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Osteoblast Precursors Respond to Cyclic Mechanical Compressive and Tensile Strains at the Bone-Implant Interface

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OSTEOBLAST PRECURSORS RESPOND TO CYCLIC MECHANICAL COMPRESSIVE AND TENSILE STRAINS AT THE BONE-IMPLANT INTERFACE

by

Andrew Phillip Noblett

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DEDICATION

I would like to dedicate this thesis to my parents and family. Their steadfast love, prayers, and encouragement have helped me through this and many other difficult endeavors.

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ABSTRACT

Bone is highly responsive to mechanical loads. Clinically this is important since mechanical loading could be a possible means of increasing osseointegration of dental and orthopedic implants. Using a custom 4-point-bend system, a culture of W-20-17 cells was subjected to daily doses of cyclic mechanical strain at 1Hz for six consecutive days on a commercially pure titanium substrate with multiple forms of strain. The preosteoblasts were strained 800με either compressively or in tension. Strains were applied both continuously (30 minutes of strain per day) and intermittently (15 minutes of strain, 15 minutes of rest, and 15 minutes of strain per day) with the aim of evaluating the response of osteoblasts precursors to compressive and tensile strains based on DNA quantification, total protein, and alkaline phosphatase (ALP) concentrations. Over the course of the experiment the preosteoblasts continued to proliferate on the titanium over the six days of consecutive straining. However, the W-20-17 cells demonstrated significantly less proliferation under tensile strain than the control. Compressive strain did not appear to effect proliferation and resulted in DNA concentrations very close to those measured from the unstrained control. Normalized ALP concentrations were higher than the unstrained control for all loading schemes after six days of straining with tensile strains providing the maximum values. The data suggest that daily doses of cyclic mechanical strain promoted differentiation and the cells responded differently depending on whether they experience compressive or tensile strain under these test conditions. The cellular responses to the applied tensile strains indicate that the cells were

differentiating rather than proliferating in an effort to minimize the experienced tensile strains. Compressive strains applied in this study also seemed to promote differentiation of the preosteoblasts, but less strongly. Future goals include applying the conditioned media collected from strained W-20-17 cells to RAW264.7 cells, an osteoclast-like cell line. Thus, it might be possible to get a general idea of how mechanical strain impacts the relationship between osteoblasts and osteoclasts and better understand the specific bone remodeling interactions at the bone-implant interface.

PREFACE

The main body of this thesis is a journal article entitled “Compressive and Tensile Cyclic Mechanical Strains Limit Proliferation and Promote Increased ALP Production in Stromal cells on Titanium”. This manuscript will be submitted to the Annals of Biomedical Engineering. However, the manuscript is not intended to be submitted until a supplemental experiment has been performed analyzing the effect of the conditioned media from strained osteoblast-like cells on osteoclast-like cells.

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INTRODUCTION

PROBLEM STATEMENT

Orthopedic and dental implants are a multi-billion dollar industry that improves the quality of life for thousands of patients each year.[1] After implantation, these devices are subjected to cyclic loading and experience strains. Bone cells, as well as the other neighboring tissues, actively respond to loading at the bone-implant interface which influences osseointegration.[2] The response of osteoblasts, in particular, has been shown to be dependent upon the cell substrate, magnitude of the applied strain, frequency of loading, and the duration that the load is applied.[3-14] Many in vivo and in vitro studies have been conducted to investigate specific responses, but few have been performed using a biomaterial, such as titanium, as the cell substrate. Also most studies only examine the effect of tensile strain, but implanted devices are subjected to both tensile and compressive strains. In this study we wished to begin examining the responses of osteoblasts precursor cells on titanium under both tensile and compressive cyclic mechanical strain conditions to help gain insight about osseointegration at the bone-implant interface.

HYPOTHESIS

Osteoblasts precursor cells will respond to daily exposure to cyclic mechanical strain on titanium. Specifically the cells will show increased proliferation, total protein, and alkaline phosphatase concentrations with increasing cycles of loading. The responses will also be larger after exposure to tensile strain than to compressive strain since bone tends to remodel itself to reduce tensile strains. Lastly, intermittent loading will result in higher proliferation, total protein, and ALP than simple continuous straining since the cells will have time to recover between cycles.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

BONE BIOLOGY

The human skeleton helps to protect vital organs such as the lungs, heart, and brain. Bones also give the body structure, allow for movement, store minerals, and generate blood cells.[15] The primary function of bone during movement is to control strains as forces are transmitted from one part of the body to another. Bone provides an adaptable structure and the mechanical strength required for movement, while structures such as cartilage, ligaments, and tendons guide the transition of forces.[16] All of these traits are the product of a unique structure and composition that has been tailored to specific needs over thousands of years of evolution.

BONE STRUCTURE

As with all body tissues, the structure of bone has adapted to meet its role in the human body. A hierarchical structure persists on the micro and macro scale creating a balance of strength and flexibility. During embryonic development, the skeleton consists of woven bone, which can form quickly and is composed of a disorganized arrangement of collagen fibers and mineral crystals. The embryonic woven bone matrix calcifies and is completely remodeled as lamellar bone after 4-5 years, but will reappear during fracture healing and is present in the epiphyseal sections of growing bone. The lamellar bone forms slowly into highly

organized parallel layers called lamellae, making it much stronger than woven bone.[16]

Macroscopically bone is non-homogeneous, porous, and anisotropic. Mature bone tissues develop into distinct areas of very high and very low porosity. Thus bone is further characterized as either trabecular or cortical bone. Trabecular bone, often referred to as cancellous bone, typically has a porosity of 50-90% and is located at the epiphysis of long bones, in cuboidal bones, and forms the interior of other bones.[15, 16] The pores are interconnected by a network of lamellar bone layers called trabeculae, which are generally 200μm thick and have the appearance of plates and web-like fibers extending out in different directions.[17] The open space between trabeculae is filled with marrow, composed of nerves, vasculature, and a variety of different cell types.

More dense bone tissue, known as cortical or compact bone, can be found surrounding sections of trabecular bone and in the diaphysis of long bones.[15] Cortical bone only has a porosity of 5-10% but has several of distinct types of pores. This compact bone is organized into bundles of concentric lamellar layers called osteons or Haversian systems. The osteons run down the long axis of the cortical bone and surround pores know as Haversian canals. At 50μm in diameter Haversian canals are the largest cortical pores, making room for major vasculature. Smaller Volkmann's canals transversely connect the Haversian canals to provide space for nerves and capillaries while even smaller pores are associated with lacunae and canaliculi.[16] Mechanically cancellous and cortical bones have very similar modulii and hardness on the nanoscale, but values for

cortical bone are typically larger macroscopically.[18, 19] However, different bones and even different regions within a single bone have large variety in porosity, collagen matrix, and mineralization so it is difficult to make general statements about mechanical properties.[20]

BONE COMPOSITION

Chemical composition also plays a major role in the multifunctionality of bone. Deviations in bone composition are associated with many serious bone diseases such as osteoporosis, chronic liver disease, or Cushing's disease and may signify poor bone health.[21, 22] The basic components of bone are hydroxyapatite, collagen, noncollagenous proteins, proteoglycans and water.[23] However, the dispersion of these components is neither homogenous nor isotropic and can vary with sex, age, anatomical location, and species.[24] In humans the inorganic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) makes up 60-70% of the total bone mass and forms the rigid framework providing high compressive strength and stiffness.[25] However, this inorganic mineral phase only constitutes 25% of the volume of human bones. The majority of human bone volume exists as in an organic phase consisting primarily of type I collagen. The less dense collagen allows for flexibility and toughness and occupies the remaining 25-30% of total bone mass.[26] Also large volumes of water can be found inside the trabecular bone enabling the diffusion of both nutrients and waste.[27]

BONE HEALING AND OSTEOGENESIS

Trauma and disease can severely damage bone tissue. Initial healing begins with fibrous tissue forming a protective layer around the site of injury. Fibrous tissue can form quickly to provide temporary stability and guard against infection. Over time new bone is remodeled by groups of cells with very specific functions.[10] The remodeling process follows a cycle of activation, resorption, and formation. Old or damaged bone is removed by osteoclasts as new bone is laid down by osteoblasts.[16] The process is governed by mechanical, hormonal, and physiological stimuli and begins on the internal surfaces of the bone matrix.[28]

Osteoblasts originate from periosteum or from the non-hematopoietic part of bone marrow where they differentiate from pluripotent mesenchymal stem cells (MSCs).[28, 29] Osteoblasts are responsible for the new bone formation.[30] These cells lay down organic bone matrix and secrete molecules that lead to mineralization. For example, active osteoblasts produce large amounts of type I collagen, alkaline phosphatase (ALP) and many other signaling molecules such as osteocalcin, osteopontin, osteoprotegrin, bone sialoprotein, and osteonectin.[31, 32] Once enough matrix material has been produced the osteoblasts on the surface become inactive, but can be reactivated through mechanical and chemical signaling. Inactive surface osteoblasts are often referred to as bone lining cells.[16, 33] Other osteoblasts become embedded in the new bone matrix and differentiate into osteocytes. Osteocytes reside in the lacunae and are responsible for maintaining the bone and communicating to the other cells through canaliculae.

Osteocytes are much less active than osteoblasts at producing matrix proteins, but they are crucial to blood-calcium homeostasis.[16]

The bone resorbing osteoclasts are derived from pluripotent hematopoietic stem cells (HSCs) and come from the marrow.[16, 30] Osteoclasts are multi-nucleate, form from the fusion of monocytes, and behave similarly to phagocytic macrophages. These cells are called to the remodeling site by hormonal and physical stimuli at the activation of the remodeling process.[34] The osteoclasts then demineralize sections of bone matrix with acid and break down the collagen with enzymes.[16] Once some of the old bone has been removed, the osteoclasts will inactivate and leave the site so that osteoblasts can come into the cavity and begin forming new bone.[34]

The process of modeling and remodeling are closely regulated by the interaction between the different cell types. All of the bone cells except osteoclasts are closely interconnected by a network of intercellular junctions.[35] Signaling molecules then further facilitate communication and cooperation between the different cell types. In fact, osteoblasts modulate the rate at which osteoclasts differentiate from the precursors through the receptor activator of nuclear factor-κB (RANK). RANK ligand (RANKL) binds to RANK to activate osteoclasts. However, osteoblasts secrete osteoprotegrin (OPG) which acts as a RANK antagonist and inhibits RANKLs ability to bind.[30] The balance between bone forming osteoblasts, maintaining osteocytes and resorbing osteoclasts is what keeps bones strong. This close knit relationship between the different types of bone cells has also been shown in vitro. For example Lau et al. recently

recorded an increase in osteoclast activity after exposing rat osteoclastic cells to conditioned media collected from mechanically stimulated osteocytes culture.[36] Each of the studies such as the one performed by Lau et al. add a little piece to the puzzle, but there are still many questions to be answered.

MECHANICAL REGULATION OF BONE

WOLFF'S LAW

Bone is a highly adaptable tissue that can rearrange itself to cope with different situations. Julius Wolff was one of the first pioneers of adaptational bone research with his book *The Law of Bone Remodeling* in 1892. He proposed that the internal architecture and then the external structure of a bone will transform to meet the needs of its loading in a healthy person, which became known as Wolff's law.[37] In other words, an active person will have stronger bones than a less active person to deal with the increased stresses and strains. Similarly bones that remain unloaded will begin to resorb and decrease in mass so that the body may use the nutrients elsewhere rather than maintaining the unused tissue.[38] Some of the mathematical relationships and findings in Wolff's initial work have been proven incorrect, but his work is still very instrumental in changing the way researchers approach bone since he suggested that the stimulus triggering bone remodeling was related to mechanical loading.[39]

SENSING MECHANICAL LOADS

Bone remodeling is triggered when detected stimuli, physical, hormonal, or mechanical, reach the appropriate threshold. Determining which regions in bone detect the stimulus and then initiate bone remodeling has been the subject of many different studies in the past 20 years. Bone remodeling is reliant upon the activities of specific cells so mechanical signals are most likely interpreted by each individual cell or by specific sensor cells.[34]

Evidence suggests that osteocytes might be the primary mechanosensors responsible for initiating bone remodeling, but osteoblasts have also been show to be sensitive to mechanical stimulation.[4, 5, 35, 40-44] If osteocytes detected mechanical stimuli then the connected cellular network could transmit the signal to the bone lining cells and osteoblasts.[35] Also the matrix materials and processes of osteocytes are not calcified so they form a three dimensional network which would be ideal for sensing mechanical signals.[45] Osteocytes have been recorded expressing mRNA for important factors such as osteocalcin, insulin-like growth factor I (IGF-I), and many others during in vivo studies.[46] In vitro studies have also shown that osteocytes become more metabolically active after exposure to mechanical stimuli such as the physiological strains associated with locomotion.[13, 47-49] Similarly production of alkaline phosphatase (ALP) and other proliferation and mineralization markers in osteoblasts are highly responsive to mechanical loads.[3-6, 41-44, 50-52] Thus both cell types may play a role in sensing mechanical stimuli before the remodeling process is initiated. The glycocalix, a surface proteoglycans layer, is thought to be the primary sensor site but many questions

still need to be answered to determine the exact contributions of each cell type and all of the pathways involved.[53]

STIMULATION BY MECHANICAL STRAIN

Over the last few decades, researchers also have been trying to determine which aspects of mechanical loading regulate bone remodeling. Many different techniques and protocols have been developed over the years applying a variety of different stimuli. During mechanical loading, interstitial fluid flows through the lacunae and canaliculi as a result of pressure gradients.[54, 55] Thus fluid flow and hydrostatic pressure have become popular ways of stimulating osteocytes and osteoprogenitor cells in vivo.[56] Loaded bone also experiences mechanical strains in vivo due to the bending, axial, and torsional loads resulting with normal movement. During the physiological loads of locomotion, these strains are typically between 0.04–0.3%.[14] The average peak tibial strains experienced by a walking human have measured around 400με.[57] However, strains have been measured to reach values up to 3000με during more strenuous activities.[12] Stimulation by mechanical strain is applicable to osteoblasts residing on the surface, as well as osteocytes and osteoprogenitor cells.[34, 56]

Harold Frost proposed that bone only responds within certain intervals of strain with his mechanostat hypothesis.[58] As long as experienced strains remain within the expected physiological range, bones will remodel normally. He defined this range as being between 50με and 1500με, which could change due to

disease and other physiological situations. Being exposed to strains outside of these thresholds will drive the process of resorption and new bone formation at specific sites until the experienced strains are once again within expected boundaries.[58] From ideas such as the mechanostat hypothesis, research began to look at the relationship between the magnitudes of cellular strain and cellular responses. For example, Mosely et al. noticed changes in rat ulna architecture and periosteum from different magnitudes of strain.[9] Axially loaded rat ulnas placed under moderate strain, peak strains between 500 and 2000με, resulted in reduced bone curvature with reduced periosteal bone formation when compared to unloaded controls. On the other hand, peak strains around 4000με resulted in reduced bone curvature with improved periosteal bone formation.[9] In vitro, osteoblasts seem to be virtually unaffected by strains below 300με or above 24,000με.[3, 6] However, Winter et al. recorded significant levels of ALP from a rat osteogenic cell line after 24hrs of cyclic mechanical strain at 400με and higher levels of ALP from loading at 1000με.[3]

Brand and Stafford expanded upon Frost's ideas with their trigger theory.[11] They surmised that stimuli outside of the expected physiological responses were processed temporally and that tissues could become desensitized to the stimulus over time.[11] Thus exposing bone to strains outside of the normal expected range for long periods of time may not result in increased cellular responses. In fact, Lanyon et al. saw that cyclic loading produces ulna bones with significantly larger cross sections than static loading.[8, 59] This begs the question as to whether osteoblasts and their precursors have a frequency and loading

duration at which they will cause maximum bone formation. To answer this question, Kaspar et al. strained human bone derived osteoblastic cells on silicone dishes for several different durations and at varying frequencies.[5] At 1Hz the cells had increasing proliferation with increasing cycles up to 1800 cycles in a dose dependent manner. However, proliferation began to decrease after 3600 cycles. When exposed to a constant number of cycles the cells had maximum proliferation at 1Hz, but proliferated similarly to unloaded samples when exposed to cyclic loading at a higher frequency of 30Hz.[5]

Interestingly, it has recently been reported that low magnitude high frequency vibrations can help increase bone mass in vivo and increase osteocyte activity in vitro.[7, 36] Low magnitude high frequency vibrations present a promising testing platform because it can be applied clinically much more easily than higher magnitude strain. Allowing cells time to rest in between cycles or mechanical strains around 1000με, intermittent loading, has also shown to increase the effects witnessed in vitro. Winter et al. stretched osteoblast cells for equal numbers of cycles but loaded some cell continuously while allowing other cells to rest between cycles. The intermittent loading scheme resulted in significantly higher ALP, calcium, and DNA concentrations after one hour of loading than continuous loading.[4] In summary, the response of bone to in vitro mechanical stimulation seems to be dependent on the magnitude, duration, frequency, and loading scheme of the applied loads.

CYCLIC MECHANICAL STRAIN SYSTEMS

The systems developed to mechanically stimulate cells in vitro, as described in the studies above, vary widely. Each system is different with varying benefits and limitations. Some systems apply multi-axial strain while others are uniaxial. Flexcell® International Corp. is a major producer of multi-axial strain systems. Their systems typically apply strain through vacuums and/or positive pressure in a multi-well, automated package. Others systems apply multi-axial strain mechanically with multiple actuators stretching the substrate to induce strain. However, the cell substrates are generally not materials readily used for clinical implants.[60] Systems have been developed to accommodate biomaterials but they still have limitations, including problems maintaining homogeneous strain fields.[61]

Maintaining homogeneous strain in one direction is much easier but the situation is obviously simplified from the multiple strains and multiple directions experienced in vivo. Grip displacement systems are the simplest models.[61] The cell substrate is gripped between two loading arms and pulled apart in tension. This setup is limited because it works best with elastic, non-biomaterial substrates and can only apply tensile strain loads. Four-point bending provides an alternative and allows one to mechanically strain cells under either tensile or compressive strain due to the Poison effect.[61] Few studies have been done comparing the effects of tensile versus compressive strain and fewer still have done so on biomaterial substrates. Thus using a four-point bend system is the best option for exploring the aims of this project.

BONE-IMPLANT BIOMATERIALS

When an injury is too severe to heal naturally, implanted devices help to restore, augment and/or replace the function of the damaged or diseased bones, tissues, and joints.[62] Such devices are subjected to many loads and these loads are translated over to the adjacent cells and surrounding tissues. The choice of material for an implanted device is very important. The response of bone to a material is substrate dependent and will differ from material to material.[62]

GENERAL REQUIREMENTS FOR AN ORTHOPEDIC BIOMATERIAL

All of the materials coming in contact with living tissues or being placed inside the body must meet very specific design criteria. If these criteria are not met implants and other medical devices will fail, leading an assortment of medical complications and costly revision surgeries. To be viable for orthopedic or dental/craniofacial applications biomaterials must have excellent corrosion resistance, high fatigue and wear resistance, good biocompatability, high strength with a low elastic modulus, be ductile, and not be cytotoxic.[63, 64] Mechanical properties are particularly important in orthopedic implants due to heavy loads, large numbers of cycles, and the need to avoid stress shielding which can lead to bone resorption at the implant site.[62]

CURRENT ORTHOPEDIC BIOMATERIALS

Polymers are the largest class of materials with the widest range of properties. However, polymers are generally only given supporting roles in orthopedic applications due to limited mechanical properties. For example, polymers may be used to fabricate scaffolds promoting bone growth around an injury. Some exceptions currently being explored are polyaryletherketones (PAEKs). Poly-ether-ether-ketone (PEEK), in particular, has emerged as a potential alternative to metal implants for load bearing applications over the last few decades.[65] PEEK has an elastic modulus very close to that of cortical bone and excellent biocompatibility but, metals still remain the gold standard for orthopedic implants because they are well studied and less expensive. PEEK is currently being used clinically for spinal application and is being investigated for many additional orthopedic applications.[65] A more well known polymer widely used in orthopedics is Poly(methyl methacrylate), commonly known as bone cement. PMMA has been around for decades and has been an invaluable tool for operation rooms worldwide.[66] Other promising polymers are also in development specifically in tissue engineering applications, but it may be many years before many are clinically ready and most still require the aid of metal or ceramic fixation devices. Polymers may one day dominate the realm of orthopedic biomaterials, but for now metals provide a more trusted and economical solution to orthopedic device needs.

Ceramics are popular orthopedic implant materials, particularly in the hip, that have had great success clinically and have been around for a long time.

Biomedical applications of zirconia date back to 1969.[67] In general ceramics are very strong under compression and have incredible corrosion and wear resistance. However most ceramics are weak when exposed to tensile loads, brittle, and susceptible to crack propagation.[68] Ceramics are particularly popular in dental applications but materials such as zirconia and alumina have been used for many load bearing joint replacement applications.

Metallic materials are the most common choice for the fabrication of medical implants due to their high mechanical properties. The most prevalent metals in implants are 316L stainless steel (316LSS), cobalt chromium alloys (Co-Cr), and titanium and its alloys.[62] Stainless steel is subject to crevice and stress corrosion so it is not an ideal long term implant material. As a result, 316LSS is typically only used for spinal fixation and temporary fracture repair applications rather than for long term implants.[69] Co-Cr alloys have many more biomedical applications than stainless steel because they have extraordinary wear characteristics. This high resistance to wear makes Co-Cr suitable for applications such as total joint replacements.[69] 316LSS and Co-Cr are very strong, but have much higher elastic modulii than human bone. As a result, the majority of the stress from daily loading is absorbed by the metal and is not transferred to the native bone. This stress shielding effect leads to bone resorption and implant loosening.[70] Another limitation of 316LSS and Co-Cr alloys is that they can be prone to emit harmful elements as they corrode inside the body.[71] These limitations led researchers to look for a new metallic material

capable of overcoming these short comings. The search eventually turned to titanium and titanium alloys.

TITANIUM AS A BIOMATERIAL

Titanium and titanium alloys have been used clinically with great success for many years and have now become the standard material for many orthopedic applications. It is a wonderful metal that can be given a wide range of properties depending on how it is treated and what it is alloyed with. In general titanium has high strength, low density, good biocompatability, and a relatively low modulus of elasticity when compared to other metallic biomaterials.[72] The specific strength of titanium makes it stand out from other implant materials because its alloys can have a strength comparable to stainless steel yet be 55% less dense.[73] Titanium and titanium alloys also have a much lower elastic modulus than many other implant materials, which lowers the risk of stress shielding around an orthopedic implant. For example the elastic modulii for 315LSS and Co-Cr alloys are both over 200 MPa whereas titanium ranges from around 55 to 110 MPa.[62] These properties have led to successful implants in dental, orthodontic, joint replacement, spinal, bone fixation, and cardiovascular applications.

The two most common forms of titanium used in implants are commercially pure (cp) titanium and Ti-6Al-4V ELI (Ti64, extra low interstitial).[62] Ti64 has better strength and corrosion resistance than cp titanium, but there are some concerns with long-term leeching of aluminum and

vanadium.[74] Vanadium is toxic in both oxides and the elemental state, but the elemental release has become less of a problem over the years with better fabrication techniques.[62] The excellent biocompatability of titanium and titanium alloys is a result of the dense 2-6nm thick oxide layer which forms on the surface. This layer has low solubility in body fluid, is chemically inert, and remains thermodynamically stable.[75]

OSSEOINTEGRATION

For a load bearing implant to be successful clinically, osseointegration is crucial. Osseointegration has been defined differently by many researchers.[76-78] Some of the most notable definitions are from Branemark et al.[79, 80] They initially defined osseointegration as "a direct structural and functional connection between ordered living bone and the surface of a load-carrying implant" but later refined their definition to say that "an implant is regarded as osseointegrated when there is no progressive relative movement between the implant and the bone with which it has direct contact.[79, 80]

Without proper osseointegration, a weaker layer of fibrous tissue can form between the bone and the implant surface. Then micromotions can lead to implant loosening and failure.[81] Several studies have shown that applying mechanical loads early after implantation can improve how well bone tissue integrates with an implant surface.[82, 83] However, it is important to avoid overloading which can lead to bone resorption.[84] Current load bearing orthopedic

and dental implants are designed to promote osseointegration and as mentioned above, many are fabricated from titanium and titanium alloys. As a result, mechanically straining bone tissues on non-implant material substrates is not enough. Many cyclic mechanical studies have been conducted to examine the subject but there are still many unanswered questions concerning bone tissue cellular responses and interactions. By gaining a greater understanding of the exact response of bone tissue to physiological loads at the bone-implant interface may provide new insights and allow for better implant designs. This project examines the titanium bone-implant interface in vitro with multiple cell lines and multiple loading schemes in the hope of answering some of the unanswered questions.

CHAPTER 3

JOURNAL ARTICLE TO BE SUBMITTED TO THE ANNALS OF BIOMEDICAL ENGINEERING

Compressive and Tensile Cyclic Mechanical Strains Limit Proliferation and Promote Increased ALP Production in Stromal cells on Titanium

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Abstract - Stromal cells, W-20-17, were subjected to daily 30 minute doses of 800με mechanical strains at 1Hz for six consecutive days on a commercially pure titanium substrate with a custom 4-point-bend cell strain system to simulate the bone-implant interface in vivo. The loading schemes include continuous and intermittent (15 minutes of strain, 15 minutes of rest, 15 minutes of strain) dosing in both compressive and tensile strain. Cell lysates and media were collected after 12hrs on the plate and after 1, 3, and 6 day of straining. DNA, total protein, and ALP concentrations were assayed to assess the results of the different strain. Overall the stromal cells proliferated similarly to the unstrained control but produced increased concentrations of ALP, when compared to the unstrained control, suggesting that the cells are undergoing differentiation. Compressive strains had little effect on cell proliferation and only a small impact on ALP activity. Interestingly tensile strains resulted in the largest normalized ALP activities, 68% increased response to continuous tension over unstrained control, with the least amount of proliferation. These data suggest that the relationship between tensile and compressive responses may be complicated and dependent on the other straining parameters. Also the W-20-17 cells under the strain conditions of this study chose to differentiate rather than proliferate in response to the tensile strain following the tendency for bones to remodel to minimize tensile strains. Also intermittently straining the cells did not appear to cause an increased response when compared to continuous strain.

Keywords - osteoblasts, osseointegration, osseogenesis, stromal, strain, titanium,

Introduction

Orthopedic and dental implants are a multi-billion dollar industry that improves the quality of life for thousands of patients each year.[1] After implantation, these devices are subjected to cyclic loading and experience compressive and tensile strains. Bone cells, as well as the other neighboring tissues, actively respond to loading at the bone-implant interface which influences osseogenesis and osseointegration.[2]

Bone remodeling is reliant upon the activities of specific cells so mechanical signals are most likely interpreted by each individual cell or by a group of specific sensor cells.[34] The remodeling process follows cycles of activation, resorption, and formation. Mature or damaged bone is removed by osteoclasts as new bone is laid down by osteoblasts.[16] The process is governed by mechanical, hormonal, and physiological stimuli and begins on the internal surfaces of the bone matrix. Osteoblasts lay down organic bone matrix and secrete molecules that lead to mineralization. Once enough matrix material has been produced, the osteoblasts on the surface become inactive, but can be reactivated through mechanical and chemical signaling.[16, 33] Some osteoblasts become embedded in the new bone matrix and differentiate into osteocytes. The osteocytes reside in the lacunae and are responsible for maintaining the bone and communicating to the other cells through canaliculae.[16] Evidence suggests that osteocytes might be the primary mechanosensors responsible for initiating bone remodeling, but osteoblasts have also been show to be sensitive to mechanical

stimulation.[4, 5, 35, 40-44] Thus, both cell types may play a role in sensing mechanical stimuli before the remodeling process is initiated.

Applying cyclic mechanical strain is one popular method for investigating the dynamic bone-implant interface in vitro. Stimulation by mechanical strain is physiologically applicable to osteoblasts residing on the surface, as well as osteocytes and osteoprogenitor cells.[34, 56] Alkaline phosphatase (ALP) and other proliferation and mineralization markers have been shown to be highly responsive in mechanically stimulated osteoblasts.[3-6, 41-44, 50-52] The magnitude of these responses is dependent upon the substrate, magnitude of the applied strain, frequency of loading, and the duration that the load is applied.[3-14]

Few cell strain studies have used compressive and tensile strains and fewer still have used biomaterial surfaces as the cell substrate. The system utilized in this study allows cells to be cultured on titanium and subjected to either unilateral compressive or tensile strain with continuous or intermittent cycles. The goal of this study is to further investigate bone tissues' cellular responses to both types of strain at the bone-implant interface of titanium implants and the effects on osseointegration using a mouse stromal cell line and multiple loading schemes.

Materials and Methods

Strain Inducing Device

A four-point bending system (Fig. 1) has been designed to administer controlled cyclic mechanical strains to bone cells cultured on titanium. The system is based on initial design by Winter et al.[4] and incorporates improvements made by Allen[41] and Smith[85]. Improvements include the use of a pneumatic linear actuator (Model PFC-092-XL, Bimba Manufacturing Company, Monee, IL) for the precise control of plate flexion and strain generation, accommodates adjustable loading arms allowing for cells to be stretched with tensile strain or compressed with compressive strain. Commercially pure titanium (cp Ti grade 2, Titanium Industries, Rockaway, NJ) serves as the cell substrate of the system in the form of 0.114 cm thick 41.91 x 25.4 cm rectangular plates. During loading the uniaxial surface strain applied to the titanium substrates is measured by a wide range strain indicator (Model 3800, Vishay Intertechnology, Inc., Malvern, PA) with a central general purpose strain gage (350 Ω □Resistance, Cat. No. CEA-06-250UN-350, Vishay Intertechnology, Inc., Malvern, PA) attached to the underside of the plates. Since the testing platform is using four-point bending the single strain gage is sufficient because all points between the two central supports experience homogenous uniaxial strain. This homogeneity as well as the uniaxial nature of the applied strain has been confirmed by Allen through the application of fourteen strain gages across both surfaces of a titanium plate.[41]

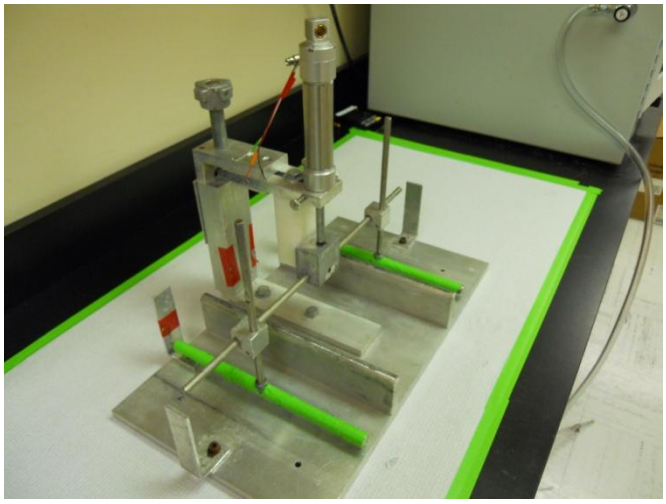

Figure 1. Photographs of Pneumatic Cell Strain Device. 4-point-bend cell strain device shown without titanium substrate plate

Substrate Preparation

Five titanium plates were wet ground and polished with SiC sandpaper up to 1200 grit and degreased with 70% isopropanol to provide a relatively smooth surface for culturing cells. Both sides of the titanium sheets were polished to reduce stress risers on the surface which might affect the shear strain. Once the plates were clean, 35 x 10 mm cell culture dishes (Corning 3294) with the bottom TCP surfaces removed were adhered to each titanium plate with 100% silicone sealant (100% Silicone Aquarium Sealant, All-Glass® Aquarium Co., Inc., Franklin, WI) to create twelve separate 9.62cm^2 growth areas (Fig. 2). All culture wells were filled with 70% ethanol and sterilized under UV light for 24 hours, rinsed with sterile PBS, and allowed to incubate with media for 15 minutes before cell seeding.

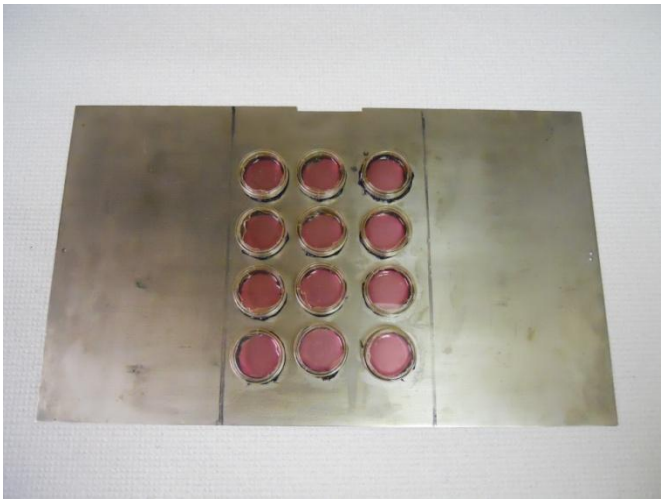

Figure 2. Photograph of Cell Substrate. Commercially pure titanium substrate plate with attached media filled cell culture wells

Cell Culture

W-20-17 cells (CRL-2623™, ATCC, Manassas, VA) are a murine osteoblast precursor cell line derived from a stromal line with the ability to differentiate in to osteoblasts.[86, 87] These preosteoblasts are often used to evaluate concentrations of BMP since they express a dose dependent response of ALP to BMP.[87] The cells were initially cultured in growth media, DMEM/high glucose (HyClone Laboratories Inc., Logan, UT) supplemented with 10% fetal bovine serum (Standard Fetal Bovine Serum, Hycone, Logan, UT) and 1% antibiotic/antimycotic (PSA, 10,000 I.U./mL penicillin, 10,000 μg/mL streptomycin, 25 μg/mL amphotericin, MP Biomedical, Solon, OH) and maintained in $75cm^2$ at 37^{oC} with 5% CO_2 till 90% confluence with medium changes every two days. Upon confluence the cells were trypsinized and seeded into culture wells of the titanium plates at a concentration of $5x10^4$ cells/cm^2 in

mineralizing media. Mineralizing media consists of growth media supplemented with 50 μg/mL ascorbic acid (Malinckrodt Baker Inc., Phillipsburg, NJ), 10 mM β−glycerophosphate (BGP, EMD Biosciences, Inc., La Jolla, CA), and 10nM dexamethasone (DEX, MP Biomedicals, Inc., Solon, OH).

Cell Straining Protocol

Once the plates were prepared and seeded, the cells were allowed to attach to the titanium plates for eighteen hours. After the ample attachment time, media was collected from three random culture wells on each plate, the wells were then rinsed with PBS, and the attached cells were lysed with sterile molecular water (DNA grade sterile water, Fisher Scientific, Fair Lawn, NJ) for one hour with regular pipette agitation. These samples served as a baseline for the study. The remaining wells still containing cells were provided with fresh mineralizing media.

All remaining cells were then subjected to cyclic mechanical strain on the cp titanium plates once a day for six consecutive days. Four plates were mechanically strained and the remaining plate served as unloaded control. The plates that experienced the mechanical strain were each strained at 800με at a rate of 1Hz. The loading schemes are summarized in Table 1. The two continuously loaded plates were strained for one thirty minute period a day. Two other plates were subjected to intermittent straining cycles. Cells on these plates were strained for fifteen minutes, allowed to recover for fifteen minutes, and then strained for

an additional fifteen minutes. All testing was performed on a bench top outside of an incubator since the durations of daily straining were relatively short. To account for the atmospheric changes outside of the incubator on testing days as well as the motions associated with moving the other plates to and from the cell stretching device, the control plates was set aside on the bench top for thirty minutes each day but were not subjected to any mechanical strains.

Some fluid motion does occur with the media inside the culture wells while plates are being strained. To document the effect of the fluid movement, 1.3 x 1.3 cm cp titanium tiles were prepared by wet SiC paper grinding and polishing up to 1200 grit to match the substrate plate preparations. The tiles were then placed inside of 12 well microplates and seeded with W-20-17 cells according to the procedure described previously. The microplates were then set on top of a clean 41.91 x 25.4 cm titanium plate without any culture wells, the clean plate was strained following the procedure used for the intermittent tension plate. In this manner, cells on titanium would experience fluid motion from the media without experiencing any mechanical strain. Media and lysates were likewise collected from these samples using the same methods as the larger plates, but the tiles were moved to clean well plates before lysing to avoid lysing cells only attached to the tissue culture plastic.

Each plate was loaded according to its designated loading scheme once a day for six days. The culture media was only changed after three days of loading to maximize the proteins and growth factors that would gather in the culture

media. After 1, 3, and 6 days of loading the media and lysates were collected as previously described. Samples were then stored at -20^{oC} until they were assayed.

Table 1. Strain Loading Schemes

Loading Condition	Loading Duration
Control	none
Constant Compression	30 minutes
Constant Tension	30 minutes
Intermittent Compression	15 minutes of loading Rest 15 minutes 15 minutes of loading
Intermittent Tension	15 minutes of loading Rest 15 minutes 15 minutes of loading
Fluid Movement	15 minutes of loading Rest 15 minutes 15 minutes of loading

Cell Proliferation

The DNA concentrations from a lysate are relative to the number of cells that were lysed because cellular DNA concentration remains relatively constant except during mitosis. As such, the PicoGreen® dsDNA assay kit (Quant-iT^{TM} PicoGreen®, Molecular Probes, Eugene, OR) provides an easy means of

evaluating cell proliferation directly from cell lysates. The PicoGreen® dsDNA reagent is a fluorescent nucleic acid stain that is very sensitive to double-stranded DNA in solution. Single stranded DNA and RNA concentrations have very little effect on the reagent. PicoGreen reagent was added to a mixture of cell lysate and a tris-HCL-EDTA assay buffer on a microplate. The microplate was then read by a fluorometer (FLx800^{TM} Multi-Detection Microplate Reader, BioTek, Winooski, VT) with excitation of 485nm and a fluorescence emission of 528nm. Concentrations were derived with a linear standard curve developed from a DNA standard (100mg/mL) provided by the supplier. The kit accurately measure dsDNA concentrations ranging from 25 pg/mL to 2000 ng/mL.

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) is a membrane bound osteoblast enzyme and a commonly used marker of osteoblastic character.[88] This enzyme is an important maker because it is expressed by mature, differentiated osteoblasts in high concentrations and cleaves phosphates for the formation of CaP during the mineralization process.[88] ALP was quantified by allowing p-nitrophenol phosphate to react with the ALP enzyme collected from cell lysates. In the presence of the cell based ALP enzyme, p-nitrophenol phosphate is catalyzed to a yellow compound p-nitrophenol and inorganic phosphate. The amount of p-nitrophenol generated is quantified colormetrically with a spectrometer at 405nm. The reaction is very sensitive so the Saos-2 lysates were diluted by a factor of 100 before assay.

Briefly, 80μL of diluted lysates were added to 20 μL of 0.5M alkaline buffer solution (1.5 M Alkaline buffer solution, Cat No. A9226-100mL, Sigma-Aldrich, St. Louis, Mo) with 10 mM $MgCl_2$ in a microplate. After adding 100 μL of 5mM p-nitrophenol phosphate substrate (Cat. No. 4264-83-9, Sigma Aldrich, St. Louis, MO) the microplate was incubated for 1 hour at 37^{o}C. A 0.3 M NaOH solution stopped the reaction and the microplate was read by a spectrophotometer (SpectraMax Plus, Molecular Devices Corp., Sunnyvale, CA) at 405 nm. An ALP powder from MP Biomedicals Inc. (Cat. No. 100180) was used to make a standard curve to calculate concentrations of ALP enzyme in the range of 25-2000 μg/ml. The final calculated concentrations were normalized by their associated DNA concentrations to determine the relative ALP activity per cell.

Total Protein Production

*C*ell proliferation and metabolic activity were assessed with total cellular protein measurements from the cell lysates. Bone cells can produce large quantities of extracellular matrix proteins so total protein levels are not linearly related to cell number but should follow similar trends.[88] Measurements were taken using a bicinchoninic acid kit (Pierce® BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). Proteins reduce Cu^{+2} to Cu^{+1} in an alkaline solution. The BCA reagent is highly sensitive and selective for Cu^{+1} and responds by forming a purple product which can be read colormetrically with a detection range of 20 to 2000 μg/mL. The assay was preformed according to the provided

instructions with an albumin standard curve. All measurements were read at 562 nm on a spectrophotometer (SpectraMax)

Statistical Analysis

All data were analyzed with a two factor ANOVA followed by a Holm-Sidak post-test, which is similar to the Bonferroni correction method, to determine statistical significance. A p-value < 0.05 was considered significant.

Results

Cell Proliferation

DNA levels of the W-20-17 cells, based on the PicoGreen® assay, were significantly altered by exposure to cyclic mechanical tensile strains over the six day testing period (Fig. 3). Two factor ANOVA showed that there were significant differences in the number of cells over time (p-value < 0.001) and subjected differences to the different mechanical strain conditions (p-value = 0.002). There was also significant interaction between time and mechanical strain conditions (p-value < 0.001) indicating that the change in cell number was dependent in part to the type of strain experienced. In general, cells increased significantly over time within each testing group, except for the samples subjected to either continuous (p-value = 0.050) or intermittent (p-value = 0.050) daily tensile strains in which cell numbers did not increase after day 3.

18hrs after cell attachment and after one day of mechanical strain, there were no differences in cell number between mechanical strain test conditions. On day 3 cells subjected to the continuous (20% lower than control, p-value = 0.006) and intermittent (22% lower than control, p-value = 0.005) tension and intermittent compression (22% lower than control, p-value = 0.006) exhibited significantly lower DNA concentration as compared to the non-strained controls. By day 6 the DNA concentrations in cells exposed to tensile strains, either continuously (21% lower than control, p-value $\leq$ 0.010) or intermittently (24% lower than control, p-value $\leq$ 0.007) exhibited significantly lower DNA concentrations than the control or compressively strained samples. Lastly, no significant differences were recorded throughout the experiment between continuous and intermittent loading schemes within either strain type (p-values $\geq$ 0.541).

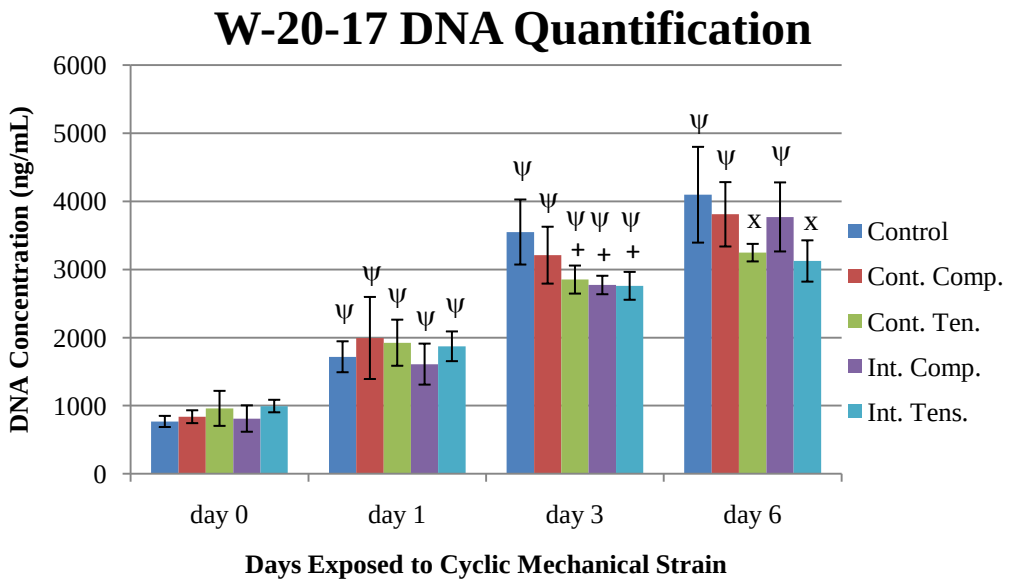

Figure 3. W-20-17 DNA Quantification. ($p < 0.05$) All groups experienced significant increases in DNA concentrations with increasing days of loading. + indicates significant difference from the control within that time point. X indicates significant difference from the control, continuous compression, and intermittent compression within that time point. ψ indicates significant difference between a value and the previous day's value of the same treatment group. n=3

The W-20-17 fluid shear control DNA concentrations were normalized to surface area and compared to the unstrained control plate (Fig. 4). Significant differences in cell number resulted due to both culture time (p-value < 0.001) and the applied strain conditions (p-value < 0.001). DNA from the cells on the fluid shear tiles followed the pattern of increasing proliferation with increasing days of culture. After day 1, DNA measurements from the fluid shear tiles significantly increased daily just like the unstrained control plate. The values measured after 1 day (43% lower than control, p-value = 0.023) and 3 days (55% lower than

control, p-value < 0.001) of fluid shear exposure were significantly lower than the relative values on the unstrained control. However, by day 6 the relative DNA numbers between the two controls were not significantly different (p-value = 0.177).

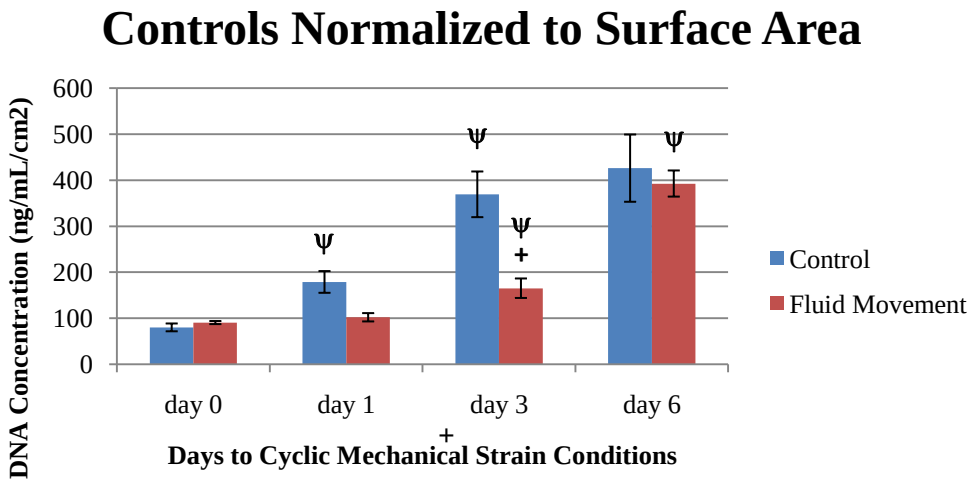

Figure 4. W-20-17 Fluid Movement DNA Quantification Normalized to Surface Area. ($p < 0.05$) + indicates significant difference from the control within that time point. ψ indicates significant difference between a value and the previous day's value of the same treatment group. n=4

Total Protein

Total cellular protein concentrations from the Pierce BCA Protein Assay kit were similar to DNA concentrations (Fig. 5). Again significant differences were found to exist over time (p-value <0.001). Differences in the mean values among the different strain treatment groups, on the other hand, were not great enough to exclude the possibility that the differences might be just due to random

sampling variability (p-value = 0.242). Likewise there was no significant interaction between time and the different treatment groups (p-value = 0.082). Following the same pattern as DNA concentration total protein values of all treatments groups had significantly larger recorded values on day 6 than day 0. Measurements for all treatments groups were very similar within each experimental time point with the only significant difference existing between total protein measurements taken on samples collected on the same day for continuous compression and intermittent compression on day 6 (continuous compression 36% larger than intermittent compression, p-value = 0.005).

Total protein concentrations were plotted versus DNA concentrations (Figure 6) to see how closely the two measurements tracked one another. The plots are nearly linear suggesting a strong correlation between the two quantities. The strongest correlation existed between intermittent compression ($R^2 = 9.48$) while continuous compression had the weakest correlation ($R^2 = 0.8719$).

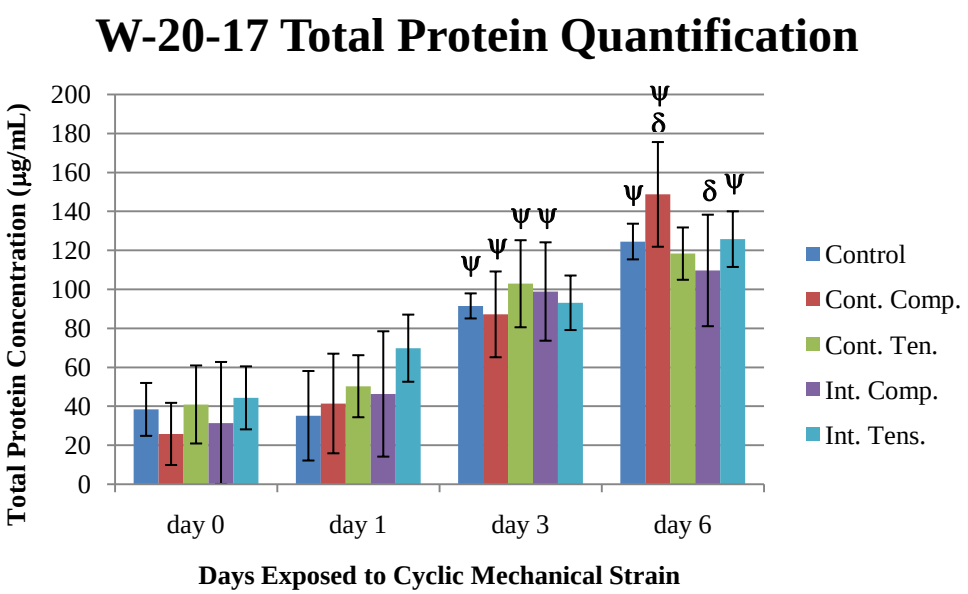

Figure 5. W-20-17 Total Protein Quantification. ($p < 0.05$) Measurements are significantly larger than prospective day 0 measurements for all groups after day 3 of culture. δ indicates that the sample is significantly different from the other marked sample(s). ψ indicates significant difference between a value and the previous day's value of the same treatment group. n=3

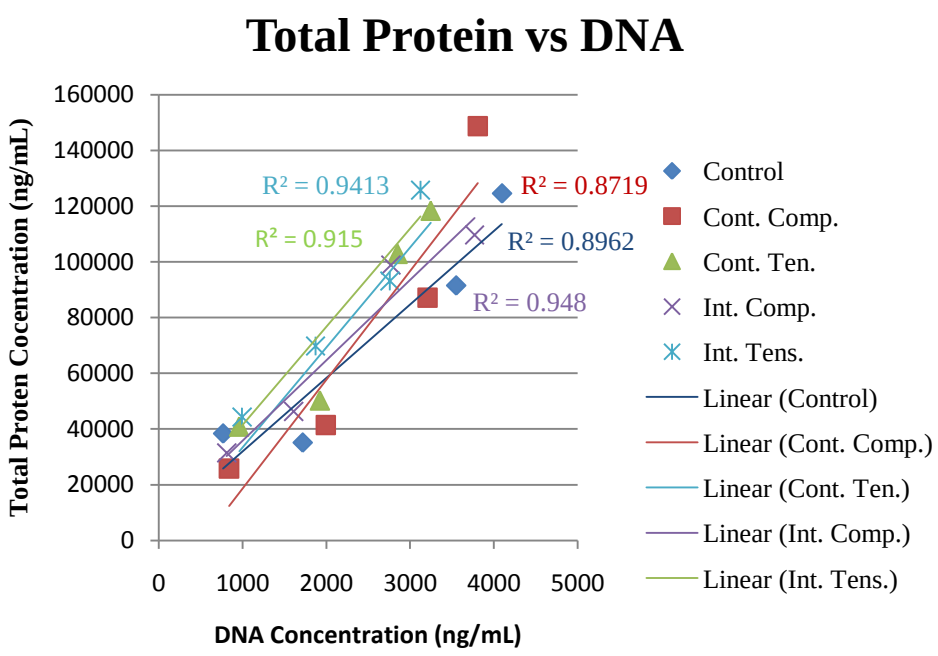

Figure 6. W-20-17 Total Protein V.S. DNA

Alkaline Phosphatase Activity

ALP concentrations were normalized to DNA showcasing the relative amounts of ALP per cell (Fig. 7). As expected, culture times were a major factor in ALP enzyme concentrations (p-value < 0.001). Significant differences (p-value < 0.001) in normalized ALP concentrations also existed between different strain treatment groups. Like measured DNA concentrations, there was a significant interaction (p-value < 0.001) between time and mechanical strain conditions applied to the cells, suggesting that the changes in ALP concentrations per cell were also dependent in part to the type of strain experienced. There were no significant differences within time points over the first three days but there was a

significant decrease in the ALP concentrations expressed by all treatment groups from day 0 to day 1 ($0.007 \leq$ p-values ≥ 0.017) followed by an increase in activity from day 1 to 3. Despite the increasing trend, ALP concentrations failed to be statistically between 1 day and 3 days of straining except for cells that were subjected to continuous tension (63% increase, p-value = 0.047) and intermittent compression (71% increase, p-value = 0.013). The increasing ALP tend continued so that all of the day 6 measurements were significantly higher than their day 1 values for each type of strain ($0.009 \leq$ p-values ≥ 0.010).

All strain groups exhibited significantly different ALP concentrations than the control on day 6, reinforcing the additive effect tensile and compressive strains have on the differentiation of preosteoblasts. Intermittent compression had a larger average normalized ALP measurement than the control (22% larger average) but the measurement was not significant with a p-value = 0.072. . The largest measurements were recorded for the samples strained under intermittent tension (92% increase over control, p-value = 0.005) followed by those exposed to continuous tension (68% increase over control, p-value = 0.006) although continuous tension was not statistically larger than continuous compression group (p-value = 0.094) which was also larger than the control (31% larger than control, p-value = 0.010).

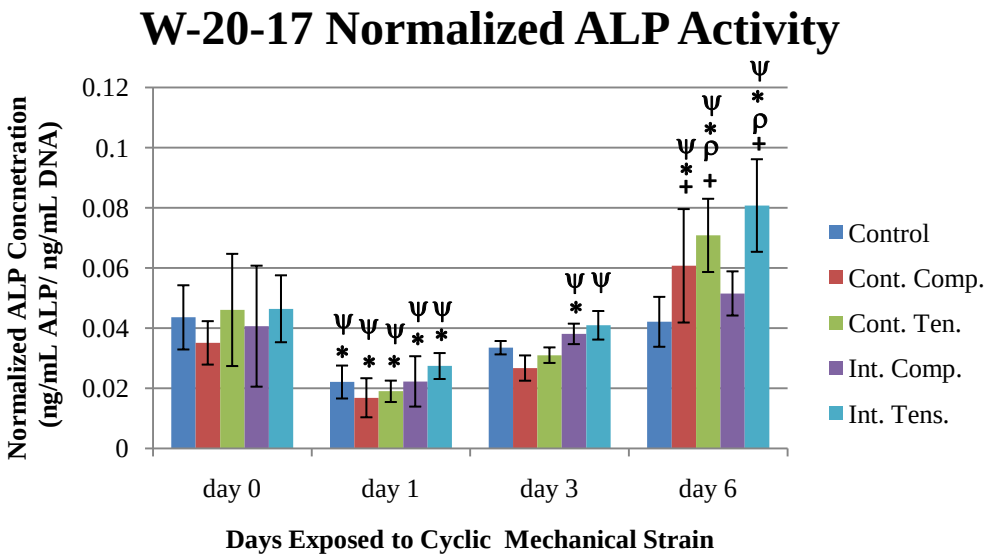

Figure 7. W-20-17 Normalized ALP Activity. ($p < 0.05$) + indicates significant difference from the control at that time point. ρ indicates that the sample is significantly different from the intermittent compression group at that time point. * indicates significant differences from the day 0 values of the same treatment group. ψ indicates significant difference between a value and the previous day's value of the same treatment group. n=3

Discussion

For a load bearing implant to be clinically successful, osseointegration is crucial. Several studies have shown that applying physiological mechanical loads early after implantation can improve osseointegration at the bone-implant interface.[82, 83] However, overloading may lead to bone resorption.[(84)] With non-optimized mechanical loading, a weaker layer of fibrous tissue can form between the bone and the implant surface. The fibrous tissue layer and poor osseointegration can cause micromotions which can start the process of implant loosening and failure.[81] Gaining an enhanced understanding of the cellular

biomechanical relationships at an implant surface will impact to optimization of osseointegration and the clinical performances of orthopedic and dental implants.

The cell straining parameters employed for this study were selected to maximize the effects related to osseointegration as seen in previously published studies.[4,5,12,89,91,93] First, 800με was chosen as the strain magnitude across all strained treatment groups because it is the largest strain that can be applied uniformly and consistently in both compression and tension by the currently used system. Physiological strains during human locomotion have been measured between 400με and 3000με, so 800με is a clinically relevant magnitude.[12] Many distinguished works implement 1000με as a physiological magnitude for in vitro studies.[4,3,89]

The loading duration and frequency were chosen for similar reasons. Kaspar et al. strained human derived bone cells in tension at 1Hz for 1800 cycles on silicone dishes by 4-point-bending and measured an over 100% increase in cells number versus unstrained controls. However, straining cells under the same conditions for 3600 cycles only resulted in cell numbers 40% higher than the control demonstrating that bone cells may be overloaded or over mechanically stimulated.[5] 1800 cycles at 1Hz translates into 30 minutes of loading, the daily dose for all treatment groups in this experiment. Kaspar et al. also reported even higher cell numbers after straining the human derived bone cells at 10Hz, but this is not a physiological applicable rate. 1Hz, on the other hand, is typically thought of as the gate frequency and is a good frequency for relating this study to the interactions at the bone-implant interface.[(5)]

Under the chosen straining parameters, the W-20-17 cells demonstrated dose dependent increases in proliferation over the 6 days of this experiment with stable total protein production. Proliferation was lower for cells subjected to daily tensile strains as compared to the non-strained controls or compressive strains, which had no effect on proliferation. ALP concentrations, on the other hand, were enhanced by both tensile and compressive strains. The lower proliferation of the cells subjected to tensile strains was associated with the most significantly increased levels of ALP expression as compared to cells subjected to compressive strains or non-strained controls. These data suggest that the tensile and compressive strains applied in this study stimulated cell differentiation, but the tensile strains promoted differentiation more strongly than the applied compressive strains. These results are different from previous studies which implemented different straining parameters and have generally reported that cell proliferation was enhanced by tensile strains and ALP expression reduced.[4, 93]

One such study by Kaspar et al. documented similar results with a 30% increase in the proliferation of human derived osteoblasts in response to a single dose of 1800 cycles of 1000με at 1Hz, but also recorded a 10% decrease in osteocalcin synthesis and a 20% decrease ALP activity after 48 hours of culture.[89] Winter et al. reported similar trends eight days after straining rat bone marrow cells.[4] It is important to note that these works of Winter et al. and Kaspar et al. apply mechanical strain to the cells only one time and then followed the cells for several days without additional mechanical strain.[4,89] In agreement with the W-20-17 cells' responses from this study, Di Palma et al. reported an increase of

ALP expression after subjecting osteoblastic cells growing on ceramic implant surfaces to 600με of tension at 0.25Hz for up to five consecutive and the same trends again on titanium.[90, 91] Our results coupled with the two studies from Di Palma et al. may suggest that responses of cells to daily loading events may be more reflective of the clinical situation since real loading rarely occur only once.[90, 91] It may be that consecutive days of straining modulate cell behavior differently than a onetime application of cyclic mechanical strain. Using the in vivo turkey ulna model, Lanyon et al. compared cyclic to static loading concluding that cyclic loading led to significantly larger bone cross-sectional area suggesting that loading bones repeatedly can result in larger impacts on the remodeling process.[8]

Also Di Palma's strain magnitude at 600με was below the 1000με used by Kasper et al. and Winter et al. as was the strain magnitude for in this study. Interestingly, Ushida et al. strained rat bone marrow cells at 18000με for four days and recorded an increase ~17% in cell number and an increase of ~50% in ALP concentration by day 4.[92] This collaborative increase in cell number and ALP in response to mechanical strain differs from all of the studies examined so far. Thus, strain magnitude coupled with frequency and duration may be responsible for prompting cells to proliferate or differentiate. The variation in results of studies applying different strain magnitudes is consistent with the mechanostat theory proposed by Frost in which critical combinations of strain magnitude and strain cycles are important to the bone remodeling processes.[10] Frost defined this range as being between 50με and 1500με, which could change

due to disease and other physiological situations. Being exposed to strains outside of these thresholds will drive the process of resorption and new bone formation at specific sites until the experienced strains are once again within expected boundaries.[58] Perhaps the cells in this study and those strained by Di Palma et al. are strained to the specific threshold where they begin to differentiate whereas those strained by Kasper et al. and Winter et al. reached the threshold promoting proliferation over differentiation and Ushida et al. strained enough to promote both.[4,5,90, 91,92]

Studies also report increased responses after straining cells intermittently rather than continuously. For example, Winter et al. strained harvested rat stromal cells on an elastomeric substrate under 1000με intermittent and continuous tensile strain cycles for 1 hour and followed the cellular responses for 28 days without loading to test the "trigger-like" response theory of Brand and Stafford.[4,11] DNA values from Winter et al. values were significantly higher for intermittently strained cells on days 4 and 8 than the respective continuously strained and unloaded treatment groups at the same time points. Winter et al. also reported lower ALP concentration than the controls after straining with continuous and intermittent loading schemes.[4] However, our W-20-17 cells were only strained at 800με for 30 minutes a day with a different cell substrate. Following the study over more time points and having more replicates may manifest a difference between intermittent and continuous loading. However, it is also possible that Winter et al. was overloading his cells with 1 hour of continuous loading where as the 30 minutes of this study did not.[4]

Cyclic forces in either tension or compression have been reported to induce modeling and growth changes to bone in vivo.[93] Such was the case for this study; both tensile and compressive strains resulted in increased ALP activity per cell. It has also been reported in vivo that bone tends to remodeling to limit tensile strains.[94] In fact, clinicians have reported that a bones set improperly at an angle after a bad fracture may straighten over time by adjusting the amounts of mineralized matrix material on either side and bones exposed to excessive loads will be larger to reduce the associated stresses.[95, 96] Interestingly this in vitro study follows the same trend, tensile strains resulted in the lowest proliferation after day 6 but the highest normalized ALP concentrations. The data suggest that after being exposed to tensile strains for six days the osteoblast precursors are differentiating into osteoblast rather than proliferation to lay down more bone matrix and reduce experience tensile strains. Additional studies measuring calcium levels and matrix proteins would have to be conducted to evaluate if the daily straining protocols implemented in this study are indeed increasing the amount of mineralized matrix. If so the cyclic mechanical straining would indeed be inducing osseogenesis at the bone-implant interface.

Lastly the fluid movement inside of the wells utilized by the cell strain device did seem to have an effect on proliferation, but by day 6 the control and the fluid shear control were not statistically different. The lysates from the fluid movement control tiles were analyzed for total protein and ALP content using the same methods as the larger substrate plates. However, none of the samples had any significant concentrations of total protein or ALP. It is possible that cells

were non-responsive to the limited fluid shear in these areas, but is also possible that the concentrations of the collected samples were simply below the resolution of the two assays. Again additional time points with more replicates would have to be performed to make any definitive conclusions confirming or denying impact of the fluid movement in the cell straining system.

Conclusion

From this work it appears that both tensile and compressive cyclic mechanical straining on titanium cause W-20-17 cells to proliferate continually but not at elevated levels while promoting increased ALP enzymatic activity (31-92% more that the control). Intermittent loading did not appear to cause significantly different reactions from continuous loading overall. It is likely that the cells were not strained beyond either the magnitude or the cycle threshold at which intermittent straining is effective. Both tensile and compressive strains promoted differentiation of the preosteoblasts, but tensile strains did so more strongly. Tensile straining resulted in the highest average normalize ALP concentrations (68-92% larger than control and 17-33% larger than continuous compression) and the lowest DNA concentrations (20-22% lower than control) suggesting that the preosteoblastic W-20-17 cells may be differentiating rather than proliferating to lay down more mineralized matrix proteins and reduce the experienced tensile strains according to Wolff's law. It may be possible to harness this information to optimize osseointegration on load bearing orthopedic and dental implants.

CHAPTER 4

FUTURE WORK

OSTEOCLAST CELL EXPOSURE TO CONDTIONED MEDIA

RAW 264.7 (TIB-71, ATCC, Manassas VA) is the cell line that will be used for future experiments. Unlike the SAOS2 line, RAW 264.7 is a murine monocyte line. These cells are important because they can differentiate into osteoclasts in the presence of recombinant receptor activator of NF-jB ligand (RANKL).[97-103] The W-20-17 cells that were mechanically strained as described in the manuscript should have released numerous proteins and growth factors into their culture media throughout the course of the experiment. We hypothesize that the conditioned media will inhibit tartarate resistant acid phosphatase (TRAP) activity in RAW 264.7 cultures. The conditioned media will be warmed to room temperature and diluted 1:1 with fresh growth media both with and without RANKL. The RANKL positive media will be supplemented with 200 ng/mL RANKL (mouse recombinant RANKL expressed in E. coli, Sigma-Aldrich, St. Louis, MO) to achieve a final concentration of 100 ng/mL when mixed with the conditioned media. A total of 52 different media types will result from these mixtures (Table 2).

Table 2. Types of Conditioned Media

Loading Scheme	*Days of Loading*	*RANKL Concentration (ng/mL)*
Control	*0, 1, 3, 6*	*100*
	0, 1, 3, 6	*0*
Continuous Tension	*0, 1, 3, 6*	*100*
	0, 1, 3, 6	*0*
Continuous Compression	*0, 1, 3, 6*	*100*
	0, 1, 3, 6	*0*
Intermediate Tension	*0, 1, 3, 6*	*100*
	0, 1, 3, 6	*0*
Intermediate Compression	*0, 1, 3, 6*	*100*
	0, 1, 3, 6	*0*
Fluid Shear	*0, 1, 3, 6*	*100*
	0, 1, 3, 6	*0*
Mineralizing Media	*N/A*	*100*
	N/A	*0*
Growth Media	*N/A*	*100*
	N/A	*0*

RAW 264.7 cells will be seeded into a 96 well microplate at a concentration of $1x10^5$ cells/cm^2 with the media types summarized in the table above, as well as RANKL positive and negative mineralization and growth media controls. The two controls should account for any responses due to the FBS or additional supplements in the media. The cells will be allowed to proliferate for four days under these conditions before being assayed for tartarate resistant acid phosphatase (TRAP).

TRAP is a major marker of osteoclastic character. A TRAP K-Assay kit (TRAP Staining Kit, Cat. No. KT-008, Kamiya Biomedical Co., Seattle, WA) has

been purchased for its ability to quantitatively assess TRAP concentrations from osteoclastic cell lines. Following the instructions suggested by the kit, aliquots of the four day old culture supernatants will be moved to a new microplate and mixed with a chromogenic reagent and a buffer containing tartrate. The mixture will then be assessed with a spectrophotometer at 540nm.

Unfortunately the K-Assay kit could not be delivered in time to be included as part of the results for this project, but the procedure will be completed soon after this thesis has been defended. Also the manuscript will be rewritten to include the new data. If the results are positive and additional reagents are still available in the kit then the procedure may be repeated using the conditioned media from the strained SAOS-2 cells as well.

ADDITIONAL STUDIES

Although there are many cell straining studies available in the literature, it is often difficult to compare one study to another. However, many of the protocols utilized in other mechanical straining studies could be explored using the cell straining system used for this project. The simplest and possibly the most noteworthy follow up for this research would be to use additional cell lines. There are several additional osteoblastic and stromal cell lines available which could provide a wealth of comparable data. However, the most interesting cell lines to explore would be osteocytes and mesenchymal stem cells. Straining osteocytes with this 4-point-bend system could lead to definitively identifying the

primary bone mechanosensor that have been much sought after. Another option would be to continue using the cell lines examined for this project but change the loading schemes. As mentioned several times above, the responses of bone to mechanical loading is dependent on the substrate, magnitude of the applied strain, frequency of loading, and the duration that the load is applied.[3-14] Altering any of these factors can change the experimental findings and provide additional useful data. The difficult task lies in deciding which parameters to choose for a given experiment. There are also many other important bone formation markers such as runx, osteocalcin, collagen, osteopontin, and many others. These proteins and markers could be analyzed using the same experimental parameters used in this study to expand the available data. Lastly the commercially pure titanium substrates could be covered with various surface coatings. Applying a polymeric surface coating, such as chitosan or another hydrogel, to the titanium would be clinically relevant and relatively simple to do. Difficulties might arise in ensuring uniform strain transitions across the coatings, but these additional variations of this research could be an interesting problem to investigate.

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APPENDICES

ADDITIONAL INFORMATION ABOUT THE CELL STRAIN SYSTEM

A four-point bending system has been designed to administer controlled cyclic mechanical strains to bone cells cultured on titanium. The system is based on initial design by Winter et al.[4] and incorporates improvements made by Brandon Allen.[41] Currently the system uses a pneumatic linear actuator for precise strain generation, accommodates adjustable loading arms allowing for tensile or compressive strains, and allows for a larger surface growth area than the original design.[41]

The system is controlled by a custom LabVIEW (Version 8.0, National Instruments, Austin, TX) computer program, also developed by Brandon Allen.[41] This program functions by generating an analog voltage wave form and outputting this signal through a data acquisition card (PCI-6024E, National Instruments, Austin, TX) to a Bimba Pneumatic Control System (PCS-1, Bimba Manufacturing Company, Monee, IL). The LabVIEW program allows the user to specify the type of waveform, frequency, and amplitude, so the system is highly adaptable for different experimental protocols. A compressed air tank emitting a pressure of 80 psi drives a Bimba Position Feed Cylinder (Model PFC-092-XL, Bimba Manufacturing Company, Monee, IL), which serves as the linear actuator of the system and is regulated by the PCS.

The base of the device is composed of aluminum measuring 46 cm x 29 cm x 38 cm. The linear actuator is fixed vertically at the top of this base and connected to an aluminum block. An aluminum rod runs through the block perpendicular to the actuator to create an adjustable track for the two loading

arms. Both aluminum loading arms are suspended vertically from the rod on either side of the actuator and can be positioned anywhere along the tracking rod. Horizontal struts fixed to the ends of each loading arm provide the first two points of contact for 4-point bending. Lastly, two vertical support struts provide the final two points of contact from the bottom. Like the loading arms, the positions of the bottom support struts are adjustable. The entire system is designed to fit inside of an incubator (Model 3158, Forma Scientific, Marietta OH) to accommodate experiments with long durations of loading. All of the components associated with the system can be seen below in Figure 8.

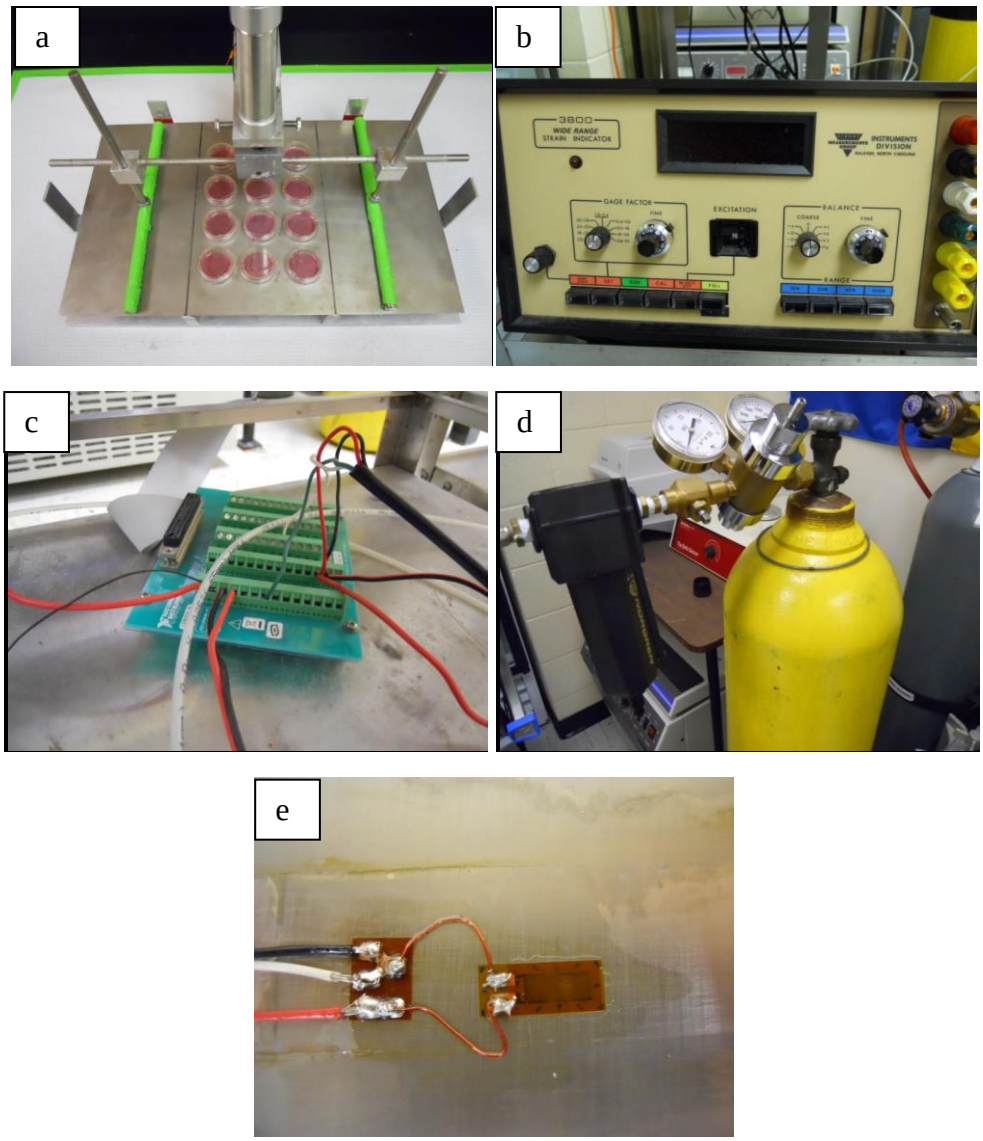

Figure 8. Cell Strain Device Components: (a) device fitted with a titanium plate (b) wide range strain indicator (c) DAQ card (d) air tank with regulator (e) strain gage mounted on titanium plate

Commercially pure titanium (cp Ti grade 2, Titanium Industries, Rockaway, NJ) serves as the cell substrate of the system. A 0.114cm thick titanium sheet was cut into 41.91 x 25.4 cm rectangular plates. When a plate is

deflected, one side of the plate experiences compressive shear strain while the other undergoes tensile shear strain. Adjusting the positions of the upper loading arms and lower support struts, allows one to control which sides of plate experience which types of shear strain, as shown in Figure 9. For the duration of these experiments the loading arms were positioned so that the upper struts were always 25 cm apart. The lower support struts were positioned so that they were either 14 cm apart for tensile strain or 34 cm apart to induce compressive strain.

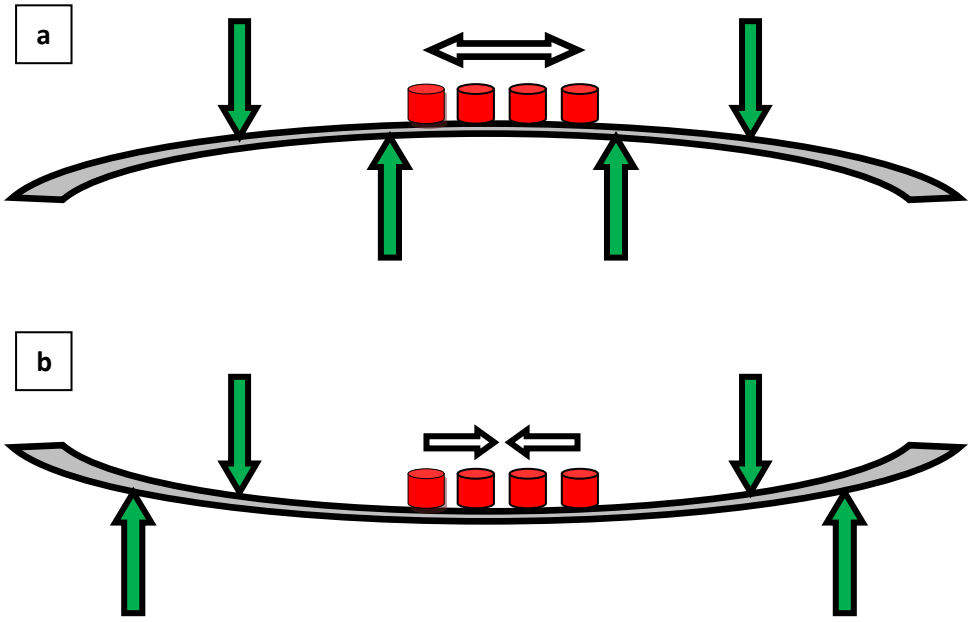

Figure 9. Cell Strain Device Orientations: (a) Ti plate shown oriented to apply uniaxial tension shear strain to the top culture surface. (b) Ti plate oriented to apply uniaxial compressive shear

SILICONE TOXICITY

Early tests used a clear GE window and door sealant (Silicone I GE012A, GE Sealants & Adhesives, Huntersville, NC) to attach the culture wells to the titanium substrate without incident. However, during multiple tests the DNA concentrations suddenly began to decrease sharply. Unfortunately, it took multiple failed tests to realize that GE made a small change in the composition of their sealant, which resulted in increased toxicity to cell cultures.

To investigate the problem SAOS-2 cells were seeded onto GE silicone, tissue culture plastic (TCP), and a silicone sealant used for aquariums (100% Silicone Aquarium Sealant, All-Glass® Aquarium Co., Inc., Franklin, WI). The cells were allowed to grow for 24hrs and were then examined under a microscope at 40x and 100x. The GE silicone resulted in complete cell death after just 24hrs. Cell on the aquarium sealant, on the other hand, had very high viability and continue to proliferate similarly to cells on TCP. Sample photos of the test results can be seen below in Figure 10.

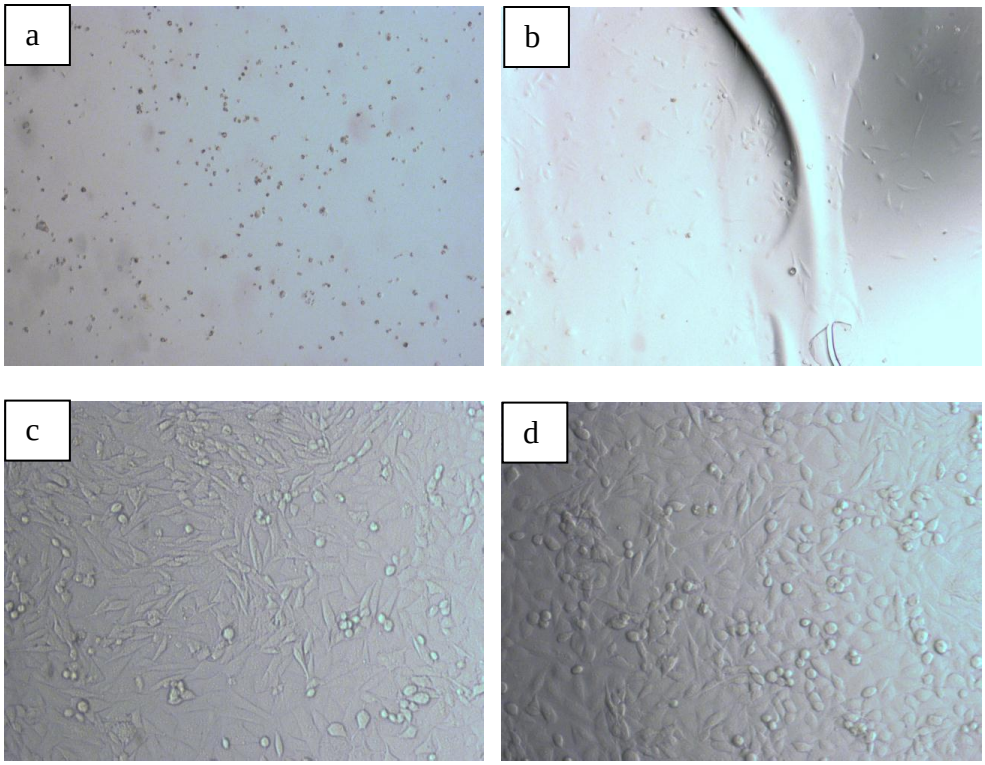

Figure 10. Silicone Sealant Toxicity of SAOS-2 Cells: (a) 24hrs on GE silicone 40x (b) 24hrs on aquarium silicone 40x (c) 10 days on TCP 100x (d) 10 days on aquarium silicone 100x

PRELIMINARY CELL STRAIN DATA

Three commercially pure (cp) titanium plates were wet ground with SiC sandpaper up to 1200 grit and then thoroughly cleaned with distilled water and isopropanol. SonicSeal® slide wells were attached to the plates with a biocompatible silicone rubber sealant. SAOS2 cells were then seeded into each well at a density of $5x10^4$ cells/cm^2 in growth media (McCoys 5A® supplemented with 10% FBS and 1% antibiotic/ antimyocotic). After 24hrs the growth media was switched to mineralizing media (McCoys 5A® supplemented with 10% FBS, 1% antibiotic/ antimyocotic, 50mg/ml ascorbic acid, and 10mM β-glycerophosphate).

The Ti plates were then subjected to cyclic strains of 900με at a rate of 1Hz for 30minutes a day for 7 days with a custom built pneumatically controlled 4-point bend machine. One plate was strained under tension, another was strained under compression, and the third plate was not strained and served as the control. Since testing was done outside of the incubator, the control plate was set aside on a bench top for 30 minutes a day to compensate for atmospheric conditions. Samples were lysed with RNAase free water after 0, 3, 5, and 7 days of loading.

Cell proliferation was assessed by DNA quantification of the lysates using a Picogreen® assay kit. Alkaline phosphatase (ALP) levels, a commonly measured marker of osteoblastic mineralization, were also measured on the lysates with an assay kit. The ALP concentrations were normalized to DNA for analysis.

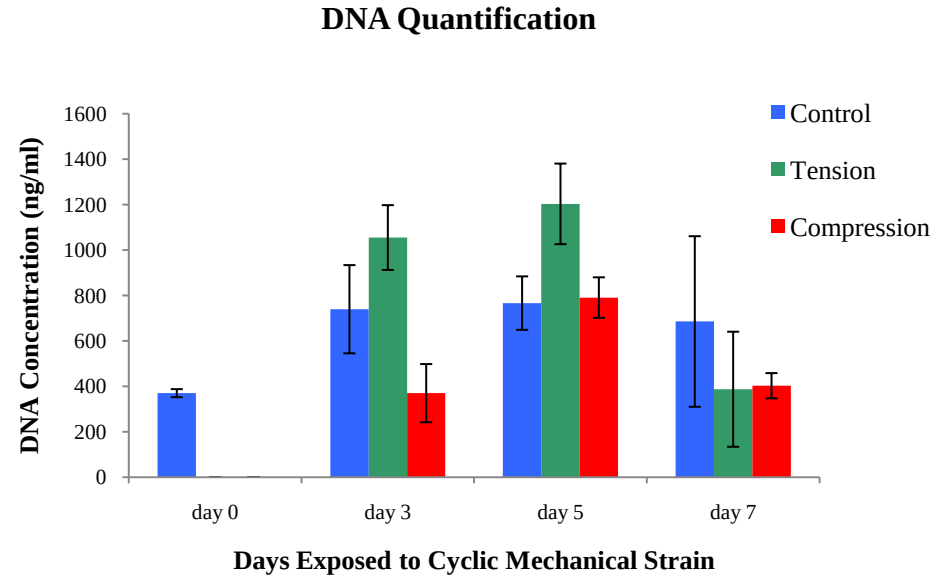

Figure 11. Pilot DNA Quantification

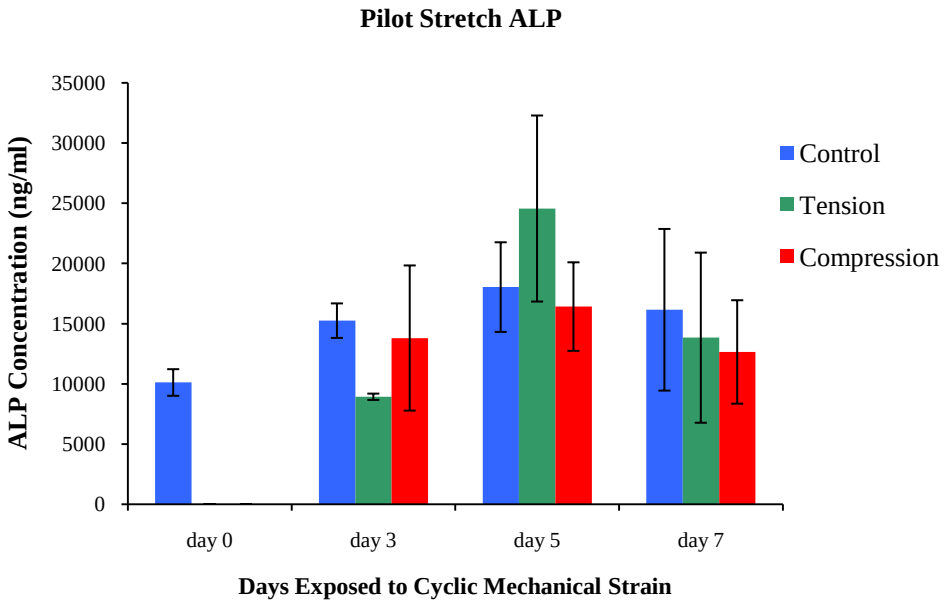

Figure 12. Pilot ALP Activity

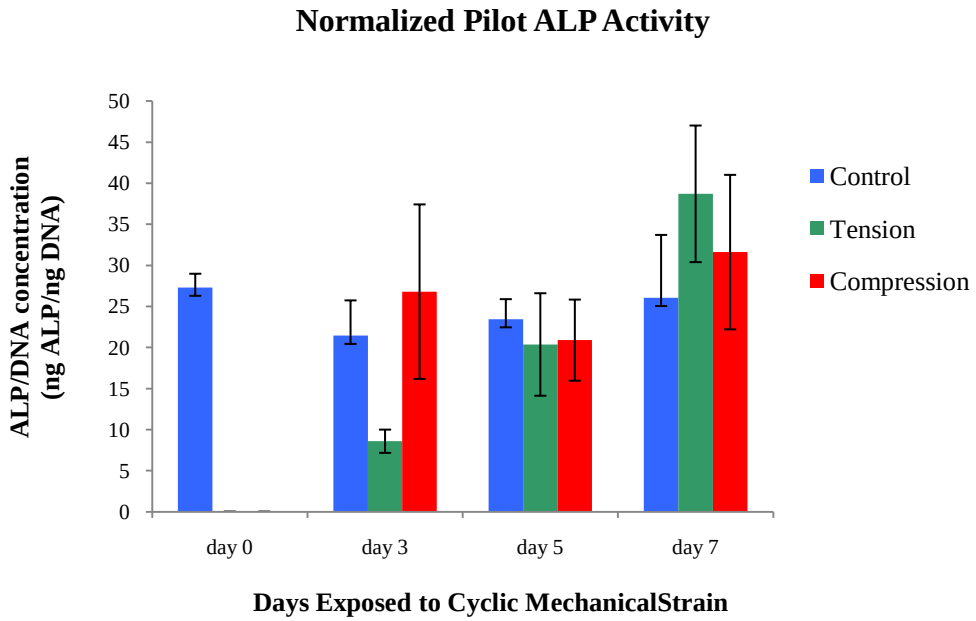

Figure 13. Normalized Pilot ALP Activity

The SAOS-2 Cells responded differently to cyclic tensile and compressive strains over the 7 days. According to DNA quantification, tensile strains resulted in higher cell proliferation than the control after days 3 and 5 days of loading. Compressive loading resulted in maximum DNA concentrations at day 3, but never reached higher values than those of the control plate. Both loading conditions showed low DNA concentrations at day 7. ALP concentrations increased incrementally with additional days of cyclic tensile strain. The cells on the control plate and those subjected to compressive strains, on the other hand, resulted in relatively constant ALP expression.

Tensile and compressive cyclic strains did seem to affect osteoblast-like cells differently. The data suggest that tensile strains promoted cell proliferation whereas compressive strains had less effect on cell number. Previous studies

have shown similar trends in DNA and ALP expression after cyclic tensile straining, but upon further investigation it was discovered that the tensile and compressive strains were not equal. Compressive strains in the study were 200-300με lower than the applied tensile strains which may account for the large differences in the data. But the basic premises and lessons learned from behind this pilot study were carried into the main experiments presented in this thesis.

SAOS-2 TENSION AND COMPRESSSION FOLLOWUP

Five titanium plates were prepared exactly as described in the journal article to create culture areas for testing osteoblast-like cells. The osteoblast-like cell line used was the Saos-2 line (HTB-85, ATCC, Manassas, VA), a human osteosarcoma line that is commonly implemented to model the mineralizing behavior of osteoblasts. These cells are easy to culture and can lay down a lot of bone matrix under the proper conditions.[104, 105] The goal of this study was to have a comparative cell line to go along with the W-20-17 cells in the manuscript.

The Saos-2 cells were cultured using the same protocol as the W-20-17 cells, using the same growth media. Upon confluence the Saos2 cells were seeded into the culture well of 5 titanium plates at a concentration of $5x10^4$ cells/cm^2 in Saos2 mineralizing media, W-20-17 mineralizing media but did not require the addition of dexamethasome. The Saos-2 cells were strained following the exact same protocol as the W-20-17 cells. However, these osteoblast-like cells did not proliferate on the titanium nearly as much as the W-20-17 cells.

Baseline measurements taken on day 0 for both cell types were very similar, but the Saos-2s did not continue to proliferate at the same rate as the W-20-17 cells.

From Figure 14 only the samples exposed to intermittent tension (p-value = 0.009) and the control plate (p-value = 0.013) were significantly different between their respective day 0 and day 6 values. In fact, measurements from the cells exposed to continuous tension never differed significantly from the initial baseline measurements. Also there were no persistent trends of increasing or decreasing DNA concentrations over time. In fact, samples from the continuous compression (p-values = 0.009, 0.010) and intermittent compression (p-values = 0.013, 0.009) groups had significantly lower DNA levels on day 3 than day 0, yet showed significant increases between days 3 and 6. Lysates sampled from the continuous compression group had the lowest measured DNA concentrations at the end of the experiment, which were significantly lower than all other treatment groups except continuous tension (p-value = 0.007).

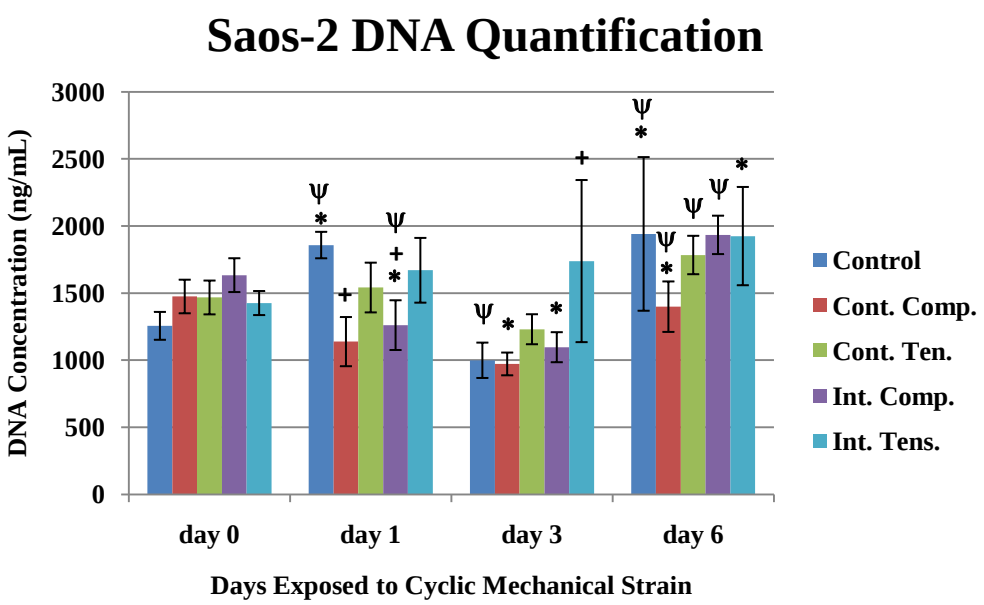

Figure 14. Saos-2 DNA Quantification. ($p < 0.05$) * indicates significant differences from the day 0 values of the same treatment group. + indicates significant difference from the control at that time point. ψ indicates significant difference between a value and the previous value of the same treatment group. n=3

The total protein trends of the Saos-2 cells in Figure 15 were not as obvious as the W-20-17 cells. However, the difference between the control group and the strained treatment groups were very obvious. In fact, most of the protein measurements from the strained plates were significantly lower than the control values which steadily increased with increasing days of culture. Day 1of the experiment, where only the intermittent tension samples were significantly different, was the only exception to the trend of significantly different control values (p-value = 0.007). The odd thing about the large differences between the

unstrained control and the four strained treatment groups was that the differences appeared even in the baseline measurements before any loads were applied to any of the plates. Also there were not significant differences between any of the strain plates at a given time point. Lastly, it should be noted that all treatments groups other than intermittent tension yielded significantly higher recorded values on day 6 than day 0 (p-value = 0.529). Also the total protein concentrations measured from all Saos-2 treatment groups other than continuous compression and intermittent tension were significantly higher after loading than their baseline values by day 3. Total protein levels did not track the DNA concentrations well, as seen in Figure 16 making it very difficult to ascertain what the cells were doing.

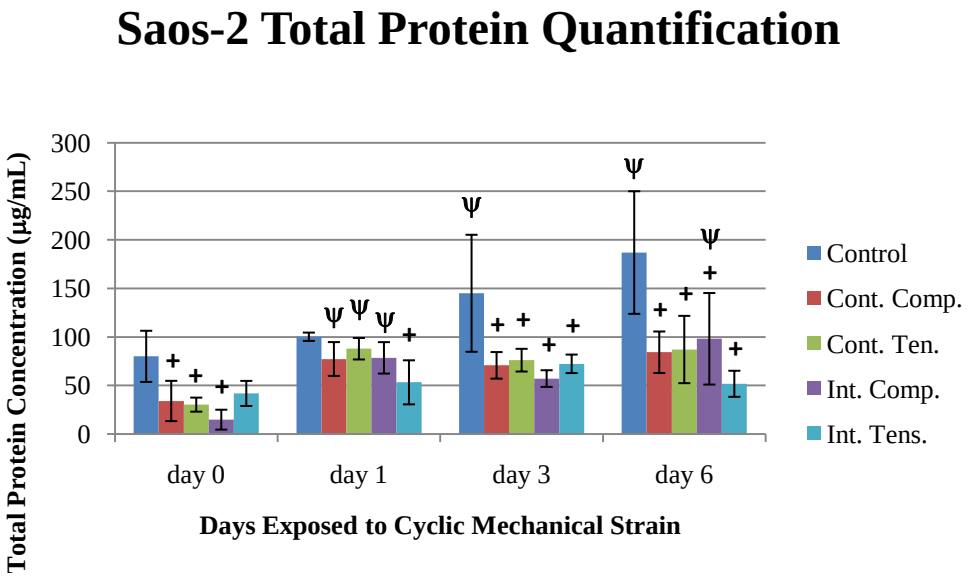

Figure 15. Saos-2 Total Protein Quantification. ($p < 0.05$) + indicates significant difference from the control at that time point. ψ indicates significant difference between a value and the previous value of the same treatment group. n=3

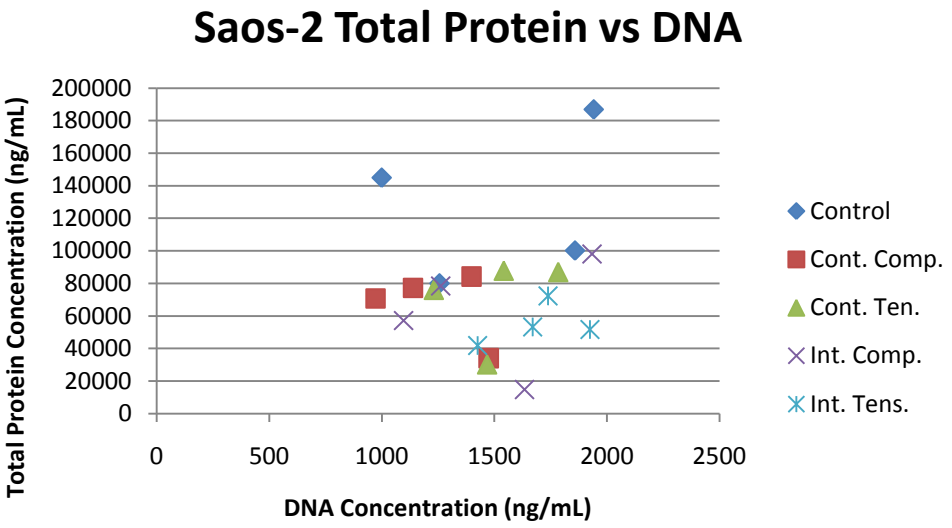

Figure 16. Saos-2 Total Protein V.S. DNA Concentration

Interestingly the same pattern was seen in the Saos-2 cells' normalized ALP concentrations on day 6, shown in Figure 9. All of the values of the strained day 6 groups had higher averages than the controls and groups exposed to tensile strains were larger than those exposed to compressive strains. However, there was larger variation within samples and not all of the differences were significant. In fact, the only significant differences within the same time point were between continuous compressive strain and the control and continuous compression and continuous tension. Within each group the Saos-2 normalized ALP concentrations increased almost linearly with increasing days of culture. This increasing trend included the measurements from the control samples suggesting that Soas-2 cells, unlike W-20-17, will mineralize without any additional stimulation other than the chemicals supplementing the mineralizing media.

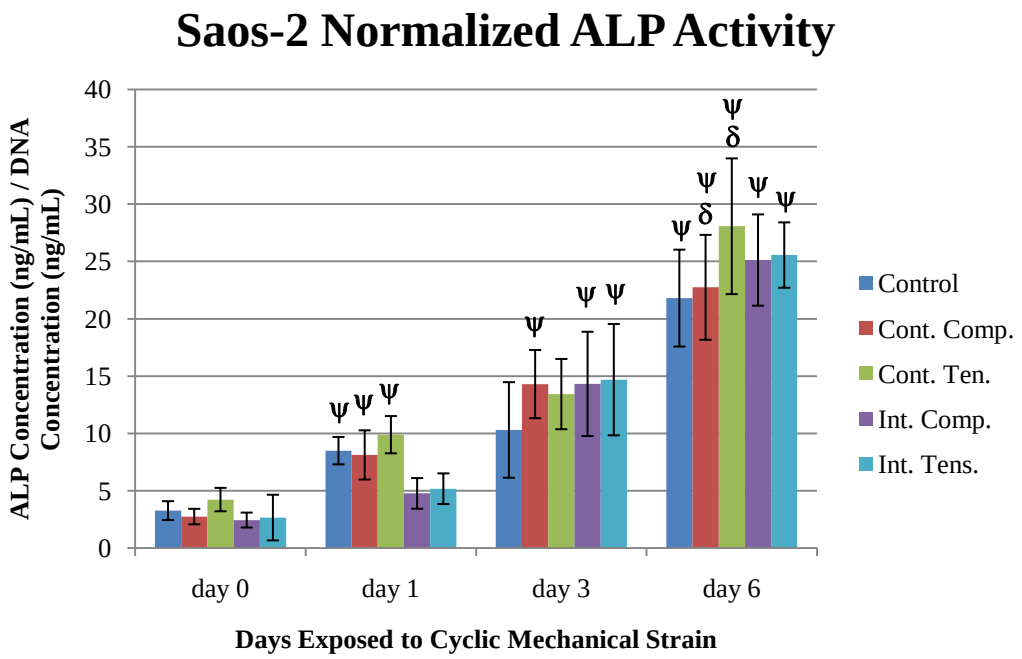

Figure 17. Saos-2 Normalized ALP Activity. ($p < 0.05$) + indicates significant difference from the control at that time point. δ indicates that the sample is significantly different from the other marked sample(s). ψ indicates significant difference between a value and the previous value of the same treatment group. n=3

In summary the Saos-2 osteosarcoma cells showed few significant changes in DNA values between the end and the beginning of the experiment and had much lower total protein production than the unloaded control. However, ALP activity steadily increased over the course of the experiment and all average strain measurements were higher than the control, even though only the measurements for continuous tension were significant. The experimental results of this project suggest that osteoblast-like cells exposed to cyclic mechanical strains on titanium are prone to mineralize more than proliferate. Kaspar et al. noted opposite findings in osteoblasts-like cell lines. Reporting increased

proliferation and collagen type 1 caboxyterminal propeptide (CICP) after mechanical strain and decreased mineralization markers such as ALP.[94] They strained their cells with tension on collagen conditioned silicone elastomer at 1000με for 1800 cycles at 1Hz but only ran their culture out to 48hr so the results may not be comparable. This combined with that fact that the DNA and total protein concentrations do not track together makes us feel inclined to repeat this experiment before trying to include it in any manuscripts. The addition a fluid shear controls may also be a worthy endeavor.