bone formation in idiopathic juvenile osteoporosis: a histomorphometric study of cancellous iliac bone. *Journal of Bone and Mineral Research*. 2000 May; 15 (5): 957-963.
[51] Tzioupis C, Giannoudis PV. Prevalence of long-bone non-unions. *Injury*. 2007 May; 38 Suppl 2 S3-9.
[52] Rachner TD, Hadji P, Hofbauer LC. Novel therapies in benign and malignant bone diseases. *Pharmacol Ther*. 2012 Feb 28.
[53] Clezardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. *Cancer Research*. 2005 Jun 15; 65 (12): 4971-4974.
[54] Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. *Lancet*. 2011 Apr 9; 377 (9773): 1276-1287.
[55] Puppi D, Piras AM, Chiellini F, Chiellini E, Martins A, Leonor IB, Neves N, Reis R. Optimized electro- and wet-spinning techniques for the production of polymeric fibrous scaffolds loaded with bisphosphonate and hydroxyapatite. *Journal of Tissue Engineering and Regenerative Medicine*. 2011 Apr; 5 (4): 253-263.
[56] Stromsoe K. Fracture fixation problems in osteoporosis. *Injury*. 2004 Feb; 35 (2): 107-113.
[57] Bickels J, Dadia S, Lidar Z. Surgical management of metastatic bone disease. *J Bone Joint Surgery, American Edition*. 2009 Jun; 91 (6): 1503-1516.
[58] Hailer YD, Hoffmann R. Management of a nonunion of the distal femur in osteoporotic bone with the internal fixation system LISS (less invasive stabilization system). *Archives of Orthopaedic and Traumatic Surgery*. 2006 Jul; 126 (5): 350-353.
[59] Schutz M, Sudkamp NP. Revolution in plate osteosynthesis: new internal fixator systems. *Journal of Orthopaedic Science*. 2003 8 (2): 252-258.
[60] ONN (2008) Orthopedic Network News,
[61] Kagami H, Agata H, Tojo A. Bone marrow stromal cells (bone marrow-derived multipotent mesenchymal stromal cells) for bone tissue engineering: basic science to clinical translation. *International Journal of Biochemistry and Cell Biology*. 2011 Mar; 43 (3): 286-289.
[62] Baroli B. From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. *J Pharmaceutical Science*. 2009 Apr; 98 (4): 1317-1375.
[63] Einhorn TA. Bone healing: little secrets. *Clin Cases Miner Bone Metab*. 2011 Jan; 8 (1): 17-20.
[64] Lane NE, Silverman SL. Anabolic therapies. *Current Osteoporosis Reports*. 2010 Mar; 8 (1): 23-27.
[65] Porter JR, Ruckh TT, Popat KC. Bone tissue engineering: a review in bone biomimetics and drug delivery strategies. *Biotechnol Prog*. 2009 Nov-Dec; 25 (6): 1539-1560.

[66] Griffith JF, Yeung DK, Ahuja AT, Choy CW, Mei WY, Lam SS, Lam TP, Chen ZY, Leung PC. A study of bone marrow and subcutaneous fatty acid composition in subjects of varying bone mineral density. *Bone*. 2009 Jun; 44 (6): 1092-1096.
[67] Shirota T, Kurabayashi H, Ogura H, Seki K, Maki K, Shintani S. Analysis of bone volume using computer simulation system for secondary bone graft in alveolar cleft. *International Journal of Oral and Maxillofacial Surgery*. 2010 Sep; 39 (9): 904-908.
[68] Hashemi HM, Javidi B. Comparison between interpositional bone grafting and osteogenic alveolar distraction in alveolar bone reconstruction. *Journal of Oral and Maxillofacial Surgery*. 2010 Aug; 68 (8): 1853-1858.
[69] De Long WG, Jr., Einhorn TA, Koval K, McKee M, Smith W, Sanders R, Watson T. Bone grafts and bone graft substitutes in orthopaedic trauma surgery. A critical analysis. *J Bone Joint Surgery, American Edition*. 2007 Mar; 89 (3): 649-658.
[70] Jager M, Herten M, Fochtmann U, Fischer J, Hernigou P, Zilkens C, Hendrich C, Krauspe R. Bridging the gap: bone marrow aspiration concentrate reduces autologous bone grafting in osseous defects. *Journal of Orthopaedic Research*. 2011 Feb; 29 (2): 173-180.
[71] Schmidmaier G, Herrmann S, Green J, Weber T, Scharfenberger A, Haas NP, Wildemann B. Quantitative assessment of growth factors in reaming aspirate, iliac crest, and platelet preparation. *Bone*. 2006 Nov; 39 (5): 1156-1163.
[72] Josse S, Faucheux C, Soueidan A, Grimandi G, Massiot D, Alonso B, Janvier P, Laib S, Pilet P, Gauthier O, Daculsi G, Guicheux JJ, Bujoli B, Bouler JM. Novel biomaterials for bisphosphonate delivery. *Biomaterials*. 2005 May; 26 (14): 2073-2080.
[73] Cattalini JP, Boccaccini AR, Lucangioli S, Mourino V. Bisphosphonate-Based Strategies for Bone Tissue Engineering and Orthopedic Implants. *Tissue Engineering Part B: Reviews*. 2012.
[74] Daculsi G, Weiss P, Bouler JM, Gauthier O, Millot F, Aguado E. Biphasic calcium phosphate/hydrosoluble polymer composites: a new concept for bone and dental substitution biomaterials. *Bone*. 1999 Aug; 25 (2 Suppl): 59S-61S.
[75] Peter B, Gauthier O, Laib S, Bujoli B, Guicheux J, Janvier P, van Lenthe GH, Muller R, Zambelli PY, Bouler JM, Pioletti DP. Local delivery of bisphosphonate from coated orthopedic implants increases implants mechanical stability in osteoporotic rats. *Journal of Biomedical Materials Research. Part A*. 2006 Jan; 76 (1): 133-143.
[76] Verron E, Khairoun I, Guicheux J, Bouler JM. Calcium phosphate biomaterials as bone drug delivery systems: a review. *Drug Discovery Today*. 2010 Jul; 15 (13-14): 547-552.
[77] Faucheux C, Verron E, Soueidan A, Josse S, Arshad MD, Janvier P, Pilet P, Bouler JM, Bujoli B, Guicheux J. Controlled release of bisphosphonate from a calcium phosphate biomaterial inhibits osteoclastic resorption in vitro. *Journal of Biomedical Materials Research. Part A*. 2009 Apr; 89 (1): 46-56.
[78] Robiony M, Zorzan E, Polini F, Sembronio S, Toro C, Politi M. Osteogenesis distraction and platelet-rich plasma: combined use in restoration of severe atrophic mandible. Long-term results. *Clinical Oral Implants Research*. 2008 Nov; 19 (11): 1202-1210.

[79] Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*. 1998 Jun; 85 (6): 638-646.
[80] Li M, Liu X, Ge B. Calcium phosphate cement with BMP-2-loaded gelatin microspheres enhances bone healing in osteoporosis: a pilot study. *Clinical Orthopaedics and Related Research*. 2010 Jul; 468 (7): 1978-1985.
[81] Miller MA, Ivkovic A, Porter R, Harris MB, Estok DM, 2nd, Smith RM, Evans CH, Vrahas MS. Autologous bone grafting on steroids: preliminary clinical results. A novel treatment for nonunions and segmental bone defects. *International Orthopaedics*. 2011 Apr; 35 (4): 599-605.
[82] Arrighi I, Mark S, Alvisi M, von Rechenberg B, Hubbell JA, Schense JC. Bone healing induced by local delivery of an engineered parathyroid hormone prodrug. *Biomaterials*. 2009 Mar; 30 (9): 1763-1771.
[83] Khan Y, Yaszemski MJ, Mikos AG, Laurencin CT. Tissue engineering of bone: material and matrix considerations. *J Bone Joint Surg Am*. 2008 Feb; 90 Suppl 1 36-42.
[84] Shi C, Zhu Y, Ran X, Wang M, Su Y, Cheng T. Therapeutic potential of chitosan and its derivatives in regenerative medicine. *Journal of Surgical Research*. 2006 Jun 15; 133 (2): 185-192.
[85] Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. *Biomaterials*. 2000 Dec; 21 (24): 2529-2543.
[86] Bostman O, Pihlajamaki H. Clinical biocompatibility of biodegradable orthopaedic implants for internal fixation: a review. *Biomaterials*. 2000 Dec; 21 (24): 2615-2621.
[87] Jiang T, Nukavarapu SP, Deng M, Jabbarzadeh E, Kofron MD, Doty SB, Abdel-Fattah WI, Laurencin CT. Chitosan-poly(lactide-co-glycolide) microsphere-based scaffolds for bone tissue engineering: In vitro degradation and in vivo bone regeneration studies. *Acta Biomaterialia*. 2010 Mar 20.
[88] Dawson JI, Oreffo RO. Bridging the regeneration gap: stem cells, biomaterials and clinical translation in bone tissue engineering. *Archives of Biochemistry and Biophysics*. 2008 May 15; 473 (2): 124-131.
[89] Mourino V, Boccaccini AR. Bone tissue engineering therapeutics: controlled drug delivery in three-dimensional scaffolds. *Journal of the Royal Society, Interface*. 2010 Feb 6; 7 (43): 209-227.

CHAPTER TWO

A CO-CULTURE CHARACTERIZATION OF PRECURSOR CELL RATIO FOR OSTEOCLAST AND OSTEOBLAST DIFFERENTIATION

2.1 Introduction

Bone is a complex and dynamic tissue with physiological properties and three-dimensional (3D) organization to maintain bone health and functionality. Bone cells are constantly communicating with each other to instantaneously respond to physical, biological, and endocrine stimuli. A signaling cascade causes coordinated cell activity, leading to renewal and/or repair depending on the specific internal and external mediators [1-3]. Upon damage due to traumatic injuries or mechanical stress exceeding physiological healing conditions, therapeutic intervention is required to facilitate good bone union. Common therapeutic practices are rigid fixation and bone graft implantation, which provide a mechanically and biologically favorable environment for healing [4-6]. Current clinically available bone grafts are autografts and allografts, i.e. bone tissue retrieved from the patient or retrieved from donor tissue, respectively [7].

Within the United States, the ≥65 population of baby boomers will exceed 77 million in 2020; this population has the highest prevalence for osteoporosis and fractures due to low bone mass. Consequently, the demand for bone graft implantation will exceed the allograft supply, and autograft retrieval can be complicated for older patients [8, 9]. Low biological activity and regenerative capacity of allografts and autografts necessitate alternate therapeutic options [10-12]. Therefore, bone tissue engineering strategies are sought to develop bone substitute materials that can induce bone healing with their

inherent chemical, biological, and mechanical properties [5, 6]. However, ceramic, polymeric, and composite materials have limited strength, architecture, degradability, and biocompatibility, as compared to native healthy bone, with respect to facilitating the healing cascade [11]. Current investigations focus on material enhancements using regenerative medicine principles of incorporating isolated cells, chemical factors, and growth factors via adsorption and absorption.

Bone substitute materials bioactivity is limited to mediating bone regeneration within in the normal bone environment and does not address compromised healing reactions of diseased bone which is susceptible to non-unions. Advancements in bone tissue engineering and regenerative medicine must address cellular and metabolic abnormalities of bone diseases to effectively treat fragility fractures. In order to characterize the bioactivity of substitute designs, *in vitro* and *in vivo* testing systems must simulate normal and diseased bone physiology.

Transgenic animals have advanced the understanding of the dynamic complexity of native bone cell coordination and of the differentiation into the osteoclast and osteoblast lineages [13, 14]. However, *in vitro* simulation of bone pathology is lacking, due to the complexities and inconsistencies in current co-culture systems that attempt to highlight osteoclastic and osteoblastic metabolic coupling. The coordination is exemplified in the intertwined signaling pathways of hematopoietic and mesenchymal stem cell differentiation along the osteoclastic and osteoblastic lineage. Secreted cytokines and factors from either lineage can mediate maturation and activity of the other [15, 16]. For example, receptor activator of nuclear factor kappa-B ligand (RANK ligand)

is the key factor produced by osteoblasts to signal osteoclast differentiation through the RANK pathway, leading to activation of transcription factors [17, 18].

The coordinated partnership is also evident in the bone remodeling cascade, with a balance in bone resorption and deposition needed to maintain bone health at a defined ratio of cell concentration and mediators [14, 19]. Any deviation from homeostatic bone remodeling and balance in cell ratio can result in bone diseases like osteoporosis and osteopetrosis [20-22]. Histomorphometric evidence of bone biopsies of healthy and diseased bone exemplifies the morphology and cell ratio differences of osteoclast and osteoblast cells. For Paget's disease and bone metastasis from breast carcinoma, the cell ratios of osteoclast to osteoblast were calculated to be 1:23.1 and 1:1.6, respectively, while healthy biopsies exhibit a ratio of 1:15 [23, 24]. Consequently, a co-culture approach to evaluate bone cell response to bone substitute bioactivity is necessary, and understanding the consistency and relevancy of culture parameters to native bone metabolism is essential.

The objective of this study was to determine the effect of specific culture parameters, i.e. precursor cell ratio and differentiation supplement mediator (soluble RANK ligand), on osteoclast and osteoblast differentiation. Co-culture was conducted, with three different cell ratios of RAW monocytes and D1 stromal cells under osteogenic conditions, to monitor differences in lineage maturation. This study also focused on hematopoietic differentiation in osteogenic and RANK ligand-supplemented conditions to examine the necessity of the additive in co-culture conditions, where RANK ligand is secreted by osteoblasts. Gene expression levels of early and late bone cell maturation

markers were quantified to monitor if *in vitro* cellular differentiation and activation was characteristic of that of native bone cells.

2.2 Methods

2.2.1 chronOS Granule Sterilization

To simulate the *in vivo* environment of bone, chronOS (Synthes) β-tricalcium phosphate cancellous bone substitute was used as the three-dimensional (3D) matrix for cell co-culture. The 150mg of granules were placed in scintillation vials (Wheaton) without caps and sterilized at 200°C under 10 psi, in a vacuum oven (VWR Symphony) for at least 2 hours. The vials were immediately transferred from the oven to the Steriguard biological cabinet to be capped with autoclaved vial tops.

Table 2.1. Experimental Setup (n=3)

Cell Ratio (RAW:D1)	Osteogenic (OS) Medium	Osteogenic Medium with RANK ligand (R)
1:1= 76000 RAW : 76000 D1	OS1	R1
1:10 = 7600 RAW : 76000 D1	OS2	R2
1:100 = 760 RAW : 76000 D1	OS3	R3
Fluorescence Imaging Samples: OS1, OS2, R1, R2 Controls: growth medium with 1:100 ratio on ChronOS		

2.2.2 Cell Culture

Murine RAW 294.7 monocytes (ATTC) and D1 stromal cells (ATCC) were seeded on chronOS granules in 24-well plates (Corning) with osteogenic medium (Dulbecco's Modified Eagle's Medium (DMEM) (Atlanta Biologics) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (ATCC), 0.2% fungizone (Invitrogen), 0.1μM dexamethasone (Sigma), 10mM β-glycerophosphate (Sigma),

5µg/ml ascorbic acid (Sigma)) supplemented with 30ng/ml RANK ligand (Pepro Tech) or no supplement. The D1 cells were co-cultured with RAW 294.7 monocytes and stimulated to differentiate into osteoblasts and osteoclasts, respectively. Each well plate contained 150mg of chronOS granules; the granules were presoaked in growth medium for 24 hours at 37°C prior to cell seeding. The experimental layout for the co-culture of RAW to D1 cell density ratios and experimental groups are shown in Table 2.1. The culture was maintained for 36 days, samples were collected at Days 8, 15, 22, 29 and 36, and the medium was changed every other day.

2.2.3 Fluorescence Imaging

Endogenous phosphatases, suggestive of bone cell differentiation, such as osteoclast characteristic tartrate-resistant acidic phosphatase (TRAP) granules and osteoblast alkaline phosphatase (ALP) were visualized using fluorescence-based ELF97 staining (ELF97 Endogenous Phosphatase Detection Kit, Molecular Probes). Additional samples (experimental groups: OS1, OS2, R1, R2) were cultured on four-well chamber slides (Lab Tek II) for better image quality (Table 2.1). ELF97 staining was performed at endpoint Day 36 following the manufacturer's protocol, with two counterstains to reveal a more defined cellular morphology. Hoechst 33342 (Invitrogen) was used to stain cell nuclei blue while AlexaFluor 546 Phalloidin (Invitrogen) was used to stain cytoskeleton actin red, to contrast with the green fluorescence of ELF97. Images were taken using the microscope (Axiovert 40 CFL, Zeiss) with attached fluorescence lamp under Hoechst/DAPI and TRITC filter sets.

Ribonucleic Acid (RNA) Isolation

To analyze gene expression of differentiation markers of cells attached to the granules, total RNA was isolated at Days 8, 15, 22, 29, and 36 using the TRIzol reagent (Invitrogen) and protocol. Briefly, medium was removed from well plates before 1 ml TRIzol reagent was added to lyse cells, then 0.2 ml of chloroform (Honeywell, HPLC Grade) was added to dissolve RNA into an aqueous phase. The RNA was collected, precipitated with 0.5 ml isopropyl alcohol (VWR), and washed with 1 ml 75% ethanol (Sigma). The ethanol was removed and the precipitated RNA was air dried before resuspending in 30µl of nuclease-free water (Promega). Next, the RNA was treated to remove any contaminant DNA, using the TURBO DNase-Free kit (Ambion). RNA was quantified and qualified using a NanoDrop 1000 spectophotometer (Thermo Scientific), then stored at -80°C until reverse transcription.

2.2.4 Reverse Transcription Real-Time Polymerase Chain Reaction (RT-PCR)

Reverse transcription was performed using the RETROscript kit (Ambion) with 1µg of isolated RNA to synthesize 25 ng/µl of complementary deoxyribonucleic acid (cDNA). QuantiTect SYBR Green kit (Qiagen) was used to perform real-time PCR with the primers listed in Table 2.2. Primers were checked for uniqueness with Primer-BLAST database and efficiency prior to purchase and use, respectively. The StepOne Plus (Applied Biosytems) was used to run PCR at a holding temperature of 95°C for 15 min, then 35 cycles of denaturing at 94°C for 15 sec, annealing at 54°C for 20 sec, and extension at 72°C for 20 sec. Melting occurred at a 70-99°C ramp to check for primer dimers. The cycle number (Ct) was obtained at a threshold of 0.1 to calculate relative

expression ratios (RER) of target genes compared to the internal standard, GAPDH, using the ΔΔCt method as follows:

$$RER = 2^{(-\Delta\Delta Ct)}$$

$$\Delta\Delta Ct = \Delta Ct_{(experimental)} - \Delta Ct_{(control)}$$

$$\Delta Ct_{(experimental)} = Ct_{(target)} - Ct_{(reference)}$$

$$\Delta Ct_{(control)} = Ct_{(target)} - Ct_{(reference)}$$

Table 2.2. RT-PCR Primers

Primers		Sequence	Reference
Internal Standard			
GAPDH	F	5'-GAACGGATTTGGCCGTATTG-3'	
	R	5'-CGTTGAATTTGCCGTGAGTG-3'	[25]
Osteoblast Early Differentiation Marker			
Runx2	F	5'-AGTGGACCCTTCCAGACCAG-3'	
	R	5'-TAATAGCGTGCTGCCATTCG-3'	
ALP	F	5'-GTAACGGGCCTGGCTACAAG-3'	
	R	5'-AAAGACCGCCACGTCTTCTC-3'	
Osteoblast Late Differentiation Marker			
Osteocalcin	F	5'-TGCGCTCTGTCTCTCTGACC-3'	
	R	5'-ATGGAAGGCTAAGGGCTCTG-3'	
Osteopontin	F	5'-AAAGAGAGCCAGGAGAGTGCC-3'	
	R	5'-TGTGGCTGTGAAACTTGTGGC-3'	
Osteoclast Multinucleated			
RANK	F	5'-GGACGTTCACACTGGTCAGC-3'	
	R	5'-TGCTTCCCTGCTGGATTAGG-3'	
NFATc1	F	5'-CTCGAAAGACAGCACTGGAGCAT-3'	
	R	5'-CGGCTGCCTTCCGTCTCATAG-3'	[26]
Osteoclast Resorption			
Cathepsin K	F	5'-CTGCCTTCCAATACGTGCAG-3'	
	R	5'-CCTTTGCCGTGGCGTTATAC-3'	

2.2.5 Western Blotting

A different study was conducted following a similar protocol to collect protein lysate for protein production quantification. The difference between the two studies was the collection time point at Days 7, 14, and 21. Samples were rinsed with phosphate buffered saline (PBS) (Sigma) twice, before 150µl of Mammalian Protein Extraction Reagent (M-PER) (Pierce) was added. The samples with M-PER were ultrasonicated (Sonic Dismembrator, Fisher Scientific) for two 3-second cycles. The lysate was collected and centrifuged (X-12R, Allegra) for 5 minutes at 14,000rpm, then stored at -4°C until the end of the study. Bicinchoninic acid (BCA) protein assay (Pierce, Thermo Scientific) was performed to quantify total protein concentration per sample for gel electrophoresis. Total protein (40µg) was diluted with Laemmli sample buffer (Bio-Rad) and distilled water, for a total volume of 30µl, and loaded onto 10% Tris-HCl Criterion gels (Bio-Rad). Proteins on the gel were blotted onto 0.45µm nitrocellulose membranes (Bio-Rad) for protein detection. Primary antibodies for osteopontin (66kDa) and Runx2 (55kDa) (Rabbit-anti-mouse, Santa Cruz), followed by secondary goat-anti-rabbit conjugated with horseradish peroxidase (HRP), were used to detect protein at specific molecular weights. Beta-actin (42kDa) conjugated with HRP (Cell Signaling) was used as the internal standard. Chemiluminescence imaging was performed using FluorChem™ M (Protein Simple).

2.2.6 Statistical Analysis

The calculated relative expression ratio (RER) average and standard error were graphed for each target gene. JMP 10 (SAS) was used to perform all statistical analyses

to detect interaction among the cell ratios, in two differentiation conditions, over time. Randomized splitplot was implemented to statistically compare gene expression differences within medium groups (wholeplot) for each cell ratio (subplot) within days. If significance in the interaction was detected ($p<0.05$), Tukey-HSD post-hoc analysis (alpha=0.05) was performed to determine significant effects of the factors. Data averages were graphed with standard error of mean.

2.3 Results

2.3.1 Endogenous Phosphatase Staining

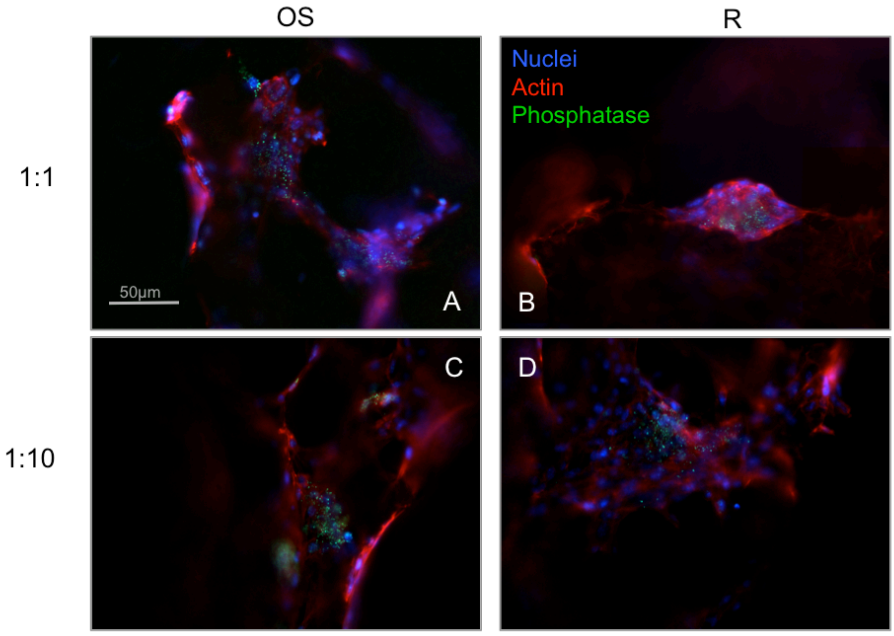

Figure 2.1. ELF97 endogenous phosphatase images for Day 36 of the study, for OS and R groups at 1:1 and 1:10 ratios. Blue, red and green fluorescence indicate nuclei, actin, and phosphatase, respectively. Positive staining for phosphatase is seen for all samples. Scale bar indicates 50µm.

Fluorescent ELF97 staining images show that phosphatases are present in cell clusters for both medium conditions of osteogenic and osteogenic supplemented with

RANK ligand, at cell ratios of 1:1 and 1:10 (ratio 1:100 was not imaged) (Figure 2.1). However, the images do not reveal morphology that allows distinguishing between the osteoclast- or osteoblast-like cells. The distribution of fluorescent phosphatases is sporadic, with no uniformity for all experimental groups.

2.3.2 Real-Time PCR

Osteoblastic differentiation and activity was monitored by the gene expression of Runx2, ALP, and osteocalcin. Under osteogenic conditions, Runx2 transcription factor for all the cell ratios demonstrated peak expression at Day 22, followed by lower expression at Day 29, demonstrating typical cellular differentiation (Figure 2.2A) ($p=0.0002$)). However, at Day 36 expression levels were higher than at Day 29. Statistical interaction of medium condition and cell ratio was not evident for Runx2 expression during the 36 days of culture. Relative expression levels for ALP demonstrated no statistical influence by medium conditions; however, cell ratios indicated differences in expression levels (Figure 2.2B) ($p<0.0001$). Cell ratio 1:100 resulted in significantly higher expression levels as compared to 1:1 and 1:10. OS1 and R1 expression levels were lowest for all days as compared to the other ratios. Relative expressions at cell ratio 1:100 (OS3 and R3) were lowest at Day 22 with a rise in expression for Days 29 and 36 ($p=0.0003$). For OS1 and OS2, levels were higher at each time point while R1 and R2 peaked at Day 29. Osteocalcin expression had no statistical interaction with medium conditions and cell ratio groups (Figure 2.2C). Statistical analysis indicated no change in expression levels from Day 8 to Day 29, with a higher expression at Day 36. Osteopontin expression showed no correlation for different

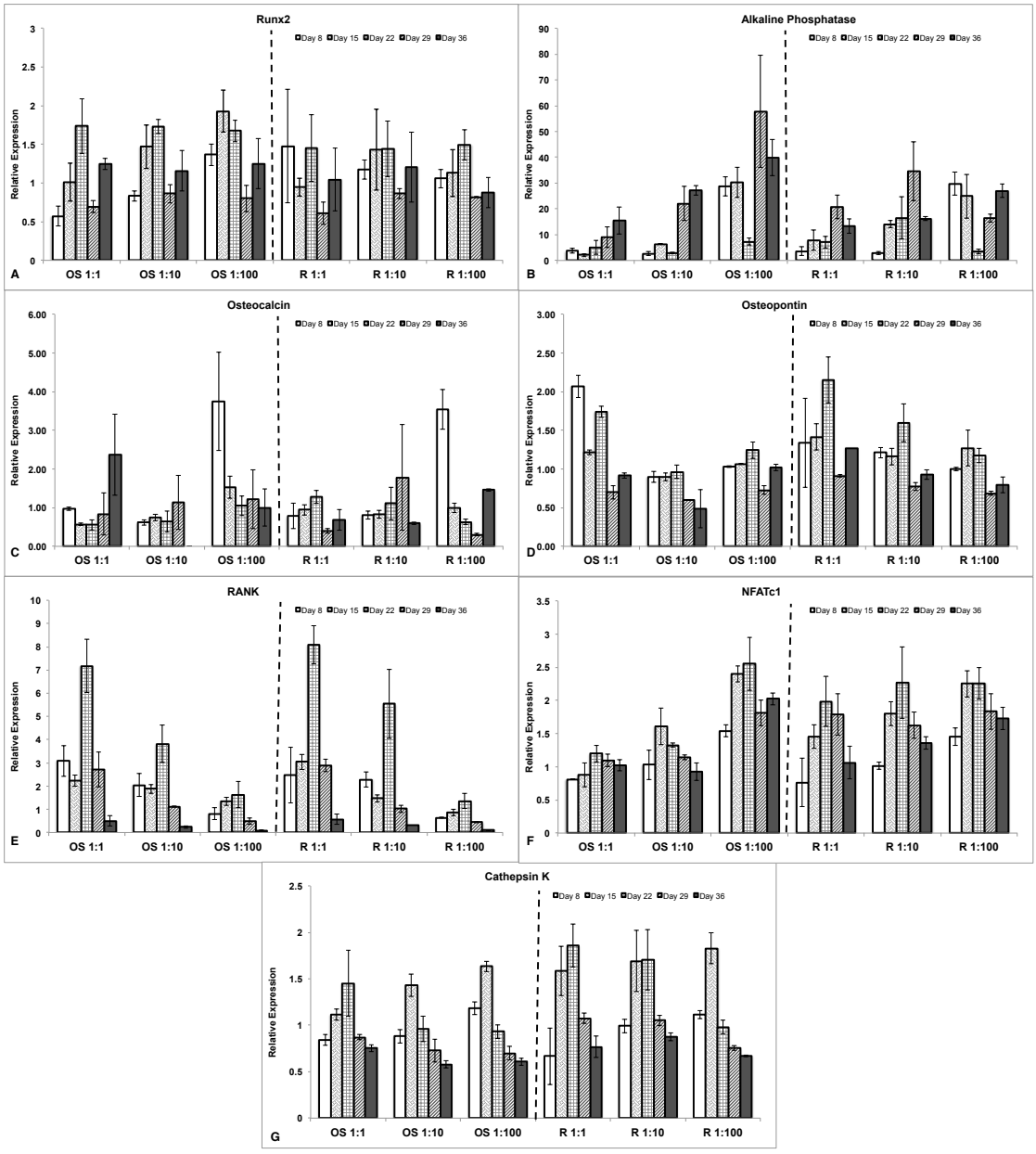

Figure 2.2. Relative expression ratio osteoblastic and osteoclastic markers of maturation and activation graphed with standard error of mean. Runx2, ALP, osteocalcin, and osteopontin are early and late markers for osteoblastic characteristics. RANK, NFATc1, and cathepsin K are markers for osteoclastic cells.

medium conditions; however, cell ratio differences were seen, in which OS1 and R1 were higher than other ratios (Figure 2.2D) ($p=0.0457$).

For osteoclastic differentiation and activity expression levels of RANK, cathepsin K, and NFATc1 were observed. The two different medium conditions resulted in similar RANK levels; however, there were significant differences between the cell ratios, with highest expression for the 1:1 group and lowest expression for the 1:100 group ($p<0.0001$) (Figure 2.2E). NFATc1 gene expression exhibited differences in medium conditions ($p=0.0041$) and cell ratios ($p<0.0001$) (Figure 2.2F). R medium condition had a higher expression of NFATc1 than the OS condition. Unlike the RANK expression, the highest NFATc1 expression was exhibited by OS3 and R3 groups, while the lowest was seen in the OS1 and R1 groups. The NFATc1 expression levels peaked at 22 days of differentiation, then decreased by Day 29. Cathepsin K expression, on the other hand, was different within medium conditions; specifically, high levels were evident for the R medium condition (Figure 2.2G)($p=0.0041$).

2.3.3 Western Blotting

Protein production of osteoblastic Runx2 and osteopontin was observed to determine differentiation and activation (Figure 2.3). Runx2 production for OS and R groups was higher from Day 7 to Day 14; however, at Day 21 the R treatment group decreased in production while OS group in all ratios sustained production. In osteopontin production, OS ratios increased in production, with Day 21 having the highest while R ratios demonstrated the high production at Day 7 with lower production on consequent days.

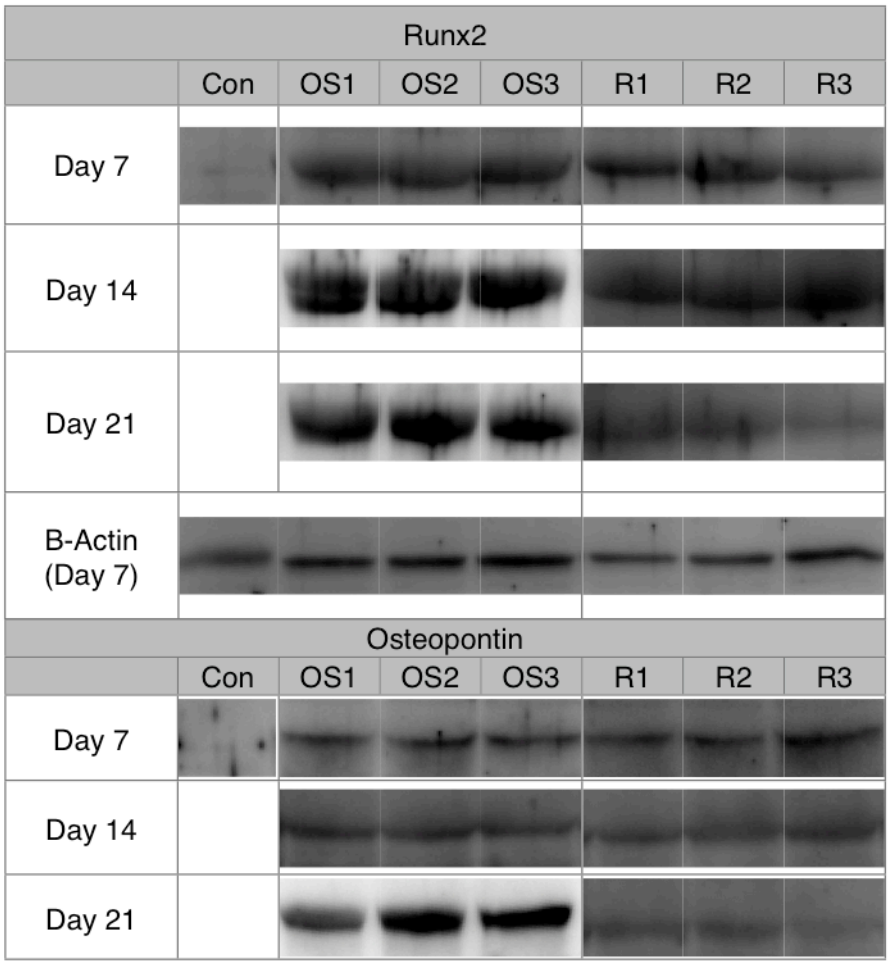

Figure 2.3. Western Blot results for Runx2 and osteopontin production during 21 days of co-culture. B-actin at day 7 was used as the internal standard.

2.4 Discussion

Biomaterials synthesized for bone substitute applications are characterized *in vitro* to determine cellular response and toxicity of the material in culture with bone cells. Some groups have co-cultured precursor osteoclasts and osteoblasts to simulate the paracrine relationship of the bone cells in regulating bone cell differentiation and bone

healing [19, 27-29]. However, considerations to the cell ratio of the two precursor cells during differentiation were limited or not mentioned at all.

Osteoclast differentiation depends on the presence of osteoblasts to initiate paracrine signaling through membrane-bound receptors (RANK) and ligands (RANK ligand). However, the recruitment of precursor osteoblasts to the site of bone resorption depends on secreted and membrane-bound factors from osteoclasts. The balance between bone resorption and deposition is a coordinated effort to maintain bone integrity and health. Increased bone mass leads to osteopetrosis, while decreased bone density causes osteoporosis. In healthy bone tissue, histological images reveal that the population ratio between osteoclasts and osteoblasts is 1:15 or 1:15.5 [23, 24]. Therefore, it is necessary to determine the physiological bone cell ratio for *in vitro* culture to simulate the equilibrium of the bone cells in natural bone tissue in order to establish a co-culture system optimal for material characterization.

A review of the literature reveals the experimental designs have a bias toward high osteoclast concentrations in co-culture conditions. This design allows increased resorption on material surfaces which, in turn, leads to more mineral deposition; however, the high osteoclast concentration contradicts conditions in native bone. In a study conducted by Bernhardt and coworkers, human monocytes and human mesenchymal stem cells in a 25:1 ratio were indirectly co-cultured on collagen tapes for 38 days in RANK ligand-supplemented osteogenic medium. Results showed no significant difference in TRAP and Cathepsin K expression between the monoculture control and the indirect co-culture, with very little detection of multinucleated cells via

scanning electron microscopy [29]. Jones and colleagues, on the other hand, conducted experiment to compare osteoblast only and osteoclast only culture with co-cultured osteoclast (primary murine monocytes) to osteoblast (MC3T3-E1) ratio of 100:1. The cells were seeded on silk fibroin, chitosan films, and poly-l-lactide films to evaluate the potential of the material to induce bone resorption. Unfortunately, results from TRAP staining for osteoclast differentiation and material surface roughness for osteoclast resorption were inconclusive [19]. Using a surface pretreatment approach, Spence and coworkers stimulated *in vitro* osteoclast resorption for 21 days, then removed the osteoclasts before seeding osteoblasts onto the resorbed surfaces of hydroxyapatite and carbonate-substituted hydroxyapatite discs. Even though the osteoclast to osteoblast cell ratio was 100:1 in an indirect co-culture, the results indicated increased collagen synthesis for osteoblasts on the resorbed discs [28]. The Tortelli group investigated 3D versus 2D co-culture conditions using a 1:1 ratio, under osteogenic conditions, for up to 60 days. Relative gene expressions and histological results revealed that 3D skelite discs stimulated enhanced osteoblast differentiation, leading to early osteoclastic differentiation [27]. These studies exemplify the inconsistencies of osteoblast to osteoclast cell ratio from study to study and that the studies do not mimic the ratios in native bone tissue; therefore, evaluations of material properties and differentiation responses are not comparable from laboratory to laboratory and with respect to *in vivo* conditions of bone. Consequently, the need for a standard *in vitro* culture model, with physiologically-relevant precursor osteoclast to osteoblast ratio, is crucial for a

systematic comparison of materials and simulation of coordinated bone resorption and deposition.

The co-culture of RAW monocytes and D1 stromal cells at three different fixed ratios under two osteogenic media conditions was evaluated to determine cell ratio and medium parameters for an *in vitro* co-culture model. Three RAW:D1 precursor osteoclast and osteoblast cell ratios, 1:1, 1:10, and 1:100, were chosen with goal of better mimicking *in vivo* conditions for clinically relevant evaluations and predictions. Osteoclast activation and osteoblast activity were observed via fluorescence imaging to assess multi-nucleation, TRAP granule production, and ALP production. Total RNA was collected and analyzed for relative gene expression of markers in osteoclast and osteoblast differentiation.

Positive ELF97 staining for samples in both medium conditions demonstrated the presence of endogenous phosphatase from either osteoclast TRAP granules and/or osteoblast ALP. In mono-culture of RAW and D1 cells (data not shown), ALP released in abundance by D1 cells, while TRAP was limited under osteogenic and growth medium conditions. Even with actin and nuclei counterstaining, cell clusters could not be distinguished to examine differences in cell morphology. Hence, ELF97 staining and counterstaining was not effective for visualizing osteoclast TRAP granules and increased ALP production due to the release of ALP from undifferentiated cells.

To evaluate osteoblast differentiation, expression levels of Runx2, ALP, and osteocalcin were measured for stromal cell differentiation and osteoblast mineralization, respectively, during 36 days in two osteogenic conditions (OS and R) and with three cell

ratios (1:1, 1:10, 1:100). Runx2 is a transcription factor in the signaling pathway directing the differentiation of stromal cells into pre-osteoblasts, then into immature osteoblasts; however, at later stages Runx2 can hinder osteoblast activity [30, 31]. Hence, a high expression of Runx2 during Day 22 of culture, coupled with a lower expression and the presence of mature and active osteoblasts at Day 29, indicates differentiation.

Alkaline phosphatase (ALP) expression suggests immature osteoblasts becoming mature osteoblasts, with increasing ALP levels during maturation and plateaus once mineralization is initiated. ALP levels for all ratios in both media conditions reached the highest by Day 29, indicating a maturation and even activation of osteoblast metabolism. The RANK ligand supplemented medium condition induced does not affect osteoblastic differentiation according to ALP expression. Cell ratio, on the other hand, does affect ALP levels, with OS1 and R1 having the lowest expression while OS3 and R3 have the highest, correlating to the presence of differentiated RAW cells. At closer inspection, the trough in ALP levels at Day 22 for OS2, OS3, and R3 is unexpected, and possibly demonstrates the cyclic nature of ALP activity during osteoblast maturation and activation.

The production of osteocalcin, a calcium-binding protein in osteoblasts, regulates mineralization and osteoclast activity; hence, expression levels are expected to be highest during mineralization or at the later time points of co-culture. The expression levels changed minimally throughout the culture period, with unpredicted peaks on Day 8 for OS3 and R3. Osteoclast differentiation is initiated, with monocyte clustering and fusion into multinucleated cells through RANK-RANK ligand signaling. The multinucleated

cells are then activated and resorb damaged or old bone by secretion of protolytic enzymes [18, 22, 32]. Osteoclast differentiation from RAW monocytes, monitored through the expression of membrane receptor RANK, indicates a cell ratio effect. The pattern of RANK expression is highest at Day 22, with a lowered expression at Day 29 and Day 36 rather than a sustained expression of RANK. The lowering of expression at Day 29 can be a result of RAW cell fusion or osteoclast apoptosis during osteoclastic differentiation; hence, a reduction in overall membrane area for the membrane receptor. The downstream signaling of RANK to RANK ligand was measured by expression of NFATc1, an early sign of osteoclast differentiation, from Day 8 to Day 22. Hence, expression of protolytic enzyme cathepsin K was high on Day 15 and Day 22, suggesting resorption as a late marker of differentiation.

2.5 Conclusions

The evaluation of early and late differentiation precursor osteoclast and osteoblast markers, with goal of establishing a physiological co-culture model of cell ratio for precursor bone cell differentiation, demonstrated that high concentration of osteoclastic RAW cells can suppress early and late osteoblastic D1 differentiation markers. Furthermore, the results provide histomorphological evidence that osteoclast and osteoblast population ratio variations can predict healthy and pathological bone conditions. During osteoclastic differentiation of RAW cells, early markers directly corresponded with RAW concentration while the late marker was higher with respect to the RANK ligand condition. Since both osteoclast and osteoblast differentiation and activation occurred within 22 days of osteogenic co-culture for both medium conditions,

similar to that seen in other *in vitro* single and co-cultures, the supplementation for osteoclastic differentiation in co-culture is nonessential. An *in vitro* co-culture test system for coordinated osteoclast and osteoblast differentiation is necessary to consistently characterize cellular response to materials potential under a healthy or diseased physiological model. Future studies will focus on confirming precursor cell differentiation and activation at the 1:10 cell ratio and will focus on validating the *in vitro* model for clinical predictions of material failure in pathological bone conditions.

References

[1] Barrere FM, TA ; De Groot, K; van Blitterswijk, CA. Advanced biomaterials for skeletal tissue regeneration: Instructive and smart functions. *Materials Science and Engineering: R: Reports*. 2008 February 29; 59 (1-6): 38-71.
[2] Giannoudis PV, Einhorn TA, Marsh D. Fracture healing: the diamond concept. *Injury*. 2007 Sep; 38 Suppl 4 S3-6.
[3] Takeda S. Central control of bone remodeling. *Biochemical and Biophysical Research Communications*. 2005 Mar 18; 328 (3): 697-699.
[4] Schindeler A, McDonald MM, Bokko P, Little DG. Bone remodeling during fracture repair: The cellular picture. *Semin Cell Dev Biol*. 2008 Oct; 19 (5): 459-466.
[5] Soucacos PN, Johnson EO, Babis G. An update on recent advances in bone regeneration. *Injury*. 2008 Sep; 39 Suppl 2 S1-4.
[6] Moroni A, Larsson S, Hoang Kim A, Gelsomini L, Giannoudis PV. Can we improve fixation and outcomes? Use of bone substitutes. *Journal of Orthopaedic Trauma*. 2009 Jul; 23 (6): 422-425.
[7] Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. *Injury*. 2005 Nov; 36 Suppl 3 S20-27.
[8] Tzioupis C, Giannoudis PV. Prevalence of long-bone non-unions. *Injury*. 2007 May; 38 Suppl 2 S3-9.
[9] Younger EM, Chapman MW. Morbidity at bone graft donor sites. *Journal of Orthopaedic Trauma*. 1989 3 (3): 192-195.
[10] Dawson JI, Oreffo RO. Bridging the regeneration gap: stem cells, biomaterials and clinical translation in bone tissue engineering. *Archives of Biochemistry and Biophysics*. 2008 May 15; 473 (2): 124-131.
[11] Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. *Biomaterials*. 2000 Dec; 21 (24): 2529-2543.
[12] Hofmann A, Konrad L, Hessmann MH, Kuchle R, Korner J, Rompe JD, Rommens PM. The influence of bone allograft processing on osteoblast attachment and function. *Journal of Orthopaedic Research*. 2005 Jul; 23 (4): 846-854.
[13] Raisz LG. Bone cell biology: new approaches and unanswered questions. *Journal of Bone and Mineral Research*. 1993 Dec; 8 Suppl 2 S457-465.
[14] Takayanagi H, Kim S, Matsuo K, Suzuki H, Suzuki T, Sato K, Yokochi T, Oda H, Nakamura K, Ida N, Wagner EF, Taniguchi T. RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. *Nature*. 2002 Apr 18; 416 (6882): 744-749.
[15] Teti A, Grano M, Colucci S, Cantatore FP, Loperfido MC, Zallone AZ. Osteoblast-osteoclast relationships in bone resorption: osteoblasts enhance osteoclast activity in a serum-free co-culture system. *Biochemical and Biophysical Research Communications*. 1991 Aug 30; 179 (1): 634-640.
[16] Matsuo K, Irie N. Osteoclast-osteoblast communication. *Archives of Biochemistry and Biophysics*. 2008 May 15; 473 (2): 201-209.
[17] Roodman GD. Cell biology of the osteoclast. *Experimental Hematology*. 1999 Aug; 27 (8): 1229-1241.

[18] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature*. 2003 May 15; 423 (6937): 337-342.
[19] Jones GL, Motta A, Marshall MJ, El Haj AJ, Cartmell SH. Osteoblast: osteoclast co-cultures on silk fibroin, chitosan and PLLA films. *Biomaterials*. 2009 Oct; 30 (29): 5376-5384.
[20] Demulder A, Guns M, Ismail A, Wilmet E, Fondu P, Bergmann P. Increased osteoclast-like cells formation in long-term bone marrow cultures from patients with a spinal cord injury. *Calcified Tissue International*. 1998 Nov; 63 (5): 396-400.
[21] Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000 Sep 1; 289 (5484): 1504-1508.
[22] Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Research and Therapy*. 2007 9 Suppl 1 S1.
[23] Pestka JM, Seitz S, Zustin J, Puschel K, Amling M, Barvencik F. Paget disease of the spine: an evaluation of 101 patients with a histomorphometric analysis of 29 cases. *Eur Spine J*. 2012 May; 21 (5): 999-1006.
[24] Vukmirovic-Popovic S, Colterjohn N, Lhotak S, Duivenvoorden WC, Orr FW, Singh G. Morphological, histomorphometric, and microstructural alterations in human bone metastasis from breast carcinoma. *Bone*. 2002 Oct; 31 (4): 529-535.
[25] Maxson S, Burg KJ. Conditioned media enhance osteogenic differentiation on poly(L-lactide-co-epsilon-caprolactone)/hydroxyapatite scaffolds and chondrogenic differentiation in alginate. *Journal of Biomaterials Science, Polymer Edition*. 2010 21 (11): 1441-1458.
[26] Egusa H, Doi M, Saeki M, Fukuyasu S, Akashi Y, Yokota Y, Yatani H, Kamisaki Y. The small molecule harmine regulates NFATc1 and Id2 expression in osteoclast progenitor cells. *Bone*. 2011 Aug; 49 (2): 264-274.
[27] Tortelli F, Pujic N, Liu Y, Laroche N, Vico L, Cancedda R. Osteoblast and osteoclast differentiation in an in vitro three-dimensional model of bone. *Tissue Engineering. Part A*. 2009 Sep; 15 (9): 2373-2383.
[28] Spence G, Patel N, Brooks R, Rushton N. Carbonate substituted hydroxyapatite: resorption by osteoclasts modifies the osteoblastic response. *Journal of Biomedical Materials Research. Part A*. 2009 Jul; 90 (1): 217-224.
[29] Bernhardt A, Thieme S, Domaschke H, Springer A, Rosen-Wolff A, Gelinsky M. Crosstalk of osteoblast and osteoclast precursors on mineralized collagen--towards an in vitro model for bone remodeling. *J Biomedical Material Research A*. 2010 Dec 1; 95 (3): 848-856.
[30] Komori T. Regulation of osteoblast differentiation by Runx2. *Advances in Experimental Medicine and Biology*. 2010 658 43-49.
[31] Komori T. Regulation of osteoblast differentiation by transcription factors. *Journal of Cellular Biochemistry*. 2006 Dec 1; 99 (5): 1233-1239.
[32] Zhao Q, Wang X, Liu Y, He A, Jia R. NFATc1: functions in osteoclasts. *International Journal of Biochemistry and Cell Biology*. 2010 May; 42 (5): 576-579.

CHAPTER THREE

MULTICELLULAR CULTURE TO SIMULATE INFLUENCE OF ADIPOCYTES ON THE OSTEOBLAST AND OSTEOCLAST ACTIVITY *IN VITRO* ON 3D BONE GRANULES

3.1 Introduction

Patients with bone diseases have the highest risk of sustaining a fracture and have high risks of non-unions and mal-unions in fracture healing. Bone diseases are the result of hyperactive or hypoactive osteoclasts and/or osteoblasts in bone formation and remodeling, leading to bone fragility. The most commonly diagnosed bone disease is osteoporosis, with high prevalence in women post-menopause resulting in increased bone resorption and thinning of cancellous trabeculae [1-3]. The purported cause of hyperactive osteoclast resorption is increased marrow fat due to aging; hence, osteoporosis is associated with the obesity of bone [2]. Other bone diseases, such as osteopetrosis, abnormal bone growth, and osteogenesis imperfecta, lack of collagen in bone formation, are due to genetic mutations and also lead to bone fragility [4, 5]. Histomorphometric analysis of patient bone tissue biopsy reveals that osteoblast and osteoclast cellular morphology and concentration vary between healthy and diseased bone [6-9]. Furthermore, patients with bone diseases undergo bone protective therapy to retard bone degeneration via inhibition of osteoblastic bone formation or osteoclastic bone resorption. The drug agents systemically target metabolic pathways with a range of specificity to bone cells which can further reduce native bone healing reactions within the pathological environment.

Current bone fracture management strategies use substitute materials such as autografts, allografts, and synthetic tissue to fill the bone fracture void for improved bone healing. The demand for engineered bone tissues will increase in the next decade with predicted increase of bone fractures due to fragile bone and limited supply of autografts and allografts [10]. Bone substitute materials are engineered to mimic gold standard autograft biological and mechanical properties. With limitations in exact mimicry, engineered substitutes are developed from ceramics, polymers, and their composites to induce bone healing and bridge the fracture gap. Commercially available bone substitute materials have limited functionality and insufficient accommodation for the pathological

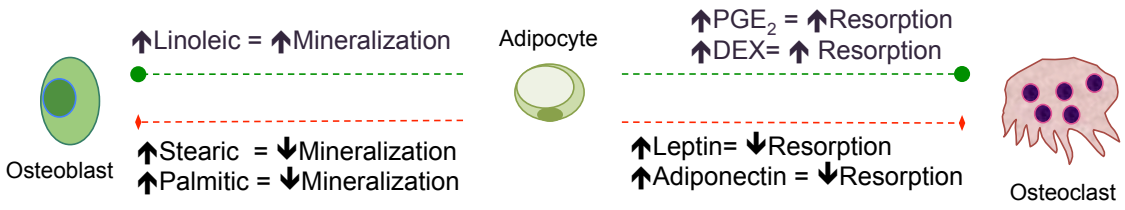

Figure 3.1. Adipogenic regulation of bone formation and resorption through fatty acid and hormonal secretions.

environment and compromised bone cell activity found in patients with bone diseases. Advancements to functionalize material constructs include incorporation of bioactive molecules, cytokines, and cellular components to locally stimulate physiological healing reaction in healthy and diseased conditions [11-13].

To complement development of targeted bone constructs, evaluations of the materials should be conducted within similar targeted conditions, diseased or healthy, to ensure efficacy of construct bioactivity. Hence, *in vitro* simulation of the bone environment must also advance to co-cultures of physiological significance, including

parameters such as osteoclast to osteoblast cell population ratio and indirect effects of other cell types. The cell population ratio of osteoclast to osteoblast can be determined with available clinical histomorphometric data of healthy and diseased bone biopsies [8, 9]. Understanding the influence of marrow fat adipocytes on bone cell maturation and activity can allow mimicry of specific aspects of the disease *in vitro* [2, 14]. Instead of understanding only individual effects of fatty acids and adipogenic hormones at various dosages on bone cell functionality, the inclusion of adipocytes is more relevant to understanding the synergistic effect of adipogenic regulation of bone metabolism [14-17]. Clinical bone density/mass to fat assessment as well as *in vitro* studies have shown fatty acids such as linoleic and stearic can regulate osteoblast mineralization, while adipogenic hormonal secretions such as dexamethasone and leptin can regulate osteoclastic resorption (Figure 3.1). Quantification of secreted fatty acids from adipocytes and within the bone environment has limited precision and reveals no significant differences between healthy and diseased bone [16, 18, 19].

The purpose of this study was to determine adipocyte influence on co-cultured differentiation of precursor osteoclasts and osteoblasts by monitoring gene expression and protein production of differentiation and activation markers. The culture system included commercially available bone cell-seeded chronOS bone granules in indirect contact with adipogenic cells. The healthy bone precursor osteoclast and osteoblast ratio was determined in a in Chapter 2 to be 1:10, respectively; this ratio also correlates to reported physiological histomorphometric evidence [8, 9]. The goal of this *in vitro* study was to determine if the indirect tri-culture system, combining adipocytes with

differentiating osteoclasts and osteoblasts, demonstrated interactions representative of diseased conditions without added chemical mediators. That is, the underlying theory was that cellular mediators secreted from adipocytes, rather than just fatty acid supplements, would have higher physiological relevance.

3.2 Methods and Materials

3.2.1 Cell culture

The culture system was designed to simulate direct interaction of precursor osteoclasts and precursor osteoblasts and indirect interaction of the bone cells with adipocytes, as shown in Figure 3.2. There are several preparation phases to obtain adipocytes to culture with precursor bone cells summarized in the timeline (Figure 3.3).

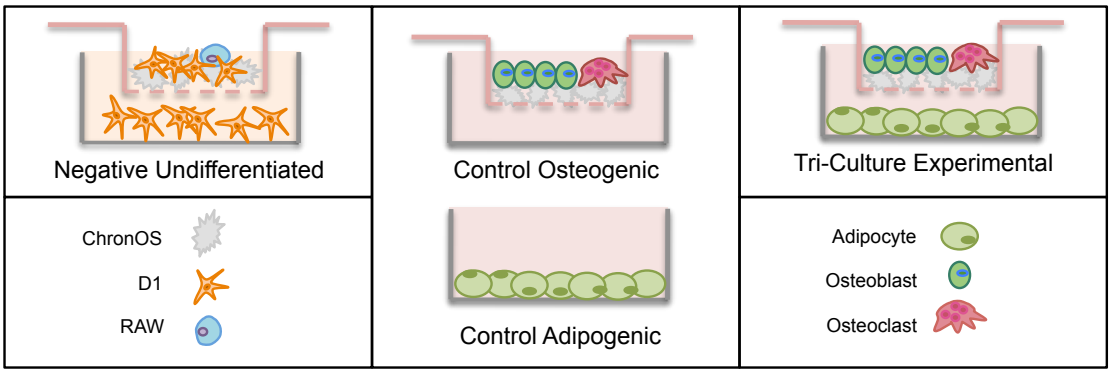

Figure 3.2. Tri-culture setup with negative control, positive control, and experimental group.

To obtain adipogenic cells, D1 cells (200,000 cells per well) (ATCC) were differentiated in 12-well plates (Corning) for 14 days in adipogenic medium (10ml growth medium ((DMEM) (Atlanta Biologics) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (ATCC), 0.2% fungizone (Invitrogen)) + 250µl human recombinant insulin (4mg/ml)(Gibco), 2µl dexamethasone (1mg/ml

ethanol)(Sigma), 1ml 5mM 3-isobutyl-1-methylxanthine(Sigma)) prior to tri-culture with bone cells.

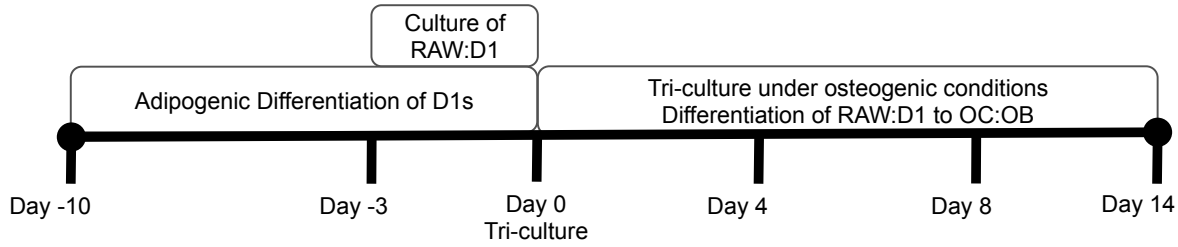

Figure 3.3. Timeline for tri-culture, with differentiation phase for adipogenic cells and initiation of tri-culture

The chronOS granules (~300mg, Synthes) were heat sterilized in disposable scintillation vials (Wheaton) at 200°C under 10psi in a vacuum oven (VWR Symphony) for at least 2 hours. The sterile chronOS granules were transferred to Netwells™ (12-well, 75μm mesh, Corning) with 2ml of medium (α-MEM, 10% fetal bovine serum, 1% penicillin/streptomyocin, 0.2% fungizone) for 2 days. Four days prior to tri-culture, RAW 264.7 monocytes (ATCC) ($1.5x10^5$ cells) and stromal D1 cells ($1.5x10^6$ cells) were seeded at a 1:10 ratio, respectively, to allow cell attachment and proliferation. Differentiation of RAW and D1 cells into osteoclasts and osteoblasts was stimulated with osteogenic medium (Dulbecco's Modified Eagle's Medium (DMEM) (Atlanta Biologics) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (ATCC), 0.2% fungizone (Invitrogen), 0.1μM dexamethasone (Sigma), 10mM β-glycerophosphate (Sigma), 5μg/ml ascorbic acid (Sigma)). Controls were differentiated in mono-culture for adipocytes and co-culture for RAW:D1 cells.

To initiate tri-culture, Netwells were transferred to appropriate well compartments for the tri-culture experimental, control osteogenic, control adipogenic, and negative undifferentiated control for 2 weeks of tri-culture (Figure 3.2). Samples (n=3) were collected at Days 4, 8 and 14 for protein and ribonucleic acid (RNA) extraction to analyze gene expression of differentiation markers with real-time polymerase chain reaction (PCR) and protein production via Western Blot. Samples were also collected for Western Blotting to determine protein production of Runx2, osteopontin, and RANK.

Table 3.1. RT-PCR Primers

Primers	Sequence		Reference
Internal Standard			
GAPDH	F	5'-GAACGGATTTGGCCGTATTG-3'	
	R	5'-CGTTGAATTTGCCGTGAGTG-3'	[20]
Osteoblast Early Differentiation Marker			
Runx2	F	5'-AGTGGACCCTTCCAGACCAG-3'	
	R	5'-TAATAGCGTGCTGCCATTCG-3'	
ALP	F	5'-GTAACGGGCCTGGCTACAAG-3'	
	R	5'-AAAGACCGCCACGTCTTCTC-3'	
Osteoblast Late Differentiation Marker			
Osteocalcin	F	5'-TGCGCTCTGTCTCTCTGACC-3'	
	R	5'-ATGGAAGGCTAAGGGCTCTG-3'	
Osteopontin	F	5'-AAAGAGAGCCAGGAGAGTGCC-3'	
	R	5'-TGTGGCTGTGAAACTTGTGGC-3'	
Osteoclast Multinucleation			
RANK	F	5'-GGACGTTCACACTGGTCAGC-3'	
	R	5'-TGCTTCCCTGCTGGATTAGG-3'	
NFATc1	F	5'-CTCGAAAGACAGCACTGGAGCAT-3'	
	R	5'-CGGCTGCCTTCCGTCTCATAG-3'	[21]
Osteoclast Resorption			
Cathepsin K	F	5'-CTGCCTTCCAATACGTGCAG-3'	
	R	5'-CCTTTGCCGTGGCGTTATAC-3'	
Adipocyte Marker			
PPARγ2	F	5'- CTCCGTGATGGAAGACCACTC -3'	
	R	5'- AGCAACCATTGGGTCAGCTC-3'	
AP2	F	5'- AGCCCAACATGATCATCAGCG -3'	
	R	5'-TCGAATTCCACGCCCAGTTTG-3'	

3.2.2 RNA isolation

At Days 5 and 14 of the tri-culture, samples were collected for RNA isolation using Trizol Reagent (Invitrogen) per manufacturer's instructions under the 600 PCR Workstation (AirClean Systems). Briefly, culture medium was aspirated from well plates before 1ml of TRIzol reagent was added. The well plates with TRIzol were placed on a plate rocker (VWR Minishaker) for 5 minutes at 200rpm to obtain cell lysates from within the crevices of the chronOS granules. Lysates were transferred to a 1.5ml RNase-free centrifuge tube (Fisher Scientific) before 0.2ml of chloroform (Honeywell HPLC grade) was added to

dissolve RNA into the aqueous phase. The aqueous layer was transferred to a new tube and 0.5ml of isopropyl alcohol (VWR) was added to precipitate the RNA. The RNA was sequentially washed with 75% ethanol (Sigma). The ethanol was removed, the RNA was allowed to air dry to remove most of the excess ethanol, then the RNA was dissolved in 30μl of nuclease-free water (Promega). Removal of DNA contaminants was performed on the RNA samples using the TURBO DNase-Free kit (Ambion). Quantification and qualification of the RNA samples were conducted using the NanoDrop 2000 spectrophotometer (Thermo Scientific), then samples were stored at -80°C until reverse transcription.

3.2.3 Reverse transcription and real time PCR

Reverse transcription with heat denaturation was performed as instructed in the RETROscript kit (Ambion) with 436μg of RNA samples (amount was limited by RNA collected). QuantiTect SYBR Green kit (Qiagen) was used to perform real-time PCR

with primers (Runx2, alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), RANK, cathepsin k (Cap K), and NFATc1) obtained from Integrated DNA Technologies. The StepOne Plus (Applied Biosystems) was used to maintain a holding temperature of 95°C for 15 minutes, then to provide 35 denaturation cycles at 94°C for 15 seconds, annealing at 54°C 20 seconds, and extension at 72°C for 20 seconds. Melting was conducted at a 70-99°C ramp to check for primer-dimers. The cycle number (Ct) was obtained at a threshold of 0.1 to calculate relative expression ratios (RER) of target genes compared to the internal standard GAPDH, using the ΔΔCt method.

The RER were analyzed via JMP 10 (SAS) to determine significant mean differences and interactions ($p \leq 0.05$) for osteoclast and osteoblast differentiation markers at various maturation stages.

3.2.4 Western blotting

To quantify protein production of Runx2 and osteopontin, Western blotting was performed on isolated protein samples. Protein was isolated using 150µl of Mammalian Protein Extraction Reagent (MPER; Pierce, Thermo Scientific) per sample using an ultrasonicator (Sonic Dismembrator, Fisher Scientific) for two 3s cycles. The lysate was collected and centrifuged for 5 minutes at 14,000rpm, then stored at -4°C. Bicinchoninic acid (BCA) protein assay (Pierce, Thermo Scientific) was performed to quantify total protein concentration per sample for gel electrophoresis. Total protein (20µg) was diluted with 6x Laemmli sample buffer and distilled water for a total volume of 45µl, and loaded onto 12-well 10% Tris-HCl Criterion gels (Bio-Rad). Proteins on the gel were blotted onto a 0.45µm nitrocellulose membrane for protein detection. Primary antibodies for

osteopontin (66kDa) and Runx2 (55kDa) (Rabbit-anti-mouse, Santa Cruz), followed by secondary goat-anti-rabbit conjugated with horseradish peroxidase, (HRP) were used to detect protein at specific molecular weights. Beta-actin (42kDa) conjugated with HRP (Cell Signaling) was used as the internal standard. Chemiluminescence imaging was performed using a FluorChem™ M (Protein Simple).

3.3 Results

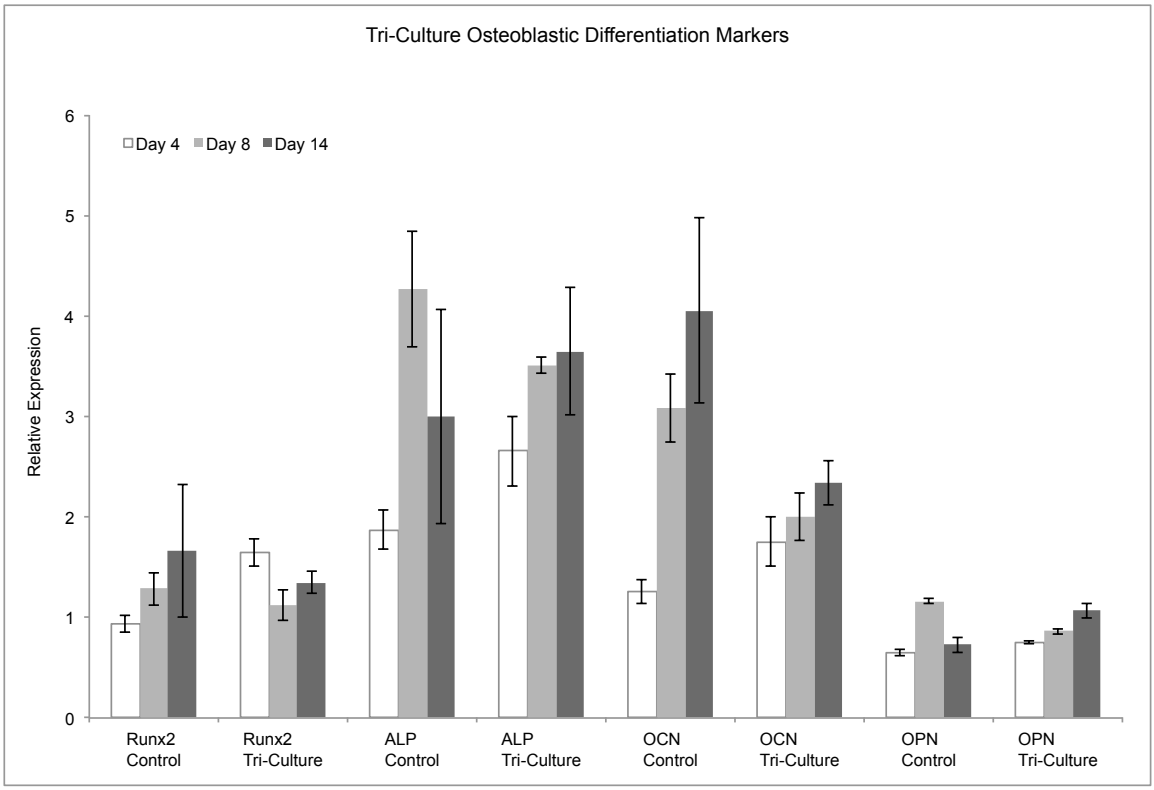

Figure 3.4. Osteoblastic gene expression for early and late differentiation markers. No differences in expression were detected between control and tri-culture group for Runx2 and ALP. Osteocalcin expression was higher for control group than for tri-culture group (p=0.428). Osteopontin expression for control and tri-culture groups was significantly different (p=0.0001)

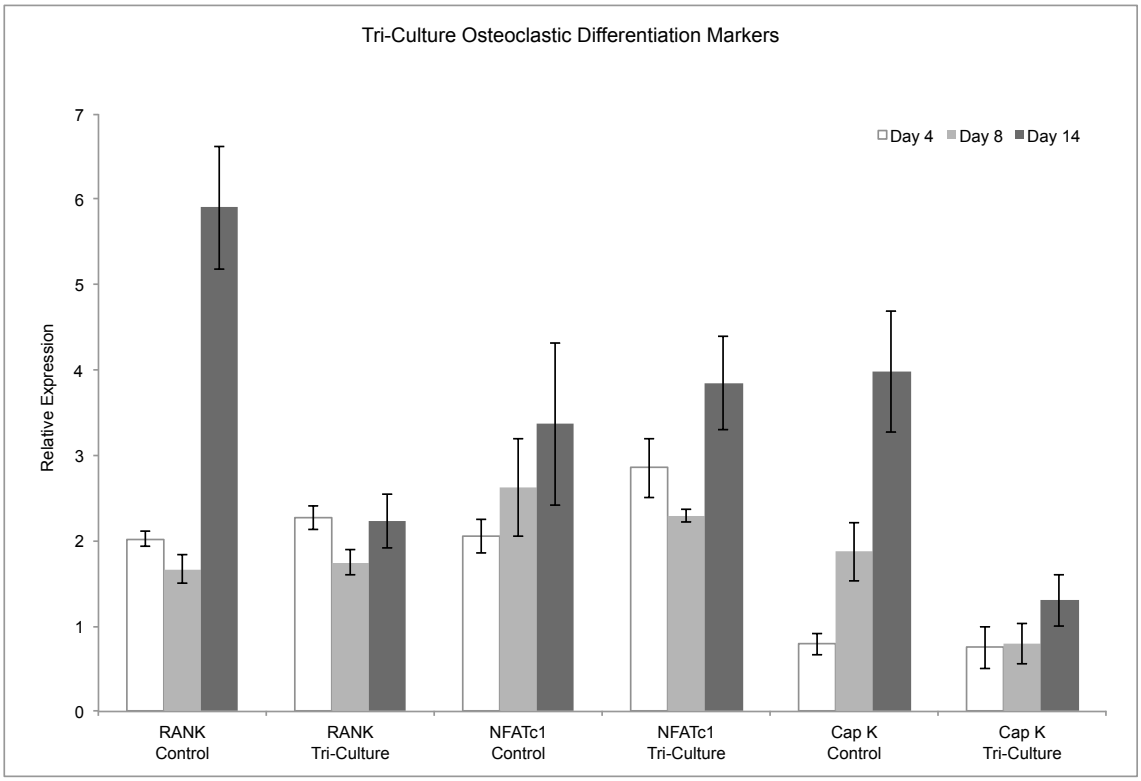

Figure 3.5. Osteoclastic gene expression of differentiation markers. A correlation was detected between expression levels and study groups for RANK (p=0.0001) and cathepsin K (p=0.044). Results show no statistically significant relationship between downstream transcription factor NFATc1 and study groups.

Gene expression was assessed to monitor differentiation of precursor osteoclastic RAW cells and precursor osteoblastic D1 stromal cells in indirect contact with adipocytes. No difference was detected in early-stage osteoblast differentiation genes when comparing osteogenic control and tri-culture through 14 days (Figure 3.4). No change was detected in Runx2 levels for either group over 14 days, while ALP expression peaked at Day 8 for the control group. Significant differences (p=0.0428, p=0.0001, respectively) were detected in osteocalcin and osteopontin, later markers of osteoblast mineralization, between osteogenic control and tri-culture groups. The control

group expressed higher levels of osteocalcin after Day 8. Osteopontin levels in the tri-culture system increased throughout the 14-day study, while control levels peaked at Day 8.

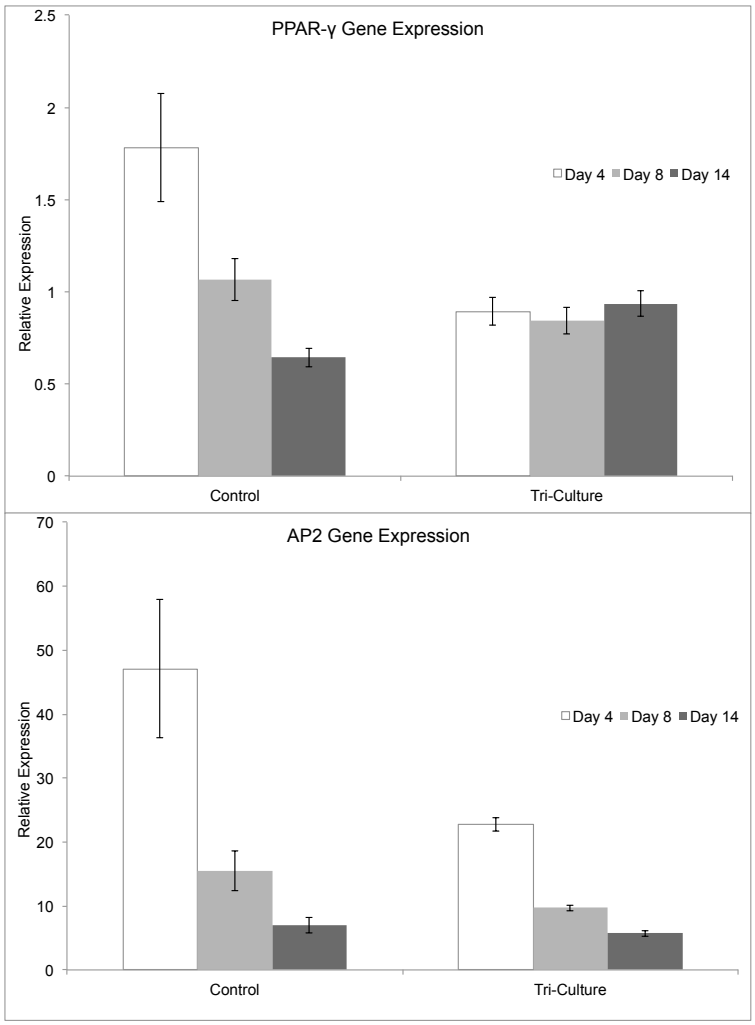

Figure 3.6. Adipogenic gene expression of differentiation markers. Control cultures expressed higher levels of PPAR-γ ($p=0.004$) and AP2 ($p=0.0001$) as compared to tri-cultures. Tri-cultures exhibited a sustained expression of PPAR-γ while control expression decreased. Both conditions showed decreased AP2 expression throughout 14 days.

Lower levels of RANK and cathepsin K, osteoclastic markers, were detected in tri-culture at Day 8 ($p=0.0001$; $p=0.004$) (Figure 3.5). However, the levels of downstream transcription factor NFATc1 in the control and tri-culture groups were not significantly different.

Adipogenic characteristics were monitored through the production of PPAR-γ and AP2 (Figure 3.6). PPAR-γ expression in the control group decreased after Day 4, while the level in the tri-culture group plateaued throughout 14 days of the study (p=0.004). AP2 expression behavior for both groups similarly decreased throughout the study; however, levels were higher for the control group at Day 4 (p=0.0001) (Figure 3.6).

Protein production of Runx2 and osteopontin, measured by Western Blot, indicated differences among all groups during the 14-day experiment. Runx2 for the tri-culture condition was higher than the positive control at Day 4, while minimal production was evident for the negative control (2D growth). Protein concentration of Runx2

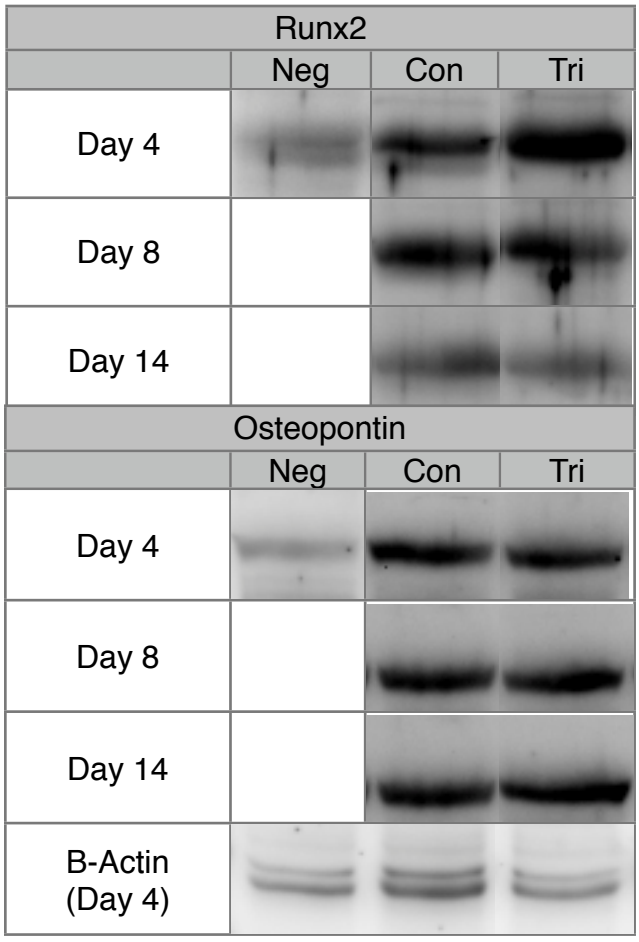

Figure 3.7. Western Blot for protein production of Runx2 and osteopontin during14 day tri-culture (Tri) with positive (Con) and negative (Neg) controls. Production of Runx2 and osteopontin are higher in tri-culture as compared to in Neg. Tri-culture levels of Runx2 are greater but the levels of osteopontin are similar to those of the positive control.

decreased with time in the osteogenic control and tri-culture conditions, similar to what one would expect *in vivo*. Osteopontin production, on the other hand, was produced in sustained fashion for all 14 days of tri-culture, similar to production patterns in the positive control. Osteopontin was produced minimally in the negative 2D control as compared to production in osteogenic conditions.

3.4 Discussion

One hypothesized cause of osteoporosis is the accumulation of marrow fat in aging, resulting in increased bone resorption and eventually low bone mass [19]. Hence, women who inherently have higher body fat have higher accumulation of marrow fat post-menopause and are predisposed to osteoporosis. However, a deeper understanding of the mechanisms of osteoporosis is necessary to improve preventative measures and pathological understanding of the condition. Even though clinical evidence from several studies reveals no significant differences in fatty acids between patients with normal and osteoporotic bone, researchers are curious about the orchestration between adipogenic and osteogenic cells [2, 15, 22]. *In vitro* studies have been designed to understand lipotoxicity and adipogenic regulation of bone cell metabolism; however, co-cultures with either osteoblastic or osteoclastic cells on a two-dimensional (2D) environment exemplify limited understanding of a disease or condition [16, 17, 23, 24]. The inverse relationship of osteogenesis and adipogenesis can be monitored by the expression of early and late markers like PPAR-γ and Ap2, respectively. Elbaz and coworkers studied primary human osteoblasts and osteoclasts in 2D for 21 days and observed lipotoxicity of stearate and palmitate, resulting in a decrease in osteoblast differentiation and activation

markers (Runx2, ALP, OCN) [16]. Similar results were observed by Liu and coworkers in their investigation of PPAR-γ and adiponectin regulation of osteoblastic differentiation [24]. As for adipogenic regulation of osteoclastogenesis, the Kunh and colleagues and Hozumi and colleagues confirmed both positive and negative effects on differentiation and activity with direct and indirect cultures [17, 24].

Studies in the literature have suggested the inverse relationship between adipogenesis and osteogenesis as a hypothesis for osteoporosis pathogenesis. However, as demonstrated clinically, cell population ratios also influence cellular activity [8, 9]. Indeed, the indirect tri-culture with adipogenic cells demonstrated influence of fat on osteoblast activation and osteoclastogenesis. Statistical analysis revealed no differences in osteoblast expression of transcription factor Runx2 or ALP, indicators differentiation. However, Runx2 production measured via Western Blot was higher in tri-culture conditions; it is known that mineralization of osteoblasts can be hindered due to excessive Runx2 signaling [25]. Osteocalcin and osteopontin expressions were higher in the control groups, suggesting suppressed osteoblastic activity in the tri-cultures. On the contrary, no difference was seen between osteopontin production in the control group and that in the tri-culture group. Osteoclast maturation and activation, confirmed via RANK and Cathepsin K levels, were also retarded by adipogenic presence during the 14-day culture period. Interestingly, the decrease in PPAR-γ evident in the control conditions is phenotypic once stromal cells commit to adipogenic lineage, while sustained levels PPAR-γ exhibited in the tri-culture condition suggested indirect regulation of adipogenic maturation by soluble factors from co-cultured osteoclastic and osteoblastic cells.

Expression difference of AP2 further supports the theory that communication within the tri-culture condition is bi-directional.

3.5 Conclusion

Soluble cytokines and factors produced by adipocytes, osteoblasts, and osteoclasts orchestrate the differentiation and activation within a tri-culture condition. Adipocytes suppress both late osteoblast maturation and mineralization and down-regulate osteoclast differentiation and resorption activity. Adipogenic activity is altered in tri-culture conditions as compared to mono-culture conditions. Recognizing the complex communication pathways between adipocytes, osteoblasts, and osteoclasts, an *in vitro* test system with clinical relevance can be established to model osteoporosis and characterize bone substitute materials for patients with bone diseases. By further altering the adipocyte:osteoblast:osteoclast cell ratio, a deeper understanding of pathological-like activity can be realized and used to evaluate the effectiveness of bone substitute materials in targeting fragility fractures.

Acknowledgements

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References

[1] Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. *Lancet*. 2011 Apr 9; 377 (9773): 1276-1287.

[2] Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? *Nature Clinical Practice. Rheumatology*. 2006 Jan; 2 (1): 35-43.

[3] Stromsoe K. Fracture fixation problems in osteoporosis. *Injury*. 2004 Feb; 35 (2): 107-113.

[4] Del Fattore A, Cappariello A, Teti A. Genetics, pathogenesis and complications of osteopetrosis. *Bone*. 2008 Jan; 42 (1): 19-29.

[5] Wekre LL, Eriksen EF, Falch JA. Bone mass, bone markers and prevalence of fractures in adults with osteogenesis imperfecta. *Archives of Osteoporosis*. 2011 Dec; 6 (1-2): 31-38.

[6] Rauch F, Travers R, Norman ME, Taylor A, Parfitt AM, Glorieux FH. Deficient bone formation in idiopathic juvenile osteoporosis: a histomorphometric study of cancellous iliac bone. *Journal of Bone and Mineral Research*. 2000 May; 15 (5): 957-963.

[7] Rauch F, Travers R, Parfitt AM, Glorieux FH. Static and dynamic bone histomorphometry in children with osteogenesis imperfecta. *Bone*. 2000 Jun; 26 (6): 581-589.

[8] Pestka JM, Seitz S, Zustin J, Puschel K, Amling M, Barvencik F. Paget disease of the spine: an evaluation of 101 patients with a histomorphometric analysis of 29 cases. *Eur Spine J*. 2012 May; 21 (5): 999-1006.

[9] Vukmirovic-Popovic S, Colterjohn N, Lhotak S, Duivenvoorden WC, Orr FW, Singh G. Morphological, histomorphometric, and microstructural alterations in human bone metastasis from breast carcinoma. *Bone*. 2002 Oct; 31 (4): 529-535.

[10] ONN (2008) Orthopedic Network News,

[11] Dimitriou R, Mataliotakis GI, Angoules AG, Kanakaris NK, Giannoudis PV. Complications following autologous bone graft harvesting from the iliac crest and using the RIA: a systematic review. *Injury*. 2011 Sep; 42 Suppl 2 S3-15.

[12] Hollinger JO, Onikepe AO, MacKrell J, Einhorn T, Bradica G, Lynch S, Hart CE. Accelerated fracture healing in the geriatric, osteoporotic rat with recombinant human platelet-derived growth factor-BB and an injectable beta-tricalcium phosphate/collagen matrix. *Journal of Orthopaedic Research*. 2008 Jan; 26 (1): 83-90.

[13] Li M, Liu X, Ge B. Calcium phosphate cement with BMP-2-loaded gelatin microspheres enhances bone healing in osteoporosis: a pilot study. *Clinical Orthopaedics and Related Research*. 2010 Jul; 468 (7): 1978-1985.

[14] Kruger MC, Coetzee M, Haag M, Weiler H. Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone. *Progress in Lipid Research*. 2010 Oct; 49 (4): 438-449.

[15] Reid IR. Relationships between fat and bone. *Osteoporosis International*. 2008 May; 19 (5): 595-606.

[16] Elbaz A, Wu X, Rivas D, Gimble JM, Duque G. Inhibition of fatty acid biosynthesis prevents adipocyte lipotoxicity on human osteoblasts in vitro. *Journal of Cellular and Molecular Medicine*. 2010 Apr; 14 (4): 982-991.

[17] Hozumi A, Osaki M, Goto H, Sakamoto K, Inokuchi S, Shindo H. Bone marrow adipocytes support dexamethasone-induced osteoclast differentiation. *Biochemical and Biophysical Research Communications*. 2009 May 15; 382 (4): 780-784.

[18] Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. *Progress in Lipid Research*. 2001 Jan-Mar; 40 (1-2): 125-148.

[19] Griffith JF, Yeung DK, Ahuja AT, Choy CW, Mei WY, Lam SS, Lam TP, Chen ZY, Leung PC. A study of bone marrow and subcutaneous fatty acid composition in subjects of varying bone mineral density. *Bone*. 2009 Jun; 44 (6): 1092-1096.

[20] Maxson S, Burg KJ. Conditioned media enhance osteogenic differentiation on poly(L-lactide-co-epsilon-caprolactone)/hydroxyapatite scaffolds and chondrogenic differentiation in alginate. *Journal of Biomaterials Science, Polymer Edition*. 2010 21 (11): 1441-1458.

[21] Egusa H, Doi M, Saeki M, Fukuyasu S, Akashi Y, Yokota Y, Yatani H, Kamisaki Y. The small molecule harmine regulates NFATc1 and Id2 expression in osteoclast progenitor cells. *Bone*. 2011 Aug; 49 (2): 264-274.

[22] Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J Cell Science*. 1992 Jun; 102 (Pt 2) 341-351.

[23] Kuhn MC, Willenberg HS, Schott M, Papewalis C, Stumpf U, Flohe S, Scherbaum WA, Schinner S. Adipocyte-secreted factors increase osteoblast proliferation and the OPG/RANKL ratio to influence osteoclast formation. *Molecular and Cellular Endocrinology*. 2012 Feb 26; 349 (2): 180-188.

[24] Liu LF, Shen WJ, Zhang ZH, Wang LJ, Kraemer FB. Adipocytes decrease Runx2 expression in osteoblastic cells: roles of PPARgamma and adiponectin. *Journal of Cellular Physiology*. 2010 Nov; 225 (3): 837-845.

[25] Komori T. Regulation of osteoblast differentiation by Runx2. *Advances in Experimental Medicine and Biology*. 2010 658 43-49.

CHAPTER FOUR

A LOOK AT BIOMEDICAL ENGINEERING PRINCIPLES AND MEDICAL DEVICES THROUGH BONE DISEASES AND BONE IMPLANTS

4.1 Introduction

Within biomedical engineering (BME), there are multiple concentrations/foci requiring a variety of engineering and science expertise and collaboration. Medical device technologies developed by biomedical engineers range from imaging machines (x-ray, magnetic resonance) to diabetic monitors to pacemakers to bandages. The focus of this teaching module is on bone tissue engineering, in which engineers/scientists strategize to resolve complications in bone healing, bone fractures, and bone diseases with medicinal agents, medical devices, and engineered tissue or substitute materials. Hence, bone tissue engineering is an interdisciplinary field of biology, anatomy and physiology, chemistry, mechanical engineering, physics, and material science. The introduction of BME through implementation of this bone fracture education module will demonstrate to the students the core ideas of engineering design and practices according to the *Next Generation Science Standards* (NGSS). The education module distills BME concepts, with focus on bone tissue engineering, into a 1-hour demonstration with a hands-on activity of simulated bone and bone implants that is easily translated to a classroom setting for all grade levels. The presentation introducing BME, bone biology, and the activity can be downloaded from DropBox (www.dropbox.com/s/uwk7qfpzzed5khb/Biomedical%20Engineering%20Workshop.ppt

x) and can be tailored for a specific grade level according to NGSS performance expectations.

Bone implant design is a relatable example as children are at risk for bone fractures, leading to casting for simple fractures and bone implants and surgery for complex fractures. Depending on the location of the complex fracture, bone implants can be a system of screws and plates (long bones), intramedullary nails (long bones), rods and screws (spine), and joint replacements (knees and hips). Bone implants are devices used to mend two pieces of bone, and are similar to hardware supplies used to build a house. Plates and nails/screws are used to join to planks of wood in fixed fashion, while hinges and screws are used in joints on doors to allow movement. The material, size, and shape of the plates, screws, and hinges depend on such factors as the type of wood, the load the house frame needs to support, the forces that the house frame will encounter. Similarly, factors such as age, health, and gender will influence implant selection for a patient. Many implants are designed to be removed following bone healing. For example, if a child receives an implant, such as screws and plates, to mend a complex fracture in the humerus, the screws and plates will likely be removed once the fracture is healed to allow further bone growth and avoid bone deformity.

The module will allow students to experience the process of implant development – designing, testing, redesigning – and determine necessary features for a successful bone implant system to accommodate the clinical need, patient specifications, and surgical feasibility. The long bone fracture is simulated using cardboard tubes as cortical bone for shape similarity and accessibility while the styrofoam and foam insulation represents

cancellous/spongy bone (Figure 4.1). As the students learn about BME, they should also understand that medical device design means balancing requirements in various criteria:

- Biology Criteria
  - Supports the body and/or body functions
  - Causes minimal to no immune response
  - Causes no further damage
- Patient Criteria
  - Accommodates all sizes and shapes
  - Allows quick recovery
  - Promotes good healing
  - Minimizes costs after insurance
- Surgeon Criteria
  - Facilitates minimal surgical trauma
  - Accommodates short implantation time
  - Comprises minimal parts or pieces
  - Has low chance of failure

In house construction, the location (geographic region, immediate surroundings), the budget, and the potential homebuyer preferences determine the type of material, the architecture, and the foundation. That is, the home should withstand weather conditions, be aesthetically pleasing, and be accommodating.

Even though an engineer can have the best design to address the biology criteria, if the implant does not meet the surgeon's criteria, then it is a failure. The process of commercializing an implant takes years due to testing, retesting, and approval procedures. An engineer designing a biomedical product, such as a bone implant, must wear a variety of thinking caps in order to meet the demands of the customers (the patients and surgeons). Therefore, during the hands-on activity or the engineering challenge, inquiry questions (Table 4.1) stimulate students to seek different answers depending on the cap they are wearing. Follow-up questions are asked that lead the

students to think about compromises and compare the importance of one criterion against the other.

4.2 Understanding Bone Function and Physiology

The 206 bones in the adult skeleton provide protection for the internal organs, support to keep the body upright, and movement at joints. Without the skeleton, the body would be floppy skin, organs, and muscles, found in a pile on the ground, unable to withstand gravity. The architecture of bone is a complex network of trabeculae, even more advanced and geometrically efficient than the scaffolding of the tallest skyscraper. The synergistic combination of inorganic (hydroxyapatite) and organic (collagen) components gives bone high compressive and tensile strength, respectively. Cortical, or compact, bone has higher compressive strength than cancellous, or spongy, bone; hence, cortical bone is arranged on the exterior of the bone while cancellous bone is on the interior. The spongy interior of long bone serves as a storage and source for bone marrow rich in essential red and white blood cells.

Bone has the unique ability to heal minor bone defects without leaving a scar; that is, bone continuously remodels and repairs small unnoticeable damages a person might sustain. Unfortunately, bone diseases and complex fractures do occur, leading to compromised or no healing. One of the most common bone diseases worldwide is osteoporosis, or low bone mass, which decreases the mechanical strength of bone. Since many patients with bone diseases develop brittle bone, they have the highest risk for fractures. Biomedical engineers have been designing and redesigning bone implants to resolve this problem.

When a person breaks a bone, the doctor evaluates the fracture to categorize it to ensure the best treatment for good bone healing. The preferred treatment would be a simple external cast made of plaster, but for patients with complex fractures, internal fixation with implants is necessary to realign the bone. For patients with bone diseases, such as osteoporosis, the brittle bone can be damaged with the implantation of a screw or other implants, leading to a fracture. Some engineers have recognized this design problem and are focused on balancing the biological and mechanical criteria for bone implants. Commercially available plate and screw options include titanium, stainless steel, and cobalt alloys; the selected system must allow mechanical strength to support body weight while the fracture heals. Clinically, a plate is implanted to hold the fractured bone in place; screws are placed some distance away from the fracture gap, at varying angles, to stabilize the plate.

During design and development of bone implants, engineers brainstorm to consider implant material, chemistry, implant shape, implant mechanical and biological properties (compressive strength, manufacturability, biocompatibility), clinical application, ease of implantation, and fracture site. Once a product design is conceptualized, a prototype is produced for testing to optimize implant properties and eliminate possible failures. If the design survives testing and the clinical approval process, the time from concept design to commercialization is years. This hands-on activity allows students to implant plates and screws using a simulated fracture of normal and osteoporotic bone and demonstrates the engineering design process, from prototype to testing to redesigning, for bone with biological and mechanical differences.

4.3 Education Module Implemented at Girl Scout Event 2013

A BME education module was developed to implement at a 1-day event that focused on introducing various engineering and science fields to a group of ~50 girl scouts (6^{th} – 8^{th} grade) from the Upstate region of South Carolina. The BME module was one of three science or engineering modules, each geared toward hands-on and interactive teaching. Before the demonstrations started, the girls were split into three groups to rotate through each module during the day.

Table 4.1 Questions Focused on Engineering Practices

Interactive Presentation	
What is biomedical engineering?	
What are the functions of bone?	
What bone are the two types of bone?	
Who has had a broken bone?	
How does broken bone heal? Is it different for children and adults?	
What are the problems of bone disease?	
How was your bone stabilized to heal?	
What are the differences and similarities of this broken bone model to real broken bone?	
What are the limitations of the model?	
Are the materials used a good representation of bone?	
What part of the bone does the cardboard/styrofoam represent?	
Why is metal the material of choice for bone implants?	
Hands-on Activity	
Think like an engineer	What material would you use? Plastic or metal?
	How many plates/screws would you use?
	Where would you put the plates/screws?
	What material would you use for the osteoporosis fracture? Why?
	If the implant can fail, how do you think it will fail?
	How can you calculate the forces in the mechanical testing?
	If you were performing this surgery on a real person, how could you limit the surgery time?
Think like a surgeon	How many implants would you use?
	How many screws would you use?
	Does the implant need to be clean before it is implanted?

For each group of girls, the bone implant demonstration started with a presentation to introduce BME, bone biology, bone mechanics, bone health, and fracture treatments. The presentation was interactive; the girls were encouraged to respond to

questions listed in Table 4.1 during the appropriate times. The girls were asked if they had ever broken a bone or if they knew anyone who had; they were then asked how that fracture was treated and if there were complications with healing. The presentation then

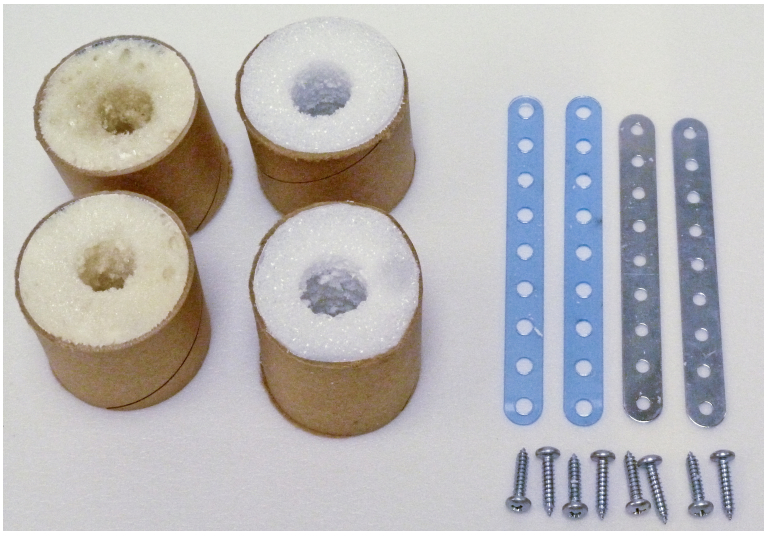


Figure 4.2. Components of the bone implant kit for two students: fake bone, screws, plastic plates, and metal plates.

transitioned into methods of treating bone fractures, with details on bone implant devices and designs. During the implant design discussion, the girls were asked what characteristics and considerations would benefit a bone implant. Once they had defined the problem and brainstormed some design criteria, a humerus fracture was introduced as the engineering challenge. The girls were given the activity kit and the tools to start their implantation, which included two models of bone fractures (normal and osteoporotic, Figure 4.1). The girls observed the differences in the color and the hardness of the two broken bone models. To stimulate their engineering thinking, the girls were asked what material they would use as an implant for the normal and osteoporotic bone (Figure 4.2).

After the girls finished their implant (Figure 4.3), they performed bend, torsion, tension, and compression tests to observe the changes and failures with their designs (Figure 4). Testing was the best part for the girls since they were allowed to attempt to break what they just designed and fixed.

For the bend test, the girls oriented their fixed bone model horizontally and bend the model holding the two bone segments. For the torsion test, the girls twisted the two

Figure 4.3. Example of fracture repair of
simulated bone fracture with ~1 inch gap for testing.

bone segments in different directions. For the tension test, the girls pulled their two bone segments apart. Lastly, for the compression test, the girls placed their bone models on end on the ground, placed a large cardboard tube (2 feet tall) around the model, and dropped dumbbell weights (3, 5, and 8 pound weights) down the tube, on top of the bone model, to test how much load their plate and screw setup could support. Groups that

finished first had time to redesign and retest their implant systems. The girls had an opportunity at the end of the session to summarize their findings to the rest of the class and suggest improvements to their design for both the normal bone and osteoporotic bone. The girls were able to take their bone implants home to remind them about biomedical engineering.

To gauge the girls' attitudes towards math, science, and engineering, before and after the events of the day, survey questions were posed targeting the girls' interest in, value of, and confidence toward the three fields. The questions followed the Likert Scale, with a 5-point rating scale ranging from 1 as "strongly disagree" to 5 as "strongly agree". Pre- and post-surveys with similar questions were given to students at the beginning and end of the day, respectively, to measure changes in the students attitudes towards math, science, and engineering. The survey data showed that the interest, perceived value, and self-confidence with respect to science and engineering statistically increased over the course of the 1-day event.

4.4 Attitude Survey Assessment

To gauge the girls' attitudes towards math, science, and engineering, before and after the events of the day, survey questions were posed targeting the girls' interest, value, and confidence of the three fields. Questions for the survey were revised from those published by Gibbons and colleagues to measure middle school students' attitude and knowledge about engineering [1]. The questions followed the Likert Scale, with a 5-point rating scale ranging from 1 as "strongly disagree" to 5 as "strongly agree". Pre- and post-surveys with similar questions were given to students at the beginning and end of the

Table 4.2 Pre- and Post- Survey Results for Girls Scout Event			
Category	Question	n	p-value
Interest	I like math	33	
	I like science	33	0.0325
	I like engineering	33	0.0005
Task Value	It is important for me to learn math	33	
	It is important for me to learn science	32	0.0181
	It is important for me to learn engineering	33	0.0041
Confidence	I am good at math	33	
	I am good at science	33	
	I am good at engineering	33	0.0044
	I can be a mathematician	32	
	I can be a scientist	33	
	I can be an engineer	33	
Expected Outcomes	Mathematicians help people	32	
	Scientists help people	33	
	Engineers help people	33	
Values indicate significant positive change (Pre-Post)			

Table 4.3 Pre- and Post- Survey Results for STEM Day Event		
Interest	I am interested in math	
	I am interested in science	
	I am interested in engineering	p=0.0005
	I want to learn more math in college	
	I want to learn more science in college	
	I want to learn more engineering in college	p=0.002
Task Value	Math will be important for my future	
	Science will be important for my future	
	Engineering will be important for my future	p=0.0005
Confidence	I am good at math	
	I am good at science	
	I am good at engineering	p=0.0027
	I have the skills to be a mathematician	p=0.0039
	I have the skills to be a scientist	p=0.0156
	I have the skills to be an engineer	p=0.0191
Expected Outcomes	Mathematicians help solve society's problems	
	Scientists help solve society's problems	
	Engineers help solve society's problems	
Values indicate significant positive change (Pre-Post)		

day, respectively, to measure changes in the students attitudes towards math, science, and engineering. Data distribution was expected to be skewed and nonparametric; hence, the

Wilcoxon Signed Rank test nonparametric data analysis for matched pair changes was employed for each question using statistical software JMP 10 (Table 4.2 and 4.3). Total sample size was n=45; however, the usable sample number was reduced to n=33 or 32 due to missing "pre" or "post" survey answers for the Girl Scouts and n=24 for the juniors. The survey data showed that the interest, perceived value, and self-confidence with respect to science and engineering increased over the course of the 1-day event. Furthermore, the positive attitude changes for juniors in high school during this science, technology, engineering, and mathematics (STEM) awareness, 1-day event indicates an immediate impact on students of varying grade levels.

4.5 Materials and Assembly

Supplies per student will cost ~$5 for 80 or more students. Preparation and assembly are required prior to activity (Table 4.4). To make the simulated normal bone,

Table 4.4 Materials List and Feasible Substitutes

Materials	Specifications	Supplier
Paper cylindrical tubes	Light-Duty Paper Tubes, 2"x20", 0.0045" thick - 23612	www.yazoomills.com
Styrofoam poles	Extruded Styrofoam poles, 2"x36"	www.thecraftplace.com
Great Stuff™ insulating foam	Insulating foam sealant- gaps, cracks	Lowes
Metal mending plates	The Hillman Group 0.5" x 4.5" x0.35"	Lowes
Plastic mending plates*	Similar to metal plates	Special made with laser cutter
Screws	The Hillman Group, 100 count 8x 3/4 zinc plated metal screw	Lowes
Equipment	**Substitutes**	**Purpose**
Band saw	Hand held saw	Cut tubes into fragment sections
Drill press	Apple corer and hammer	Hollow out center of foam
Phillips screw driver		Tighten screws
Nail	Push pin	Make hole for screws
Weights – 2,5,8 lb	Heavy books	Test the implants
*Substitutes: plastic screws, different metal plates, different thickness metal plates, or none		

insert the styrofoam pole into the cylindrical tube and cut into 2” segments, then hollow out a 1” diameter hole in the center. The osteoporotic bone segment is made by spraying insulation foam into the tube and allowing the foam to cure overnight. Once the foam has set, hollow out the center like the normal bone (Figure 4.1). Assemble the kits with the components as shown in Figure 4.2, targeting two students per kit.

The list of materials includes the basic suggestions that can be expanded upon to challenge the students or make the activity more specific to the classroom. Variation in the plates and screws would address the engineering process more as the students interact with common materials in uncommon conditions (Table 4.4).

4.6 Hands-On Activity Setup

After present the background on the activity, the students had an opportunity to look at their activity kit to discuss and assess their plan of construction. In groups of two, the students will choose and help each other to either repair the normal or osteoporotic fracture. Once the fracture has been repaired (Figure 4.3), students will make observations and predictions about their implant properties then conduct mechanical tests (Figure 4.4). The students will perform a bend, torsion, tension, and compression test with a one-inch gap between the two bone segments, similar to actual test setup and testing conducted on actual plate and screw bone implant system. The compression testing will be the most destructive test, with the heaviest weight causing implant failure. After testing, students will record what they observed through testing, noting the possible improvements to decrease implant failure.

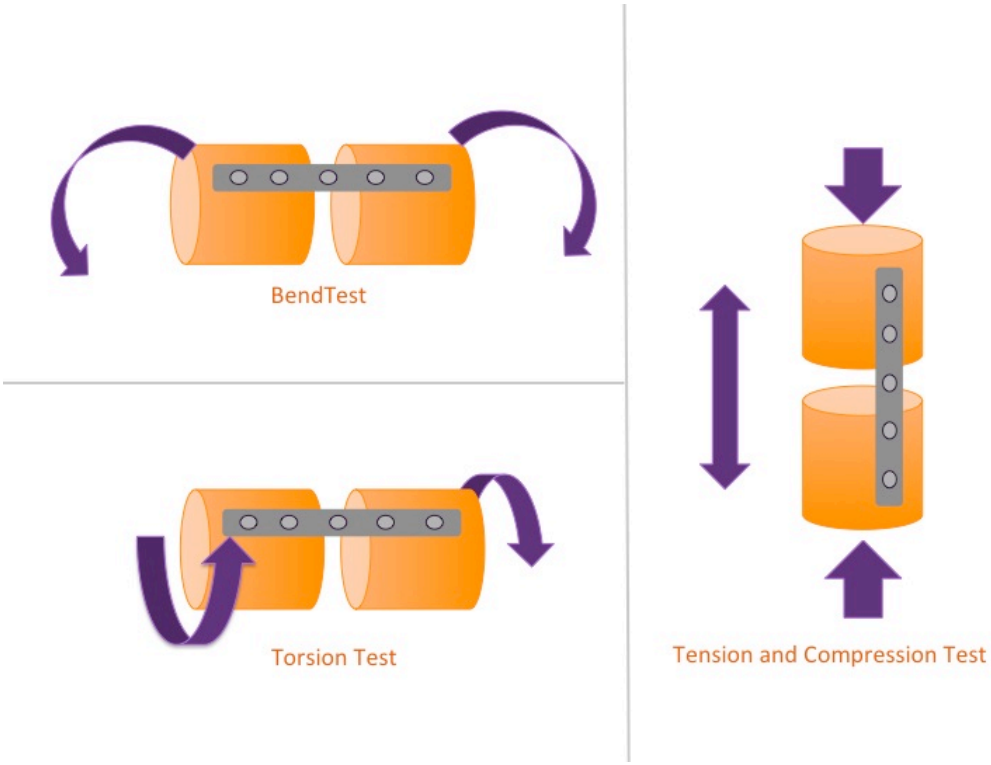


Figure 4.4. Schematic of the bend, torsion, tensile, and compression tests for the bone implant.

4.7 Teaching Assessment

The education module and activity will allow teachers to implement the eight practices of engineering and practices from the *Framework for K-12 Science Education: Practices, Crosscutting Concepts, and Core Ideas (2012)* by the National Research Council. Teachers can stimulate questions (Table 4.1) as they present the module and the activity. As the bone fracture scenario is presented to the students, they can practice their writing in defining the medical problem and devising solutions. They can do so individually then discuss with their partner for different ideas and share their interpretation of the bone model. Even though the plates and screws are provided, both teachers and students can still be creative in the materials and implementation as

summarized in Table 4.5. For example, if students are given two plates, some will decide to use one and other decide to use two. However, with two plates students can stack them together to increase the thickness and possible the mechanical strength or place the plates with distance apart. Typically students will want to drill the screws straight into the bone model but they can also try to angle the screws for stability at various angles. Students are also challenged to think differently for the osteoporotic bone versus the normal bone in consideration for the change in bone mechanical properties.

Table 4.5 Variations in Fracture Fixation

Plates	**Screws**	**Materials**
Angle	*Angle*	*Metal*
Displacement (if more than one)	*Displacement*	*Plastic*
Length	*Length*	*Wood*
Quantity	*Quantity*	
Thickness	*Threading*	
Width	*Diameter*	

The questions, listed on Table 4.1, can be modified to become assessment or evaluation questions that can focus on one particular subject area within the multidisciplinary field of BME. For example, in a biology class, teachers can expand upon the components of bone, the growth of bone, the healing reaction of bone, the functions of bone, compatibility of materials to the body. For a physics class, the teachers can focus on the mechanics (forces, stresses, momentum, etc.) of bone from the micro-scale of trabeculae in long bone to macro-scale of the bone and joints in abduction and

adduction. In a chemistry class, students can learn about material science of metals, bone matrix composition, and the sterilization procedure of implants.

Before students begin the activity they can assess the materials they have and plan out their initial design. Students can then write down their predictions, observations, and results from the mechanical testing to evaluate for the redesigning of the plates and screws to increase the mechanical stability of the implant system for both the normal and osteoporotic bone model. Observations of implant failure during the mechanical can be quantitative for math-based classes or qualitative for biological classes. Calculations of load on bone for various body types and locations in the body and forces from the compression testing to understand the restrictions of the design and improve on the redesign. Students can then evaluate the data and observations to explain why the implant was failing to reassess their model and alter their design with different ideas. At the end of the module, students can present their findings, complications, and successes to the rest of the class. At the end, the students should relate to the boarder impact of engineering design and problem solving to help society such as biomedical engineering in solving health problems through advanced medical devices.

4.8 Modifications to Meet Education Standards Specific to Grade Groups

This teaching module can more details on bone implants, bone physiology, and bone diseases in accordance to the Disciplinary Core Ideas of Progression to increase the sophistication of student critical thinking (Table 4.6). Teachers can incorporate all or some of the suggestions in the presentation provided to relate to their class syllabus.

Table 4.6 Tailoring the Presentation and Activity to Incorporate Core Ideas				
	K – 2th Grade	**3th – 5th Grade**	**6th – 8th Grade**	**9th – 12th Grade**
Physical Science	Physical differences in plastic, metal, and bone	Mechanical differences in plastic, metal, and bone	Atomic structure of plastic and metal plates and screws	Mechanical properties due to atomic structure of different plastics and metals
	Different forces bones can handle	Types of forces that cause different bone fractures	Stability of the implant system with different plate and screw lengths and displacement	Defect propagation of plastics, metals, and bone to cause failure
			Transfer of body weight onto the implant	Chemical properties of metal and plastic
				Erosion of metals in the body due to micro-motion
				Forces (stress and strain) transmitted to plates and screws
				Potential and kinetic energy supported by the implant
				Motion, energy, and forces in movement of the skeleton or extremities
Life Science	Functions of arms and legs	Function of the skeletal system and interconnect systems (muscles, tendon)	Structure and composition of bone (organic, inorganic minerals, cells)	Function of bone cells in maintaining bone health and healing bone fractures
	Importance of bone, muscle, tendon	Growth of body with a focus on bone	Functions of bone	Causes of bone diseases due to malfunctioning feedback systems
Science and Technology	Introduction of medical devices: bone implants	Difference between bone and bone implant	Function of bone implant in healing	Examples of various bone implants differing in design
	Treatment methods for bone fractures	Biological design criteria of bone implant	Clinical criteria for bone implants	Examples of biomedical technologies used to better quality of life for the users
	Importance of implant to help bone heal		Importance of biomedical engineering technology in healthcare	
Science in Personal and Social Perspectives		Importance of bone health	Development of healthy bone	Bone diseases in older population and female population
		Aging of bone	Aging of bone	Treatments for bone diseases
		Diseases of bone	Diseases of bone	
			Treatments for bone diseases	

4.9 Acknowledgements

Funding was provided by the Clemson University Programs for Educational Enrichment and Retention, the Call Me Doctor™ fellows program, and the Clemson University Hunter Endowment. A special thanks to the Women in Science and Engineering mentors for assisting with the education module and being encouraging mentors during the hands-on activity.

References

[1] Gibbons S, Hirsch L, Kimmel H, Rockland R, Bloom J (2004) International Conference on Engineering Education, Gainesvulle, FL

CHAPTER FIVE

CONCLUSION

The advancement of tissue engineering and regenerative medicine will rely on the development of bioactive tissue substitutes with specificity for diseases and effectiveness in pathological conditions. In bone tissue engineering, strategies are focused on localized delivery of bone protective agents via bone substitute biomaterials; surface coatings on internal implants improve integration and reduce osteolysis. To predict clinical outcome of these innovative designs, devices and matrices must undergo sophisticated *in vivo* and clinical testing. However, the fundamental *in vitro* testing system can also be as sophisticated with elevated mimicry of the physiological and pathological bone environment. Establishing *in vitro* culture systems focused on standard parameters of cell population ratio, seeding order, and soluble mediators for native bone metabolism will allow for screening of the effectiveness of tissue engineered designs. Various investigations to characterize bioactivity of bone substitute constructs have used mono- and co-cultures; however, culture parameters such as cell population ratio and differentiation supplements are unconsidered and inconsistent. The objective of the proposed work is to establish the parameters for cell ratio determination, using RAW monocytes and D1 stromal cells and osteogenic conditions, with or without RANK ligand supplements to induce osteoclastogenesis. Specifically the objective was to pinpoint those cultures that demonstrate phenotypic gene expression during differentiation towards osteoclasts and osteoblasts on a 3D bone substitute material. To further simulate the natural bone environment, the co-culture parameters in a tri-culture system allowed

the evaluation of the paracrine effect of adipocytes on osteoclast catabolic and osteoblast anabolic metabolism in remodeling.

The first two aims of the project focused on evaluating osteoblastic and osteoclastic differentiation, with varying cell population ratio, on a 3D bone environment to emphasize the balance in bone metabolism. Furthermore, the influence of RANK ligand on induction of osteoclast differentiation was assessed. Gene expression indicated cell ratio as a variable in regulating osteoblastic mineralization with no effect on maturation while regulating osteoclastogenesis with no effect on activation of osteoclasts. The coordination of osteoclastic and osteoblastic differentiation and activation at the 1:10 ratio is comparable to clinical observations derived from normal bone biopsy histomorphometry. As for RANK ligand addition to induce osteoclast differentiation and maturation, the relationship was not statistically significant in co-culture with precursor osteoblasts, the native producer of RANK ligand. In addition, Western blot results indicate that the addition of RANK ligand hinders production of differentiating and mineralizing proteins in osteoblasts. Hence, the use of RANK ligand should be avoided to minimize inhibition of osteoblastic activity. Within a co-culture system of precursor osteoclasts and osteoblasts, the cell population ratio needs careful consideration in order to appropriately mimic relevant aspects of physiological and pathological conditions.

The chance of the reported clinical failure of chronOS in treating a patient with scoliosis could have been significantly reduced by screening for bioactivity using a co-culture in vitro system with a 1:1 osteoclast to osteoblast ratio. The use of normal progenitor cells at an abnormal ratio would simulate scoliosis with normal bone

conditions, an imbalance in bone catabolism, and anabolism during spinal development. At the 1:1 test system ratio, early osteoblastic differentiation was lower on chronOS while early osteoclastic differentiation was higher. Minimal osteoblastic migration and abnormal osteoclastic multinucleation can lead to no bone formation or resorption, as seen on the explant.

To understand the regulatory influence of adipocytes on the differentiation of bone cells, a tri-culture system was designed to allow indirect co-culture of bone cells with adipocytes. Results indicated that the presence of adipocytes hindered osteoclastic maturation and activity while only affecting the mineral deposition of osteoblastic cells and not the early maturation. The tri-culture system can be applied as an osteoporotic test system or can be applied to other disease environments, with variations in cell ratio and cell types. The characterization of bone constructs in advanced *in vitro* test systems at the early stage of development can facilitate the inexpensive prediction of biological failures before *in vivo* testing. Hence, developing the tri-culture system to be more clinically relevant will improve the ability to predict bone construct bioactivity in an *in vivo* environment.

For diseases in which bone cell activity is abnormal, further considerations are necessary. The in vitro systems can become complex with four or more cell types and even inclusion of inflammation conditions; however, in vitro test system design should be simple and yet mimetic to provide a quick pass/fail preliminary screen that leads to continued in vivo testing or to selection of a new material.

The fourth aim of this research project focused on introducing biomedical engineering to middle and high school students with a teaching module about bone implants, bone biology, and engineering design. The teaching module was implemented at a Girl Scout event for middle school girls and a Science, Technology, Engineering and Mathematics event for high school juniors. Students were encouraged to be curious and creative during the presentation and hands-on activity, as the differences and importance in bone health regarding the simulated bone fracture were presented. They also learned about the differences in fracture management in which implants are the last resort. In their design of the implants, students gained understanding of the difficulty of implanting the bone screws and plates on even the simulated bone. During the testing of their fixed fracture, the students were reminded that the most important part of engineering design is to avoid implant failure for patients. Survey results demonstrated that the teaching modules with the incorporated bone implant lesson positively influenced both the middle school students and high school juniors in their understanding of engineering, their confidence to succeed in engineering, and the importance of engineering for society.

In conclusion, the research overall exemplified the need for culture test systems to be as sophisticated as the materials being investigated. In advancing understanding of pathological conditions and simulation of physiological conditions, fundamental *in vitro* evaluations of engineered bone devices can screen for potential incompetencies. Characterization of bone constructs can target different pathological conditions to examine specificity of bioactive molecules, cytokines, and nano-particles supplemented on the devices. The long-term goal is to significantly improve characterization of

technologies for pathological fractures by incorporating human mesenchymal and hematopoietic stem cells from bone aspirates in normal and pathological co-culture and tri-culture systems. Hence, results from this research will impact the advancement of bone tissue engineering strategies by answering the increasing demands for efficiency in engineered bone tissue and internal fixation devices. Awareness of the implications of bone diseases can be disseminated to young students or future engineers with this unique workshop that incorporates bone biology with fixation implant designs.

CHAPTER SIX

RECOMMENDATIONS FOR FUTURE WORKS

1. For clinical relevance, allografts can be obtained to co-culture cells and compare with other commercially available bone constructs. It will be important to ensure that all parameters are the same and all materials are sterilized according to company standards.
2. Once optimized, protective bone agents can be tested to understand coupling inhibition or induction responses by both osteoclasts and osteoblasts for local dosage determination and long-term systemic effects.
3. For clinical relevancy, the cell ratio study and the tri-culture study should be conducted using primary human cells. However, the test system should be optimized for murine and human cells for labs that have no access to primary cells.
4. The tri-culture can be advanced into other models of pathological bone, modeling with respect to the adipocytes, etc. Further characterization of the tri-culture will be necessary to establish it as a osteoporotic model.
5. For a more extensive understanding of osteoclastic and osteoblastic activity, a longer differentiation/maturation period for tri-culture is recommended. In addition, determine if adipogenic communication with bone cells affects activity with tri-culture of differentiated osteoclasts and osteoblasts.
6. Since tri-culture for bone cells are on a 3D bone matrix, adipocyte differentiation should be conducted on a 3D hydrogel matrix to increase physiological relevance.

However, attention should be paid to potential interactions of soluble factors between bone cells and fat cells.

7. Experiments should be conducted to determine different methods of measuring osteoclastic activity. One approach would be to assess the protease activity of cathepsin K or matrix metallopeptidase 9 (MMP-9) using zymography. Due to the 3D environment of the bone constructs, TRAP staining cannot be performed; additionally, the use of ELF97 is general for all endogenous phosphatases. For these reasons, ELISAs will need to be implemented to assess osteoclastic activity. The TRAP ELISA was conducted, but it did not detect any of the samples collected.

8. An alternative to visually qualify osteoblasts and osteoclasts on the substitute materials is to trypsinize or isolated the mature/active cells from the material to be plated on tissue culture plastic well plates. Realize that this procedure will alter cellular behavior, morphology, and population.

9. To measure adipogenic activity, a triglyceride assay and gas chromatography should be conducted to evaluate secreted fatty acid in the medium. Oil red O stain can be conducted for adipocytes in 2D culture but, for 3D culture, a different imaging method should be used.

10. Depending on the bone construct, measurement of calcium phosphate and other mineral deposition may not be possible with a chemical assay. One alternative is to use energy-dispersion x-ray (EDX) via scanning electron microscopy (SEM). Samples collected for Live/Dead imaging can be saved for SEM following sample

fixation protocols.

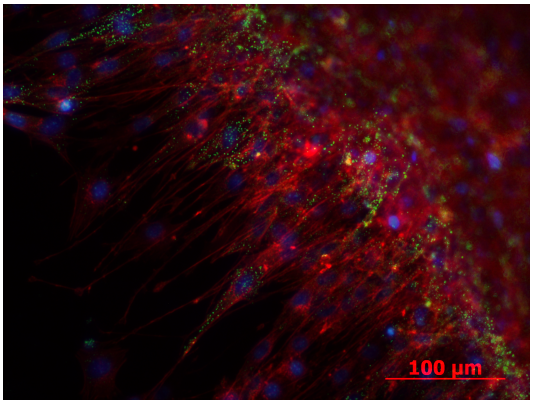
11. To improve upon the teaching model to make it more accessible for teachers and instructors, plates should be revised using household items or items that are commercially available.
12. The styrofoam for the normal cancellous bone can be changed to something less dense and stiff so that students can more easily insert screws. Also the incorporation of a hollow styrofoam will eliminate the necessity to drill a hole inside the tube.
13. Survey questions should be altered to focus on just the bone implant lesson to gain more insight on the effectiveness of the module in introducing biomedical engineering and engineering design. However, the lesson itself then needs to be expanded upon to be more than an hour to complement the entirety of the survey.
14. A follow up student event should be developed to reinforce the initial information and to study the long term effects of a long lesson versus a short 1-hour education module.

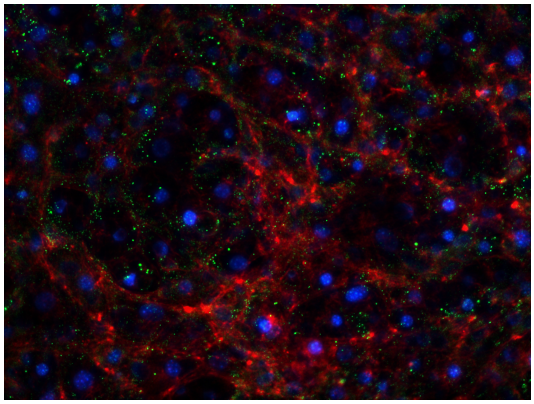
APPENDICES

Appendix A

Mono-culture Results

Comparison of differentiation in mono-culture; D1 and RAW cells differentiating into osteoblasts and osteoclasts, respectively, on 2D well plate and 3D chronOS granules at Day 14. Samples were collected at Days 4, 8 and 14.

a) 

b) 

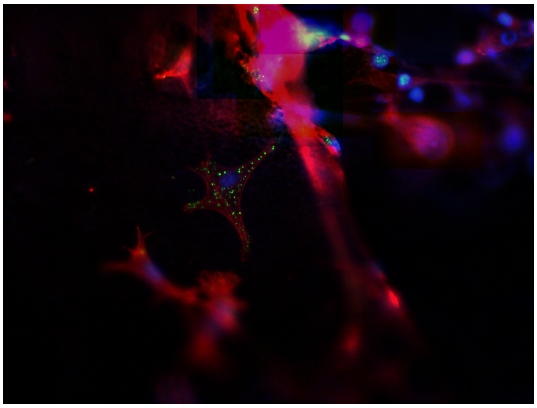
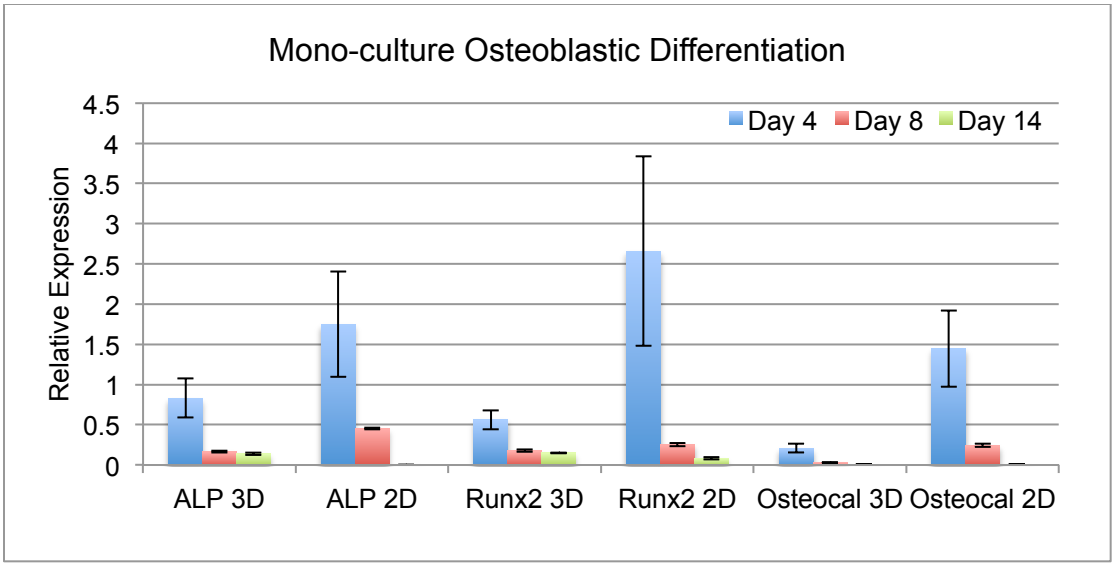
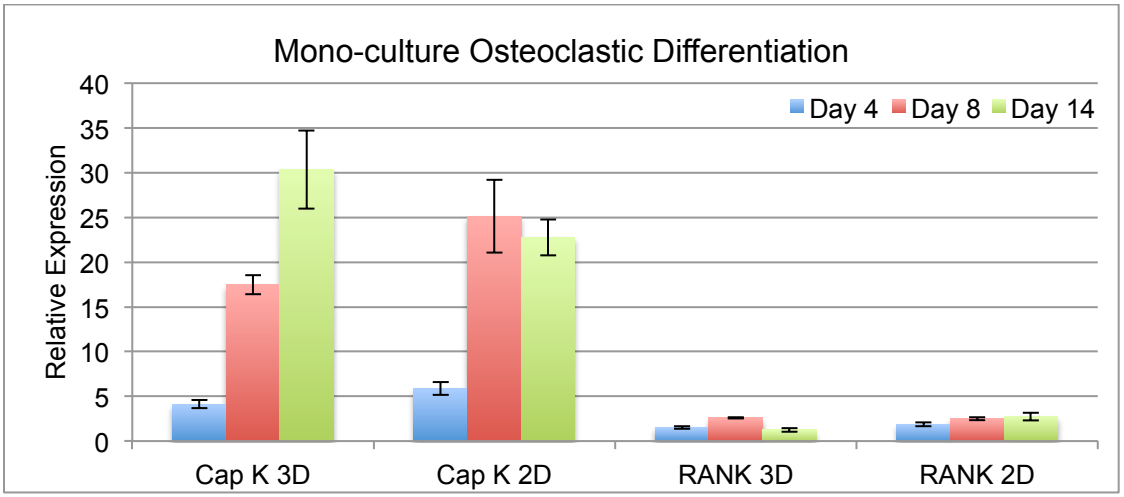
c) 

Figure A1. Fluorescent images of mono-culture of D1 cells under (a) negative control (only growth medium), (b) 2D osteoblastic differentiation with osteogenic medium, and (c) 3D osteoblastic differentiation with osteogenic conditions. Cells were probed with Hoechst nuclear stain (blue), AlexaFluor 546 actin stain (red), and ELF97 endogenous phosphatase stain (green).





Gene Expression and Significance			
Marker	Time	Matrix	Time*Matrix
ALP	0.0025	-	-
Runx2	0.0160	-	-
OCN	0.0025	0.0102	0.0180
CapK	<0.0001	-	0.0403
RANK	0.0121	0.0087	0.0144
Significance of effects and interaction of effects is shown for each marker.			

Appendix B

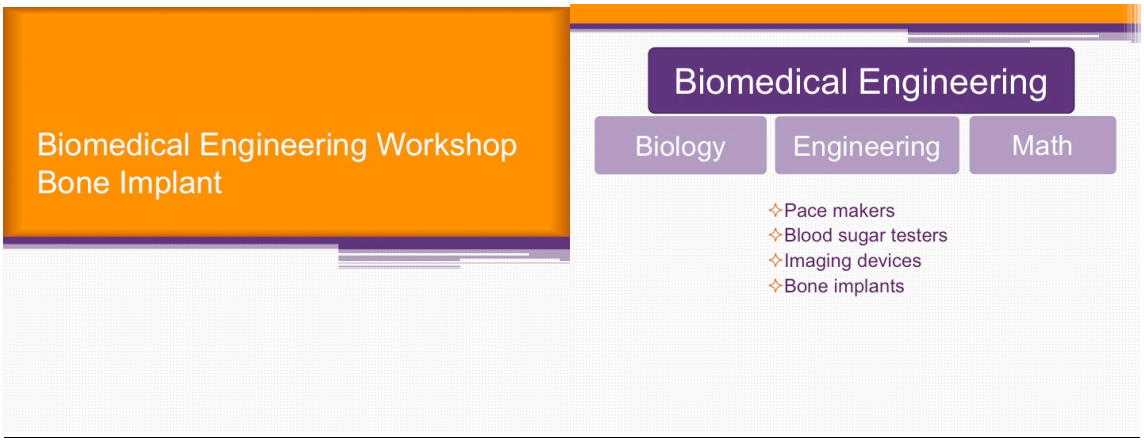
Bone Implant Teaching Module

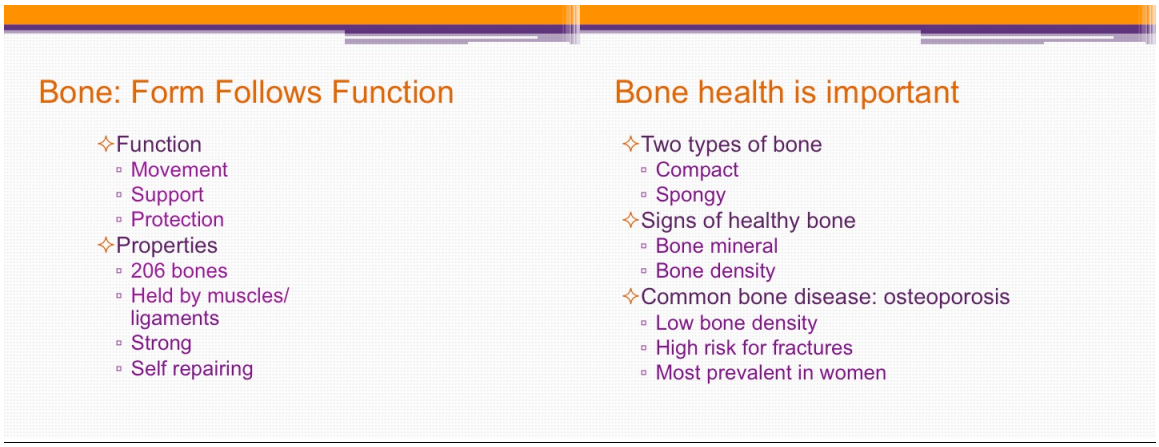
The presentation is accessible through DropBox:

https://www.dropbox.com/s/uwk7qfpzzed5khb/Biomedical%20Engineering%20Workshop.pptx

To modify presentation, enter password: BME

The presentation includes instructions, notes, and suggestions for teachers to modify to meet the standards for grade level, class subject, and class size.





Methods to fix broken bone

- Casting
  - Plaster of paris
  - Fiberglass
- Metal implants
  - Plates and screws
  - Pins
  - Intramedullary rods

Bone Implant Design

Implant Design

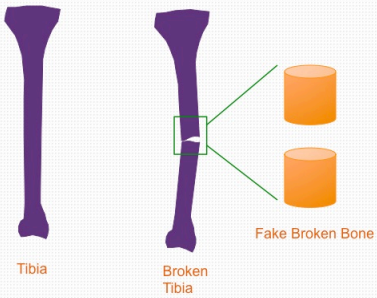
Bone Implant Design

- Health of the bone
- Material of the implant
- Location to implant
- Length of surgery
- Size of surgery opening

Engineering Design

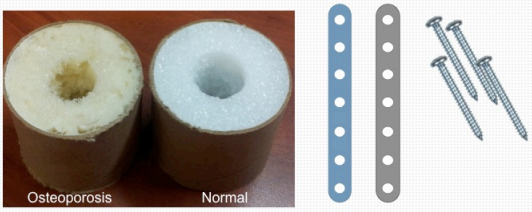
1. What are the problems?
2. What are the possible solutions?
3. How can the solutions be better?

Fix This Broken Bone



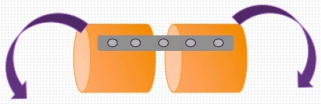
Be a surgeon
Be a bone tissue engineer

1. Patient with normal bone
2. Patient with osteoporotic bone



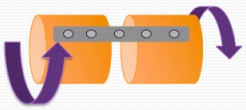
Testing = Finding ways it can fail

Bend Test



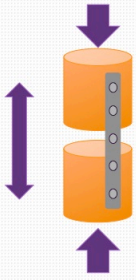
Observations

Torsion Test



Observations

Compression and Tension Test



Observations

If it failed… How can it be redesigned?

Implant Design before testing

Implant Design after testing

Lessons Learned

Recap

- Biomedical engineering = biology + math + engineering
- Bone = support + protection + movement
- Bone health is important
- Bone can break
- Broken can be fixed by surgeons and engineers

Survey Questions given to the Girl Scouts in middle school and Juniors in high school:

Please check one box for each statement.					
	Strongly Disagree	Disagree	No Opinion	Agree	Strongly Agree
I like math					
I like science					
I like engineering					
It is important for me to learn math					
It is important for me to learn science					
It is important for me to learn engineering					
I am good at math					
I am good at science					
I am good at engineering					
I can be a mathematician					
I can be a scientist					
I can be an engineer					
Mathematicians help people					
Scientists help people					
Engineers help people					

Open-ended questions:
What do mathematicians do?
What do scientists do?
What do engineers do?
How do mathematicians help people?
How do scientists help people?
How do engineers help people?

Additional questions given on post-survey (with rating scale)

I learned what electrical engineers do
I will tell my friends and family about what I learned in electrical engineering class
I learned what mechanical engineers do
I will tell my friends and family about what I learned in mechanical engineering class
I learned what bioengineers do
I will tell my friends and family about what I learned in bioengineering class