IMPACT OF MECHANICALLY-INDUCED MICRODAMAGE AND GAP JUNCTIONAL INTERCELLULAR COMMUNICATION ON MLO-Y4 VIABILITY AND SCLEROSTIN EXPRESSION

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IMPACT OF MECHANICALLY-INDUCED MICRODAMAGE AND GAP JUNCTIONAL INTERCELLULAR COMMUNICATION ON MLO-Y4 VIABILITY AND SCLEROSTIN EXPRESSION

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ABSTRACT

Bone is continually undergoing remodeling, in which old bone is resorbed by osteoclasts and new bone is laid down by osteoblasts. This process provides a mechanism for bone to respond to changes in the mechanical environment. While the role of osteoblasts and osteoclasts in bone remodeling is clear, the cellular control of the process remains unknown. One theory is that osteocytes, found in the interior of bone, plays an important role in this process. Osteocytes may play a key role as they: possess physical processes, gap junctions, which allow communication between nearby osteocytes; they heavily express proteins integral to bone remodeling such as sclerostin; and, they are located inside of bone for detecting changes in mechanical load. In this work, MLO-Y4 cells, an osteocyte-like cell line, were cultured in vitro and exposed to mechanical load. Microdamage was induced in these cells using a mechanical loading platform and verified using lactate dehydrogenase activity. Further work with MLO-Y4 cells demonstrated that sclerostin activity increased at low surface strains, and that inhibition of gap junctional-mediated intercellular communication in mechanically-loaded osteocytes led to an increase in expression at higher strains, with a statistically significant difference from when communication was present. This suggested that osteocyte sclerostin expression was mediated by strain and gap junctional intercellular communication.
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CHAPTER I
INTRODUCTION

While bone remodeling is normally coupled between resorption and formation, this balance can be disrupted by changes in mechanical environment or metabolic bone diseases such as osteoporosis. Osteoporosis is a disease in which bone loses density, leading to increased rates of fracture. 40 million Americans currently have osteoporosis, or are at risk of developing osteoporosis due to low bone density [U.S. Department of Health and Human Services, 2011]. Osteoporosis and the resulting fractures in the United States were estimated to cost $22 billion in 2008 [Blume and Curtis, 2011]. As the population ages, osteoporosis will become of even greater concern. It is estimated that by 2020, 50% of Americans over the age of 50 will develop, or be at risk of developing osteoporosis of the hip [Office of the Surgeon General, 2004]. Thus, osteoporosis is a significant public health issue needing further study, as the control of bone remodeling is not well understood on a cellular level.

Osteocytes are theorized to play a significant role in controlling bone remodeling. These cells are found inside of bone, allowing them to detect strains placed on bone. In addition, narrow channels, called gap junctions, extend between
osteocytes. These gap junctions allow intercellular communications through the exchange of small molecules. Current theory suggests that osteocytes detect changes in the mechanical environment of bone and communicate with nearby osteocytes to control the bone remodeling process. This is supported by the osteocytes expression of sclerostin, a key protein for bone remodeling [Winkler et al., 2003].

1.1: Thesis Overview:

To investigate this theory, a mechanical loading platform was developed and characterized for this work. Following, a polydimethylsiloxane (PDMS) substrate was designed and fabricated for in vitro cell culture and mechanical loading. Using a platen connected to a microactuator, the PDMS substrates were tented from below to induce a strain on the cells. This aimed to induce cellular microdamage. A lactate dehydrogenase (LDH) assay, an indicator of energy conversion, was used to examine cellular viability following loading and quantify microdamage. Following the selection of loading ranges that induced microdamage, sclerostin expression was examined through the use of immunocytochemistry. To investigate the role of gap junctional intercellular communication, a topical inhibitor was used to block this communication and quantification of sclerostin expression was repeated.
CHAPTER II

LITERATURE REVIEW

2.1: Bone Remodeling:

Bone remodeling serves an important role in maintaining bone in that it allows the repair of localized damage such as microcracks, as well as larger fractures [Taylor et al., 2007]. The process of remodeling normally involves coupled osteoclast and osteoblast activity, keeping bone at equilibrium [Parfitt, 1984]. Bone remodeling is a process that follows an Activation-Resorption-Formation (ARF) cycle. In the first stage, activation, local microdamage leads to the recruitment of osteoclasts. After being recruited, osteoclasts begin resorbing bone by forming a ruffled border and adhering to the bone surface using a sealing ring. After the formation of this ring, osteoclasts release acids and proteases to break down the hydroxyapatite mineral and the bone matrix [Zaidi et al., 2003]. Following bone resorption, the osteoclasts undergo apoptosis and osteoblasts are recruited [Roodman, 1999]. To complete the ARF cycle, osteoblasts begin laying down new bone matrix, in the form of a collagen type I (CTI) matrix [Clarke 2008; Henriksen et al., 2009]. Bone is continually undergoing remodeling through the ARF cycle in response to local damage and to changes in the mechanical environment.
In addition to repairing microcracks and fractures, remodeling also allows bone to adapt to changes in the environment, such as growing stronger when exposed to increases in load. This example of Wolff’s Law is supported by in vitro studies in which bone responds to increased loading by increasing the amount of bone which is mineralized and the size of bone [Wolff 1892]. Wolff’s Law is also demonstrated in in vivo studies of tennis players which show that the dominant arm of tennis players have more bone than the nondominant [Huddleston et al., 1980; Krahl et al., 1994]. Communication between bone cells is critical for bone remodeling, and the response of bone to changes in mechanical load [Henriksen et al., 2009]. Current theory suggests that bone remodeling is controlled by osteocytes that detect the mechanical load, and can relay this information to the osteoblasts and osteoclasts [Bonewald, 2011].

After laying down new bone matrix, some osteoblasts become trapped in the matrix and differentiate further into osteocytes [Martin & Sims, 2005]. These are the most common type of bone cells, and reside in lacunae, cavities found in the interior of bone [Noble, 2008]. While residing in lacunae, osteocyte dendritic processes extend between cells using canalicular channels, figure 2.1 [Jones et al., 1993]. These dendritic processes form gap junctions between neighboring cells and allow the transfer of small molecules such as calcium ions or phosphates [Donahue, 2000]. Osteocytes are thought to play a key role in the response of bone to increased loading [Bonewald, 2011]. Their location in the lacunae may allow them to serve as mechanosensory cells, detecting changes in the strain placed on bone or the flow of interstitial fluid [Bonewald, 2011].
The osteocyte location inside the bone matrix also allows integrins from the osteocytes to interact directly with the extracellular matrix in bone, composed mainly of CT1. This interaction with the extracellular matrix may play an important role in bone cell mechanotransduction [Mullen et al., 2013].

Figure 2.1: Display of Osteocyte Location Inside Bone. Found in lacunae, osteocytes can communicate with each other using gap junctions that extend between cells using canalicular channels. Reprinted from The International Journal of Biochemistry & Cell Biology, Volume 36, Melissa L Knothe Tate, Josée R Adamson, Andrea E Tami and Thomas W Bauer, The Osteocyte, pages 1-8, 2004, with permission from Elsevier [Tate et al., 2004].

*In vivo* studies have demonstrated that bone remodeling can be a local process in response to changes in mechanical load [Verborgt et al., 2000]. It has been proposed that gap junctions provide a mechanism of intercellular communication, and may have a role in the local bone remodeling process [Burger & Klein-Nulend, 1999; Bonewald, 2011]. Current theory suggests that as bone is loaded, osteocytes are exposed to the
mechanical loads due to their location in the lacunar cavities, figure 2.2A [Bonewald, 2011, Ott adapted by Saunders, 2010]. The microcracking of bone matrix may impact nearby osteocytes and lead to localized damage, figure 2.2B. This localized damage may take the form of osteocyte apoptosis [Cardoso et al., 2009; Verborgt et al., 2000]. This local osteocyte or dendritic process damage may be detected by nearby cells, allowing additional cells to contribute to the signaling response, figure 2.2C [Hazenberg et al., 2006; Verborgt et al., 2000]. This damage may begin a signal transduction process in nearby cells which leads to localized bone remodeling [Colopy et al., 2004].

Figure 2.2: Damage of Osteocytes and their Potential Role in Bone Remodeling. Here, osteocytes are in blue, with dendritic processes extending between them with circles representing potential gap junction coupling. Damaged osteocytes, such as those undergoing apoptosis, are displayed in red. The damaged osteocyte may communicate with local osteocytes to coordinate a response, shown in pink. The image suggests osteocytes play a role in initiating local bone remodeling. Image adapted by MM Saunders, from American Society for Bone and Mineral Research, SM Ott [Ott adapted by Saunders, 2010].
2.2: Sclerostin in Bone Remodeling:

Osteocytes release signaling factors associated with bone remodeling, such as sclerostin [Robling et al., 2008; Lin et al., 2009]. Encoded by the SOST gene, sclerostin is a protein secreted by osteocytes that has been shown to respond to changes in mechanical load. *In vivo* experiments have shown that rats exposed to unloading produce enhanced levels of sclerostin. This increase in sclerostin also leads to a reduction in bone mass [Lin et al., 2009]. Other *in vivo* studies have found that mechanical stimulation of bone leads to a decrease in sclerostin expression and an increase in bone mass [Robling et al., 2008]. These initial studies demonstrating the impact of sclerostin led to further work, investigating the mechanism of sclerostin in regulating bone mass. Initial studies have found that the sclerostin regulates bone mass using the canonical Wnt signaling pathway [Robinson et al., 2006; Robling et al., 2008; Lin et al., 2009].

The Wnt signaling pathway plays an important role in transmitting external signals into cells, and this has far reaching physiological implications. For instance, studies have shown that the Wnt signaling pathway plays an important role in human development and the progression of cancer [Logan et al., 2004; Polakis, 2000]. Previous studies have demonstrated that Wnt signaling is involved in regulating bone [Krishnan et al., 2006; Tu et al., 2007]. Of particular interest to bone remodeling is the impact of a
protein found in osteocytes, sclerostin, and its impact on the canonical Wnt signaling pathway, figure 2.3.

Figure 2.3: Display of Sclerostin and the Impact on Wnt Signaling. Sclerostin leads to the inactivation of the Wnt signaling pathway, preventing the gene transcription, shown on the left. Without sclerostin, the Wnt signaling pathway proceeds and leads to gene transcription, shown on the right. Reprinted form Bone, Volume 54, Travis A Burgers and Bart O Williams, Regulation of Wnt/β-catenin Signaling Within and From Osteocytes, pages 244-249, 2013 with permission from Elsevier [Burgers, Williams 2013].
Sclerostin has been shown to inhibit Wnt signaling by binding to lipoprotein receptor-related protein receptors 5 and 6 (LRP5/6). The Wnt molecule can bind to LRP5/6 and Frizzled (Fz), which allows beta-catenin and TAZ to accumulate in the cell. This accumulation of beta-catenin and TAZ leads to gene transcription after the molecules enter the nucleus, right of figure 2.3. When sclerostin is present it can bind to LRP5/6 and inhibit Wnt binding to LRP5/6 and Fz. This prevents beta-catenin and TAZ from accumulating, thus inhibiting the gene transcription from the Wnt signaling pathway, left of figure 2.3 [Li et al., 2005; Ellies et al., 2006]. This inhibition of Wnt signaling leads to a loss of bone mass [Kamiya et al., 2008]. The regulation of Wnt signaling by sclerostin has also been shown to play a role in the bone loss accompanied by mechanical unloading, and bone homeostasis [Kramer et al., 2010; Lin et al., 2009]. 

*In vivo* studies of rodents studying the loading of limbs found a reduction in sclerostin expression, while the unloading of limbs increased sclerostin expression, suggesting sclerostin and Wnt signaling as a key molecule in regulating bone remodeling [Robling et al., 2008].

In addition to sclerostin, other proteins of interest to this study include angiopoietin 1 and galectin-3. Previous studies have demonstrated that osteoblast overexpression of angiopoietin 1 led to an increase in bone mass [Suzuki et al., 2007]. Other work, studying chimeras of angiopoietin 1, have demonstrated increased bone morphogenetic protein induced osteoblast differentiation, and thus bone formation [Jeong et al., 2010]. While angiopoietin has been associated with osteoblasts,
osteocytes may also be of interest as they may regulate bone remodeling. Another protein of interest for this study, galectin-3, has been shown to play a role in early development of bone and bone formation [Fowlis et al., 1995; Ortega et al., 2005]. While limited work has investigated these proteins with osteocytes, they may play an important role in the bone remodeling process.

While a variety of signaling factors released by osteocytes are associated with bone remodeling, such as sclerostin and the Wnt signaling pathway, the impact of osteocyte damage and osteocyte intercellular communication in this process remains unclear. This study will culture osteocyte-like MLO-Y4 cells \textit{in vitro} and analyze the cell viability, release of sclerostin, and cell process destruction in response to cell damage from a mechanical load. The study will then be repeated with inhibited intercellular communication, thus examining the role of intercellular communication in the response of MLO-Y4 cells to mechanical overload. This study also serves as an important step towards the development of a complete \textit{in vitro} model of bone remodeling, consisting of osteoblasts, osteoclasts, and osteocytes.

2.3: Osteocyte Studies:

As osteocytes are theorized as having a significant role in bone mechanotransduction, a variety of approaches have been used to study their response to mechanical loading and unloading. A common approach is to use \textit{in vivo} models to
investigate osteocytes. *In vivo* studies commonly consist of loading animal bones prior to sacrificing the animal, and studying the change in protein expression or cellular behavior. These studies have demonstrated that bone microdamage through fatigue testing leads to osteocyte apoptosis in bone. In this study, rat ulnae were fatigue loaded until reaching a 30% reduction in bone stiffness, at a rate of 4 Hz. Ten days after loading, bones were sectioned and stained to quantify osteocyte viability. After sectioning, the results indicated osteocyte apoptosis near microcracks after loading [Verborgt et al., 2000].

Similar work has been performed with osteocyte apoptosis inhibited. Here, changes in bone remodeling following the loading were also studied, demonstrating that osteocyte apoptosis plays an important role in bone remodeling in response to microdamage. The ulnae of rats were cyclically loaded at a rate of 2 Hz until reaching a 23% reduction in bone stiffness. The rate of bone remodeling in rats with and without osteocyte apoptosis suppression were then compared by examining sectioned bone. The study found that intracortical remodeling was present with loading and osteocyte apoptosis, but not present in control samples or with inhibited apoptosis in loaded samples [Cardoso et al., 2009]. This suggests that osteocyte apoptosis may play an important role in bone remodeling.

*In vivo* studies have also been used to investigate changes in protein expression in response to mechanical loading or unloading. By comparing bones stimulated with a
mechanical loading machine with bones unloaded through disuse, reduced expression of sclerostin has been associated with mechanical stimulation [Lin et al., 2009; Robling et al., 2008]. Mechanical unloading through disuse was accomplished using a tail suspension system for 14 days prior to sacrificing the rat. Bone morphology was studied using a μCT, and protein expression was analyzed by extracting RNA and performing a real time polymerase chain reaction assay to analyze gene expression. Unloading led to an increase in sclerostin expression, and a decrease in two targets of the Wnt signaling pathway, lymphoid enhancer binding factor 1 (Lef-1) and Cyclin D1. Upon studying the impact of unloading on mice bred to lack the gene for sclerostin, the components of the Wnt signaling pathway were not impacted by unloading, suggesting the impact of sclerostin on the pathway [Lin et al., 2009]. To study the impact of loading on sclerostin, mice ulnae were loaded to approximately 2200 με 360 cycles per day at a rate of 2 Hz for 2 days prior to being sacrificed. Immunohistochemistry was then performed, and results were quantified by comparing the percentage of osteocytes expressing sclerostin. By examining sections of the ulna subjected to different strains, it was found that higher strains led to a larger reduction in the percentage of osteocytes expressing sclerostin. Real time polymerase chain reaction results comparing loaded and control ulnae also indicated a decrease in sclerostin expression in loaded bone [Robling et al., 2008].

In addition to in vivo studies, in vitro studies have been used to study osteocytes. While primary cells have been used for decades, with the development of the MLO-Y4
cell line, studies of osteocytes *in vitro* have become increasingly common [Kato et al., 1997; Rosser et al., 2012; Weinbaum et al., 1994]. With *in vitro* studies, fluid shear stresses have become a standard mechanism of loading and studying cells using parallel plate flow chambers. These chambers were commonly used to simulate the stresses of physiologic flow, as they can produce a constant shear stress over time. It has been demonstrated that osteocytes stimulated with fluid shear stresses regulated osteoblast activity through the use of gap junctions. Osteocytes and osteoblasts were cultured on discs, separated by a membrane with 1 μm pores. This pore size allows communication between the cell types, without allowing cell migration. Following culture, osteocytes were exposed to fluid shear stresses up to .44 Pa for one hour. Following loading, alkaline phosphatase activity was analyzed. This study found that alkaline phosphatase activity increased significantly in osteoblasts when cultured near osteocytes able to communicate using gap junctions, and the activity did not increase when communication was prevented due to distance between the cell cultures or the use of 18 α-glycyrretinic acid to inhibit gap junction communication [Taylor et al., 2005]. This demonstrated that gap junctional intercellular communication in osteocytes could impact osteoblast activity.

In addition to fluid shear studies, ultrasound has also been used in investigating the impact of mechanical load on MLO-Y4 cells cultured *in vitro*. Osteocytes cultured *in vitro* until confluence were treated with pulsed ultrasound at a low intensity (>30mW/cm²) for 20 minutes, followed by 6 hours of incubation. Following the 6 hours,
sclerostin expression was measured through the use of western blots. This study demonstrated a significant decrease in sclerostin expression in MLO-Y4 cells cultured in vitro, similar to previous in vivo work [Liu and Ou, 2008; Robling et al., 2008].

Further work investigated the impact of soluble factors produced by osteocytes in response to fluid flow. After culturing osteocytes, they were exposed to pulsatile fluid flow for 1 hour at a rate of 5 Hz and a mean shear stress of .7 Pa. After loading the medium was collected and added to osteoblast cultures. Following 2 days in culture, osteoblasts cultured with medium from loaded osteocytes proliferated at a significantly lower rate and exhibited significantly higher levels of alkaline phosphatase than those cultured with osteocyte medium that was not loaded [Vezeridis et al., 2006]. The same fluid flow was also used to demonstrate a reduction in osteoclast formation and bone resorption when exposed to medium from loaded osteocytes when compared to control osteocytes [Tan et al., 2007]. These results, showing the role of medium from loaded osteocytes on osteoclasts and osteoblasts, support the theory of osteocytes as a key regulator of bone remodeling [Tan et al., 2007; Vezeridis et al., 2006].

2.4: Current Work

While in vitro mechanotransduction studies have been performed, most use fluid shear stresses to induce damage. While these studies have demonstrated a great deal about osteocyte behavior, further work is needed. For example, different modes of
loading may be more physiologically relevant. While the strain placed on bone is minimal, normally less than 2000 microstrain, osteocyte lacunae may amplify the strain [Burr et al., 1996; Han et al., 2004; Rath et al., 2006]. For this study, cells were seeded on an elastic material, polydimethylsiloxane (PDMS). Previous work has demonstrated that osteocytes proliferate, remain viable, and exhibit the characteristic protein expression on PDMS [York et al., 2012]. Here, osteocyte-like MLO-Y4 cells were cultured in a PDMS well and mechanically loaded by tenting from below with a centrally contacting platen. Following loading, changes in osteocyte viability were measured using an LDH assay to confirm microdamage. Upon confirming the loading range needed to induce microdamage, sclerostin expression was measured. Finally, to investigate the impact of gap junctional intercellular communication, this communication was inhibited before again measuring the LDH activity and sclerostin expression.

2.5: Hypotheses:

Null Hypothesis 1: Levels of sclerostin expression and cell viability in MLO-Y4 cells will not be altered by mechanically-induced microdamage.

Alternative Hypothesis 1: Levels of sclerostin and cell viability in MLO-Y4 cells will be altered by mechanically-induced microdamage.
Null Hypothesis 2: Inhibition of gap junctional intercellular communication will not alter the sclerostin response to mechanically-induced microdamage.

Alternative Hypothesis 2: Inhibition of gap junctional intercellular communication will alter the sclerostin response to mechanically-induced microdamage.
CHAPTER III
MATERIALS AND METHODS

The work is divided into three sections for organizational purposes. First, a microloading platform was developed and characterized, and a PDMS well was designed for experimentation. Secondly, cellular loading experiments were performed, in which the viability and sclerostin responses to changes in mechanical load were quantified. Finally, the loading experiments were repeated with the inhibition of gap junctional intercellular communication (GJIC).

For the project, a variety of devices and attachments were necessary. These were fabricated as needed, and will be discussed in this chapter. While these fabricated pieces were results of the designs, they are found in this chapter for improved clarity for the reader.

3.1: Loading Platform and PDMS

For loading studies, a microloading platform developed in-house was used to stretch samples, tenting them from below using a centrally contacting platen. Prior to
use, the microactuator used with the platform was characterized insuring an accurate and reliable displacement for experimentation. Initial studies demonstrated that improvements for the microloading platform were needed, such as an alignment block for centering samples and a method to reduce preloading of samples. In addition, a PDMS substrate was fabricated and characterized for loading studies. Characterization included the use of finite element models to insure the PDMS would not fail during experimentation and the correlation of these finite element results with experimental data obtained using a digital image correlation algorithm which showed the surface strain of the stretched PDMS.

3.1.1: Microloading Platform

For loading, a microloading platform developed in-house was used. This platform consisted of an aluminum base plate connected to a Plexiglas® top plate using corner rods. An additional aluminum plate served as a mounting plate for a microactuator (Zaber Technologies) connected to a 250 g canister load cell (Honeywell), figure 3.1. A cylindrical stainless steel platen connected to the load cell was used for loading the PDMS substrate from below, while the load cell was used to detect initial contact with the PDMS.
Figure 3.1: Microloading Platform, Designed In-House. During loading experiments, a Zaber microactuator was connected to a load cell and cylindrical platen. As the displacement of the microactuator was increased, the platen stretched the sample sitting on top of the Plexiglas® plate.

3.1.2: Microactuator Characterization

Microactuator performance was characterized prior to experimentation, through the use of gauge blocks. The microactuator was connected to the microloading platform, with the microactuator extended, figure 3.2. After aligning the end of the cylindrical platen with the fixed surface, the displacement was decreased using a controller for the microactuator, until a gauge block of known thickness could be inserted between the end of the cylindrical platen and the fixed surface. The change in position reported by the microactuator was compared to the known thickness of the gauge block. This characterization was completed using gauge blocks with thicknesses of .0625, .1, .125, and .2 inches (1.59, 2.54, 3.18, 5.08 mm). Measurements were taken
in triplicate, on three days. The measured displacement was compared to the theoretical displacement based on the thickness of the gauge block.

Figure 3.2: A) Test Setup for Microactuator Characterization. The microloading platform was assembled and set on its side. The microactuator was fully extended and the platen was aligned with a fixed surface. The displacement of the actuator was decreased until a gauge block of known size could fit between the platen and the surface. B) Close up of microactuator characterization setup. Image showed a gauge block used to find the distance between the end of the cylindrical platen and the fixed surface.
3.1.3: Microloading Platform Improvements

Following microactuator characterization, an alignment block was designed for the top of the microloading platform and the microscope platform. These attachments were drawn using SolidWorks and designed to reproducibly position the samples on the loading platform stage during experimentation, and on the microscope for analysis. The microloading platform alignment block also reduced sample lift off as the microactuator displacement increased, insuring uniform substrate loading, figure 3.3. The microscope alignment block allowed improved control of samples while imaging, figure 3.4. Both alignment blocks were fabricated out of polycarbonate. The centering legs, made of stainless steel were designed to fit the microloading or microscope platform and center the alignment block.
Figure 3.3: Alignment Block Sketches and Images. A) SolidWorks sketch of the alignment block, with centering legs attached. The alignment block had a slot for sliding in substrates for testing, and was designed to center the sample on top of the microloading platform. B) Image of alignment block. The side opening allowed testing samples to be inserted, while the top hole allowed visualization during experimentation and allowed oxygen to reach the cells. C) Alignment block for the top of the microloading platform, shown on the platform. This polycarbonate block insured the PDMS substrate could be consistently positioned between experiments and reduced motion during experimentation. Stainless steel legs were used to reach the end of the platform, centering the polycarbonate block.
Figure 3.4: Polycarbonate Block Used for the Zeiss AxioVert Microscope. The block allowed precise and consistent positioning of the PDMS sample while imaging, and allowed the use of the manual stage controls for positioning the sample.

In addition to the alignment block, a cell culture holding system for the PDMS substrate was also designed, figure 3.5. This was designed using SolidWorks, and fabricated out of polycarbonate. The cell culture holding system was used to reduce cell loading prior to experimentation. Initial experiments showed that PDMS stuck to tissue culture plates, causing an unintended preload. The cell culture holding system allowed the polycarbonate block to be handled instead of the PDMS. This eliminated preloading of the PDMS prior to experimentation, allowing the study of the isolated tenting load.
Figure 3.5: Cell Culture Holding System Sketch and Image. A) Drawing of cell culture holding system in SolidWorks. B) Image of cell culture holding system. Designed to hold PDMS samples for loading, the polycarbonate could be handled without applying a preload to the sample. This allowed study of only the applied load and minimized preloads.

3.1.4: PDMS Fabrication

PDMS grids were fabricated using a spin coating procedure to fit a mold. PDMS base and curing agent (Dow Corning) were mixed at a 10:1 ratio and stirred to mix. A vacuum desicator was used to remove air bubbles from the solution prior to pouring the PDMS mixture onto silicon wafers or 60 mm petri dishes. After spinning the wafer and PDMS at 200 to 3000 RPM for 30 seconds, the silicon wafer and PDMS were cured for 2 hours in an oven at 60° C. Following cooling, the PDMS membrane pieces were removed from the silicon wafer and cut to shape using a razor blade.
For experimentation a PDMS substrate was designed using SolidWorks. This substrate provided a region for cell seeding and contained a positional reference grid to allow imaging of the same area before and after loading. PDMS was fabricated in three separate pieces so as to obtain the well geometry needed. The bottom PDMS piece, approximately 1.9 mm thick, had a center hole removed to allow tenting from below, figure 3.6. The center piece of PDMS, a strip approximately 200 microns in thickness, contained a positional reference grid and was used for cell seeding, figure 3.7. The top layer of PDMS was approximately 3.5 mm thick, figure 3.8. This layer was used to form a well allowing the seeding of cells prior to experimentation and analysis following loading. To create this well a section was removed from the center of the piece. The PDMS layers were aligned using reference geometries, generating the substrate, figure 3.9. The PDMS substrate was assembled through the use of a PDMS glue technique. PDMS was prepared by thoroughly mixing the base and curing agent at a 10:1 ratio, and this was spread over the top PDMS layer. The grid was then aligned with the top and pressure was applied to insure an even surface. This was then cured for 15 minutes at 60 °C before repeating the process to adhere the bottom layer to the grid and top layer.
Figure 3.6: Bottom PDMS Sketch and Image. A) SolidWorks sketch of the bottom piece for the PDMS device. B) Bottom piece for PDMS substrate. This piece contained a hole cut out using a cork borer, allowing loading with a platen from below.

Figure 3.7: Center PDMS Sketch and Image. A) SolidWorks sketch of center PDMS layer. The center of the layer was gridded, allowing imaging of the same area before and after loading. Grid sizes were 490 μm by 490 μm. B) Center piece of PDMS substrate illustrating the gridded center. This positional reference grid enabled the same location to be studied before and after experimentation, improving repeatability of studies.
Figure 3.8: Top Layer PDMS Sketch and Image. A) Drawing in SolidWorks of the top layer of the PDMS device. To fit the cell culture holding system, this was a thicker layer. B) Top piece for PDMS substrate in a petri dish before cutting to shape and removing the central hole. Fabricated in-house, a core borer was used to cut out a central hole to generate a well that allowed cell seeding and served as a medium reservoir.

Figure 3.9: PDMS Sketch, Drawing, and Detailed View. A) Sketch in SolidWorks showing the assembled PDMS device and the details of the positional reference grid. B) Assembled PDMS substrate in the cell culture holding system. The PDMS substrate had a hole in the bottom allowing loading, a positional reference grid, and a well to allow cell culture. The cell culture holding system prevented loading prior to experimentation, as the polycarbonate was handled instead of the PDMS. Inset image taken using a Zeiss microscope showed the detail of the positional reference grid.
3.1.6: Finite Element Modeling of PDMS Substrate

Prior to experimentation, the PDMS substrate was characterized using finite element analysis (FEA). Using previously published mechanical data, material properties of PDMS such as the elastic modulus and tensile strength were used to define the model [Mata et al., 2005; Schneider et al., 2009]. Load for experimentation was stimulated using a remote displacement applied to the center of the bottom of the middle layer of the PDMS substrate to simulate the experimental loading. For boundary conditions, the sides, top, and bottom of the PDMS substrate had a fixed position as the alignment block and cell culture handling system held the PDMS substrate. FEA studies were used to insure displacements used in experimentation did not lead to failure and to correlate the microactuator displacement with strain on the PDMS. A mesh size of .005 inches (.127 mm) was used and studies were run with displacements of .48, .96, 1.92, 2.88, 3.85, 5.76, and 7.2 mm, corresponding to a reported displacement from the actuator controller of 1, 2, 4, 6, 8, 12, and 15 mm respectively. Previous work had demonstrated a scale factor of .48 to convert from the controller displacement to the actual displacement.
3.1.7: Digital Image Correlation of PDMS

After assembling the PDMS substrate, digital image correlation work analysis was done to insure the loads shown by the finite element analysis model were accurate. This provided an estimate of the surface strains in the PDMS well. The PDMS substrate without cells was placed on the microloading platform. Using a load cell, the microactuator and platen were used to stretch the sample, using the centrally contacting platen, the same method of loading used for experimentation, figure 3.10. While loading, a digital camera was used to image the PDMS substrate as the displacement increased. Images were taken every 1000 microns as reported by the microactuator controller, up to a maximum of 16000 microns. This reported displacement of 16000 microns corresponded to a displacement of 7.68 mm. MATLAB code available online was used to process and analyze the images. This algorithm enabled the tracking of changes in position of the PDMS substrate during loading, and generated a strain map based on changes in position. This strain map was compared to the FEA results, insuring agreement between the experimental and theoretical results.
Figure 3.10: Digital Image Correlation Setup. With the mechanical loading platform on its side, the PDMS well was loaded with images recorded every 1000 microns. Pixel movement was tracked to characterize the strain.

3.2: Response of Cells to Loading

For experimentation cells were cultured on the PDMS substrate before being loaded using the microloading platform. Initial loading studies were done to determine optimal loading displacements and times. A LDH stain was used to observe cell viability after loading. For experimentation loads were used that led to an increased level of LDH activity, cell death in approximately 10% of the cells, cell death in approximately 50% of the cells, and the death of all cells. These ranges were used to simulate cell activation and cell damage in vitro. After determining the ranges needed, immunocytochemistry studies were completed to analyze the level of sclerostin, a protein inhibitor of the Wnt signaling pathway, at the various loads.
3.2.1: Cell Culture

Osteocyte-like MLO-Y4 cells (a gift from Dr. Lynda Bonewald) were cultured using a previously established protocol [Rosser and Bonewald, 2012]. The cells were cultured using minimum essential alpha medium (α-MEM, Gibco) supplemented with 5% fetal bovine serum (Hyclone), 5% fetal calf serum (Hyclone), and 1% penicillin/streptomycin (Pen/Strep, Invitrogen). Cells were maintained at 5% CO₂ and 37°C. For cell culture, 25 cm² flasks were coated with rat tail CTI (BD Bioscience) in .02 M acetic acid (Sigma) at a concentration of 5 μg/cm² for one hour prior to rinsing with Dulbecco’s Phosphate Buffered Saline Solution with calcium and magnesium (D-PBS, Calcium and Magnesium, Hyclone). Cells were passaged between 70 and 80% confluency, using .25% Trypsin-EDTA, and fed every 3 days.

For experimentation MLO-Y4 cells were seeded onto PDMS substrates at a density of 2 x 10⁴ cells/cm². These PDMS substrates were coated with rat tail CTI in .02 M acetic acid at a concentration of 5 μg/cm² overnight prior to experimentation. Cells were maintained in .5 ml of medium. After seeding cells onto the substrates, two Kimwipes (Kimtech) dipped in sterile water were wrapped around the interior perimeter of the plate generating a humidified chamber. These cells were seeded 4 days prior to experimentation allowing time for the cells to reach their characteristic protein expression and dendritic morphology [York et al., 2012]. This also allowed the cells to reach a higher confluency necessary for analyzing gap junction communication.
3.2.2: Loading Studies

For loading studies, PDMS substrates in the cell culture loading system were removed from the incubator, and slid into the alignment block on top of the microloading platform. This centered the PDMS substrate on top of the platform, figure 3.11. The displacement on the actuator was increased until contact with the PDMS substrate, detected by observing an increase in the force placed on the load cell. The displacement of the actuator was then increased for experimentation. Initial studies aimed to determine the loading ranges necessary to induce limited cell death, 50% cell death, and complete cell death. In addition, characterization studies were ran comparing the cell numbers in an identical area before and after loading, investigating the number of cells that detached from the PDMS well structure as part of loading. PDMS substrates were loaded for 15 minutes prior to 90 minutes of incubation, allowing time for a cellular response such as cell death.
Figure 3.11: Alignment Block and Cell Culture Holding System for Experimentation. The actuator, platen, and load cell, not shown in this image, were aligned beneath the center of the alignment block during experimentation. This allowed loading of the center of the PDMS substrate and improved consistency between experiments.

3.2.3: LDH Staining for Cell Viability

LDH staining, a measure of cell activity and energy conversion, was used to determine cellular viability following load. Following experimentation, the MLO-Y4 cells were washed with Hank’s Buffered Saline Solution (HBSS, Hyclone) prior to incubation with the reaction solution. The reaction solution contained a base solution of 5% polypep (Sigma-Aldrich), and 2 mM of gly-gly (Sigma Aldrich) in HBSS. Prior to staining, 1.75 mg/ml of nicotinamide adenine dinucleotide (Sigma-Aldrich) and 60 mM lactic acid (Sigma-Aldrich) were added and the solution was brought to a pH of 8.0. Following the
addition of 3mg/ml of nitroblue tetrazolium, cells were incubated with .5 ml of the staining solution per PDMS substrate.

Initial characterization experiments were used to quantify LDH expression by measuring average stain intensity at a range of time points. ImageJ, an image analysis program provided by the NIH, was used to quantify changes in the stain intensity. After taking images using the Zeiss microscope, pixel intensity was measured in cells obtaining the average, minimum, maximum, and standard deviation of the intensity values relative to the background. Characterization studies were completed on PDMS with samples at 0, 60, 90, 120, and 180 minutes. Additional characterization consisted of comparing varying squares in PDMS wells after loading.

LDH staining was used for measuring cellular activity in the loading studies. Following loading and incubation LDH staining was completed on the cells seeded on the PDMS substrates for 120 minutes. This was used to determine which cells remained viable or had an increased level of LDH activity following loading. In characterization loading experiments, all samples were stained for LDH activity. This LDH data was used to determine the loads necessary for cell activation, limited cell death, 50% cell death, and complete cell death.

LDH results were obtained using ImageJ to analyze the pixel intensity. For each PDMS structure, 9 loaded grids were measured. Analysis was completed on the central
positional reference square, as well as reference squares 3 (1.5 mm) and reference squares 6 (3 mm) above, below, left, and right of the central grid, figure 3.12. For each analyzed reference grid, 40 cells and 10 background selections were randomly chosen. From these, the average pixel value for each cell was found and the average pixel value for the background was subtracted from each. The average of these 40 measurements was also subtracted from the cell pixel value. This generated an average pixel value, relative to the background and the LDH staining of cells without loading in the well. For each loading condition, samples were loaded in triplicate and repeated on 3 days.

Figure 3.12: Example Mosaic from a Loading Experiment. Bordered grids were studied and analyzed for pixel density. Grids of the same pattern were grouped together as the middle, 3, and 6 squares away from the center. The middle square was patterned white, 3 squares away was patterned white with black lines, and 6 squares away was patterned black.
3.2.4: Immunocytochemistry for Sclerostin Expression

Following loading and incubation, immunocytochemistry was completed on the cells seeded on the PDMS substrates to quantify the level of sclerostin expression. The cell medium from experimentation was removed before a quick wash with phosphate buffered saline (PBS) and cells were fixed for 15 minutes at room temperature in 4% paraformaldehyde in PBS. Cells were then permeabilized by a 10 minute wash in .2% Triton-x100 in PBS. Following two 5 minute washes with PBS, nonspecific binding was blocked for 1 hour using 5% bovine serum albumin in PBS. Two more 5 minute PBS washes were completed prior to applying the primary antibody in 1% bovine serum albumin. Initial experimentation determined an optimal dilution for the antibody. Cells were incubated with the primary antibody overnight at 4°C in a humidified chamber to prevent evaporation. The next day 3, 10 minute washes in PBS were completed before applying the secondary antibody in 1% BSA and incubating for 1 hour at room temperature. Initial experimentation also optimized this dilution. After 3 more 10 minute washes in PBS mounting solution with DAPI was applied and the PDMS well was imaged.

For quantification black and white images were obtained using a Zeiss AxioVert fluorescence microscope. To insure consistency all sclerostin images were taken using the same exposure time on the microscope and the same dilution of sclerostin. Using ImageJ the background fluorescence was measured on each image and subtracted from
measurements inside the cell, giving a value corresponding to the fluorescence level above the background. These measurements were compared between each loading condition allowing the cellular sclerostin levels to be compared.

3.3: Studying Impact of Gap Junctions

Following the loading experiments quantifying the LDH activity and sclerostin levels, work was done to study the impact of gap junction communication in this response. This was done by repeating the loading studies after the inhibition of gap junctional intercellular communication.

3.3.1: Inhibition of Gap Junction Communication

To study the impact of gap junctional intercellular communication in the response to mechanical load in osteocytes, cells were loaded with and without this communication inhibited. To inhibit intercellular communication 30 μM 18α-glycyrrhetinic acid (Sigma Aldrich, G8503) in DMSO was used to block the communication. Cells were exposed to the treatment one hour before loading, providing time for the 18α-glycyrrhetinic acid to block communication during and immediately after loading.
3.3.2: Parachute Assay for Confirming Gap Junction Communication

To confirm the presence or absence of intercellular communication through gap junctions, a parachute assay was performed. This was a fluorescence assay which uses two fluorescent dyes to confirm communication. For this assay, .25% Dil (Life Technologies, D-282) in dimethyl sulfoxide, .1% Calcein in dimethyl sulfoxide, and 2% bovine serum albumin in HBSS were prepared. After syringe filtering the Dil, 20 μl of the calcein solution, 100 μl pluronic acid (Life Technologies, P-3000MP), 2 mL of 2% bovine serum albumin in HBSS, and 28 μl of Dil were added to a conical tube and vortexed to mix and prepare the dye solution.

After removing the medium from a 35 mm dish, the dye solution was added and these donor cells were incubated for 30 minutes. After incubation these donor cells were washed with HBSS prior to being trypsinized and centrifuged. Cells were counted in four position reference grid squares on the PDMS substrate and this count was used to estimate the total number of acceptor cells on the substrate. At the end of the centrifuge cycle the donor cells were collected and counted, with donor cells resuspended. Donor cells were resuspended such that the addition of 100 μL of medium containing donor cells would lead to a ratio of 1 donor cell to 500 acceptor cells on the PDMS well substrate. These double labeled donor cells were added to the MLO-Y4 cells on PDMS substrates, and incubated on the substrates for 2 hours. This allowed time for gap junctional intercellular communication.
Following two hours of incubation fluorescent images were taken of the PDMS substrates. The parachute assay used Dil and calcein AM as two fluorescent dyes in the donor cells. Donor cells pipetted onto the PDMS substrate were identified based on the Dil staining red and the calcein AM staining green. A cyanine 3 (Cy3) filter from Zeiss, with an excitation wavelength of 544 nanometers and an emission wavelength of 605 nanometers was used to detect the Dil. A green fluorescent protein (GFP) filter also from Zeiss, with an excitation wavelength of 470 nanometers and an emission wavelength of 525 nanometers was used to detect calcein AM. When images taken with the two filters were overlayed, donor cells stained red and green as they contained Dil and calcein AM. These donor cells were allowed two hours to communicate with the acceptor cells on the PDMS substrate and communication was tracked by imaging the calcein AM, which stained green. Acceptor cells fluoresced green as the calcein AM could move through the gap junctions while the Dil could not. The parachute assay was completed to confirm the inhibition of functional intercellular communication by the 18α-glycyrrhetinic acid treatment.

3.3.3 Cytokine Antibody Arrays

Following mechanical loading medium was collected from loaded wells for later analysis. Cytokine antibody arrays were used to study a variety of factors, including decorin and calbindin D. Samples of fresh medium, medium from cells that were not loaded, medium from cells that were not loaded but were treated with 18α
glycyrrhetinic acid, and from the 6 loading conditions were collected and analyzed. .5 mL of medium was collected from 3 samples in each condition, and this 1.5 mL was averaged together before 1 mL was analyzed using the antibody array. This allowed the measurement of the average response at each of the conditions tested.

The antibody array was a sandwich ELISA based technique. Cytokine specific antibodies were bound to the glass slide and incubated with the samples for analysis. After cytokines were bound to the antibodies, a second incubation using antibodies bound to biotin was used to insure specific detection of the cytokine using a cytokine-antibody-biotin complex. Streptavidin labeled with Cy3 was then added for a third incubation. The streptavidin could bind to the cytokine-antibody-biotin complex and the Cy3 was detected using a laser scanner. This allowed detection and quantification of precise concentrations of cytokines.
CHAPTER IV

RESULTS

Similar to Chapter III, Chapter IV is organized in three sections. The first section discusses the microloading platform characterization and PDMS well. The cellular loading experiments examining viability using the LDH assay and the sclerostin expression are discussed in the second section. The final section discusses the results of inhibiting gap junctional intercellular communication using 18α-glycyrrhetinic acid.

4.1: Loading Platform and PDMS Substrates

Before cellular studies, the microloading platform was fabricated and its actuator characterized. In addition, the PDMS well was designed and characterized computationally using a finite element analysis model and experimentally using a digital image correlation algorithm.

After fabricating and setting up the microloading platform, the microactuator was characterized using gauge blocks. The theoretical thicknesses from the gauge blocks were compared to the experimental displacements from the microactuator. Figure 4.1 displays the actuator controller’s displacement vs gauge block thickness. An
exact correlation would have a slope of 1, indicating an excellent correlation between
the experimental and theoretical data for the actuator. Given that the actuator was
used for loading with displacements of 3.84, 5.76, and 7.2 mm, this actuator correlated
well for the experimental needs.

![Graph showing linear relationship between displacement and thickness]

Figure 4.1: Actuator Controller Displacement vs Gauge Block Thickness. Measurements
were recorded in triplicate on three separate days by aligning the end of the actuator
with a fixed surface and decreasing the displacement until a gauge block could fit
between the actuator and fixed surface.

Following the PDMS well design a finite element analysis model was developed
and ran to confirm the PDMS would not fail over the desired loading range. Table 4.1
contains the properties used for the finite element analysis, table 4.2 contains numerical
results from the finite element analysis studies, table 4.3 contains approximate strains
at areas examined in later experimentation, figure 4.2 displays example resulting
images, and figure 4.3 displays strains quantified in areas studied in later
experimentation.
Table 4.1: List of Properties Used for Finite Element Analysis. Material properties were taken from previously published studies on the mechanical properties of PDMS fabricated from Sylgard 184 (Dow Corning) [Mata et al., 2005; Schneider et al., 2009].

<table>
<thead>
<tr>
<th>PDMS Material Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model Type</td>
</tr>
<tr>
<td>Elastic Modulus</td>
</tr>
<tr>
<td>Poisson’s Ratio</td>
</tr>
<tr>
<td>Mass Density</td>
</tr>
<tr>
<td>Tensile Strength</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FEA Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodes</td>
</tr>
<tr>
<td>Elements</td>
</tr>
</tbody>
</table>

Table 4.2: Finite Element Analysis Results. Results indicated PDMS was not predicted to fail in the loading range desired for experimentation and that maximum strains increased with increased displacement. These two properties indicated the PDMS well would perform as necessary for the loading studies.

<table>
<thead>
<tr>
<th>Actuator Controller Displacement</th>
<th>Max Stress (vonMises) (MPa)</th>
<th>Max Displacement (mm)</th>
<th>Max Strain (mm/mm)</th>
<th>Max P1 (MPa)</th>
<th>Min P1 (MPa)</th>
<th>Max P3 (MPa)</th>
<th>Min P3 (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mm</td>
<td>.33</td>
<td>.54</td>
<td>.16</td>
<td>.39</td>
<td>-.20</td>
<td>.17</td>
<td>-.39</td>
</tr>
<tr>
<td>2 mm</td>
<td>.65</td>
<td>1.07</td>
<td>.31</td>
<td>.78</td>
<td>-.39</td>
<td>.35</td>
<td>-.78</td>
</tr>
<tr>
<td>4 mm</td>
<td>1.30</td>
<td>2.15</td>
<td>.63</td>
<td>1.55</td>
<td>-.79</td>
<td>.69</td>
<td>-1.55</td>
</tr>
<tr>
<td>6 mm</td>
<td>1.95</td>
<td>3.22</td>
<td>.93</td>
<td>2.33</td>
<td>-1.18</td>
<td>1.04</td>
<td>-2.33</td>
</tr>
<tr>
<td>8 mm</td>
<td>2.61</td>
<td>4.30</td>
<td>1.25</td>
<td>3.10</td>
<td>-1.57</td>
<td>1.38</td>
<td>-3.10</td>
</tr>
<tr>
<td>12 mm</td>
<td>3.91</td>
<td>6.45</td>
<td>1.87</td>
<td>4.65</td>
<td>-2.36</td>
<td>2.08</td>
<td>-4.65</td>
</tr>
<tr>
<td>15 mm</td>
<td>4.89</td>
<td>8.06</td>
<td>2.34</td>
<td>5.81</td>
<td>-2.95</td>
<td>2.60</td>
<td>-5.81</td>
</tr>
</tbody>
</table>
Table 4.3: Local Finite Element Estimates. Using the strain images, the color pattern was adjusted to study the local strains predicted by the finite element model in each of the areas examined in later studies. The results confirm an increase in strain as the displacement increased, and as the area of interest moved towards the edge of the platen used for loading.

<table>
<thead>
<tr>
<th>Actuator Controller Displacement</th>
<th>Strain in Middle Square</th>
<th>Strain 3 Squares Away</th>
<th>Strain 6 Squares Away</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 mm</td>
<td>.00007</td>
<td>.01</td>
<td>.66</td>
</tr>
<tr>
<td>12 mm</td>
<td>.0002</td>
<td>.011</td>
<td>.97</td>
</tr>
<tr>
<td>15 mm</td>
<td>.0003</td>
<td>.013</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Figure 4.2: Example Finite Element Analysis Results for a Displacement of 15 mm. A displays stress, B displays displacement, and C displays strain. Results indicated a stress and strain peaking at the edge of the platen used to tent from below, and a displacement peaking directly above the platen, as expected.
Figure 4.3: Example Local Finite Element Analysis Results. The legend on the strain image from the finite element analysis results was modified to estimate the local strains in areas studied in later experiments. The area near the edge of the platen was studied in this image by adjusting the legend such that the area corresponded to the bright green, and an estimated strain of 1.17.

Following the finite element analysis a digital image correlation algorithm using MATLAB code available online was used to estimate strains placed on the surface of the PDMS well. Shown in figure 4.4, figure 4.5, and table 4.4, the results indicate strains increased with increased displacement. Results also indicate an increase in strains near the edge of the platen, as predicted by the finite element model. Finally, an estimate of local strains was obtained. Figure 4.6 displayed the PDMS well used for experimentation with labels for the areas examined in later experiments.
Figure 4.4: Average Strain Across PDMS Well. A) Average strains along a vertical line passing through the center of the platen at varying displacements. B) Graph displaying average strain across a horizontal line passing through the center of the platen. Averaging the two yielded an average strain across the well of 9% at a displacement of 3.84 mm (image 9), 21% at 5.76 mm (image 13), and 47% at 7.2 mm (image 16).
Figure 4.5: Strains Estimated by the Digital Image Correlation Algorithm. A corresponds to image 9 and a displacement of 3.84 mm, B to image 13 and 5.76 mm, and C to image 16 and 7.2 mm. The circle added to each figure corresponds to platen position, while squares represent areas examined in later cellular studies. Results indicate an increase in strain with an increase in displacement, and increased strains near the edge of the platen, as the final element model predicted.
Table 4.4: Estimated Strains Obtained from Digital Image Correlation Algorithm. 3 squares away and 6 squares away refers to the positional reference grid squares, figure 4.6.

<table>
<thead>
<tr>
<th></th>
<th>Middle</th>
<th>3 Squares Away</th>
<th>6 Squares Away</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.84 mm</td>
<td>5%</td>
<td>8%</td>
<td>10%</td>
</tr>
<tr>
<td>5.76 mm</td>
<td>15%</td>
<td>19%</td>
<td>34%</td>
</tr>
<tr>
<td>7.2 mm</td>
<td>35%</td>
<td>54%</td>
<td>60%</td>
</tr>
</tbody>
</table>

Figure 4.6: Example of PDMS Well Used for Experimentation. Regions examined are highlighted with squares with the center of the well in white, 3 squares away from the center in black and white, and 6 squares away from the center in black near the edge of the platen used for loading.
4.2: Response of Cells to Loading

Following characterization of the microloading platform and PDMS substrate, cellular loading studies were completed. These studies first used a LDH assay to determine if the microloading platform could induce detectible microdamage to the MLO-Y4 cells when cultured in the PDMS well. Further work investigated the load and the impact on sclerostin expression.

Initial LDH studies used a range of staining times at 0, 60, 90, 120, and 180 minutes. This first study aimed to confirm the effectiveness of the technique for detecting changes in LDH expression. Initial work was done using PDMS with a constant cross section. The minimum pixel value relative to the background corresponded to the most intense LDH stain, figure 4.7.
Figure 4.7: Pixel Intensity of Cells in LDH Characterization on PDMS. Different time points were used to compare staining intensities as the color change was quantified from 60 to 180 minutes. All comparisons were statistically significant with a p value of .05, except 120 and 180 minutes. This indicated that 120 minutes was sufficient for further work.

Following the culture of MLO-Y4 cells on the PDMS well structures for 4 days, PDMS well structures were loaded using the microloading platform. Initial loading studies were run to demonstrate the effectiveness of analyzing the pixel intensity of the LDH activity with results shown in figure 4.8. Additionally, cell detachment rates were analyzed by counting cell numbers in the same area before and after loading, figure 4.9.
Figure 4.8: Initial LDH Results in PDMS Well Structures. Results indicated intense LDH staining near the center of the well, in squares H9 and H7, along the right side of the image. Moving away from the center led to higher strains and a reduction in LDH activity. Near the edge of the studied area, squares D9 and D7, staining intensity was further decreased.

Figure 4.9: Cell Adhesion Experiment. Cells were imaged before and after loading in the same positional reference grid square. 76 cells were counted before loading and 73 after, indicating 96% of cells remained attached after the mechanical load.
Loading was completed using a load cell to detect when the actuator made contact with the PDMS. Upon contact loads generated by 3.84, 5.76, and 7.2 of actuator displacement mm were used, corresponding to values of 8000, 12000, and 15000 microns on the microactuator controller. Following loading cells were incubated for 90 minutes prior to 120 minutes of LDH staining and imaging. Initial LDH studies had demonstrated higher staining intensity with a decrease in pixel value and that after subtracting the background values, the LDH staining pixel values were negative. Figure 4.10 showed an example LDH stain.

Figure 4.10: Example LDH Staining of MLO-Y4 Cells on PDMS. The purple staining was characteristic of the assay. Cells near the top left of the image stained a darker purple indicating cells with higher levels of LDH activity and thus increased energy conversion.
After confirming that the LDH assay could quantify cellular activity, loading experiments and data processing began. After loading and completing the LDH assay, the 9 regions of interest (middle square, 3 squares away in the 4 cardinal directions, and 6 squares away in the cardinal directions) were imaged. These images were analyzed using Fiji, a distribution of ImageJ. 40 cells were manually selected in each of the 9 regions of interest and the mean pixel intensity in each cell was recorded, yielding 360 mean pixel intensities in each PDMS well substrate. 10 background measurements were also taken in each region of interest to account for differences in the background intensity. The cell pixel intensity relative to the background was obtained by subtracting the mean background intensity from each of the 40 cell measurements, yielding 40 background adjusted cell intensity values in each region of interest, and 360 in each PDMS well. For the LDH assay, these values were multiplied by negative one. This led to higher pixel values corresponding to higher levels of LDH activity.

After obtaining the background adjusted cell intensity values, the data was compared to controls to obtain results for statistical analysis. Controls for this work consisted of cells on unloaded PDMS wells that had been stained for LDH activity. Two equations were used for data processing and analysis. Equation 4.1 was used to quantify how many pixels more or less intense the background adjusted cell intensity values were relative to the control. The control was set to have a mean value of 0 using this equation. Cells that were staining more intensely than the control, indicative of higher levels of LDH activity, had positive values after using equation 4.1.
A second equation was developed to allow percentage comparisons. Equation 4.2 was written to subtract the minimum background adjusted cell intensity value from the numerator and denominator, setting the minimum result of the equation to 0. After dividing the background adjusted cell intensity value by the control, the result was multiplied by 100. This generated an equation that set the control value to 100 and allowed the recording of cell activity as a percentage above or below the control level of expression.

Equation 4.1: \[ \text{Pixel} = \text{background adjusted cell intensity value} - \text{control} \]

Equation 4.2: \[ \text{Percentage} = \frac{(\text{background adjusted cell intensity value} - \text{minimum})}{(\text{control} - \text{minimum})} \times 100 \]

Using the estimated strains from the digital image correlation in table 4.4, LDH results using equation 4.1 can be found in figure 4.11, and results from equation 4.2 can be found in figure 4.12. Figure 4.13 displayed the LDH results from figure 4.12 when broken down by location and displacement. Statistical comparisons between different estimated strains can be found in table 4.5. All conditions were tested for normality using the D’Agostino & Pearson omnibus normality test. As not all samples were normal, all analysis was completed using the Mann-Whitney U test for nonparametric statistical analysis. Results indicate that varying strains impacted LDH activity. These results depicted 9360 cells, as shown in table 4.6.
Figure 4.11: Pixel Display of LDH Staining Using Equation 4.1. This had a control value of 0. Results indicated an increase in LDH activity at strains of 5%, 8%, and 15%, corresponding to an increase in metabolism. Strains of 10%, 19%, 34%, 35%, 54%, and 60% led to a decrease in staining, indicative of cell microdamage or possible death.

Figure 4.12: Percentage Display of LDH Staining Using Equation 4.2. This used a control value of 100. Similar to figure 4.10, increases in staining were seen at strains of 5%, 8%, and 15%, with decreases at 10%, 34%, 35%, 54%, and 60%.
Figure 4.13: LDH Results Broken Down by Region and Displacement. Top left showed the middle square, top right 3 squares away, middle left 6 squares away, middle right actuator displacement, and bottom left the location. Results indicated increased LDH activity at a displacement of 3.84 mm, and a decrease at a displacement of 7.2 mm. Results also indicated a decrease in activity moving away from the center of the well, towards regions of higher strains.
Table 4.5: Statistical Analysis of LDH Data. Table used the percentage results from equation 4.2 and figure 4.13. Comparisons throughout this thesis used Mann-Whitney U tests as not all distributions were normal. Results indicate many statistically significant differences, indicating the ability to induce microdamage. *'s indicate statistical significance with a p value of .05, and NS indicates not significant.

<table>
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Table 4.6: Number of Cells Analyzed for the LDH Graphs. Each well structure had 360 cells with 40 in each area of interest. Table indicates that a total of 9360 cells were loaded and had their LDH activity analyzed.

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<tr>
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<th>34%</th>
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</thead>
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<td>Number of cells</td>
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<td>4160</td>
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</table>
After demonstrating that cell damage could be induced by varying levels of strain, sclerostin activity was examined using the same displacements and thus the same strains as were used with the LDH. Figure 4.14 displayed an example of the immunofluorescence staining of sclerostin in osteocytes; figure 4.15 showed the data results from equation 4.1, 4.16 the data from equation 4.2, 4.17 the data from equation 4.2 when broken down by region and displacement; table 4.7 listed statistical comparisons; and, table 4.8 listed the number of cells analyzed.

Figure 4.14: Example Immunofluorescence Staining of Sclerostin in Osteocytes. Osteocytes expressed sclerostin throughout the cell and gap junctions in a punctate pattern.
Figure 4.15: Pixel Results of Sclerostin Expression After Loading. Results indicated increased activity at strains below 15%, and decreased or no change in expression above 19%.

Figure 4.16: Sclerostin Intensity Data at Varying Strains. Results indicated a large increase in sclerostin expression at low strains, and a smaller increase relative to the control at high strains.
Figure 4.17: Sclerostin Expression Broken Down by Region and Displacement. Top left showed the middle square, top right 3 squares away, middle left 6 squares away, middle right actuator displacement, and bottom left the location. Results indicated a significant increase in sclerostin expression at a displacement of 3.84 mm and a return to near baseline levels at 5.76 mm and 7.2 mm.
Table 4.7: Results of Mann-Whitney U Test of Sclerostin Data. This was completed using the percentage results from equation 4.2 and figure 4.17. Comparisons were statistically significant, with the exception of groups of similar strains, such as: 5, 8 and 10%; 19, 34, and 35%; and, 54 and 60%. 19%, 34%, 35%, and 54% were not statistically different from the control values.

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Table 4.8: Number of Cells Analyzed for Sclerostin Graphs. Similar to the LDH cell number, 360 cells were counted in each cell substrate.

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4.3 Studying Impact of Gap Junctions

Following LDH and sclerostin studies, further work examined the impact of gap junctional intercellular communication. 30 μM of 18α-glycyrrhetinic acid was used to inhibit communication 1 hour prior to experimentation. To confirm the inhibition of communication a parachute assay was completed. Figure 4.18 displayed images with and without intercellular communication, confirming effectiveness of the inhibition treatment.

Figure 4.18: Example Parachute Assay Images. A) Parachute assay without the inhibition of communication. Donor cells, containing Calcein AM and Dil fluoresce green and red. In this image they were the 3 bright cells. Nearby cells that they communicated with fluoresced a faint green, with no red. Here, they fluoresced faintly. B) Parachute assay with the inhibition of communication. All cells fluorescing contained Calcein AM and Dil, indicating they were donor cells and no communication occurred.
Following the confirmation of the inhibition of gap junctional intercellular communication, the previous sclerostin loading study was repeated on cells with the 18α-glycyrrhetinic acid treatment. Using the same displacements and strains results can be found in figure 4.19 using equation 4.1, figure 4.20 when using equation 4.2, and figure 4.21 when using equation 4.2 and broken down by region. Table 4.9 contained the statistical analysis results while table 4.10 contained the number of cells analyzed.

Figure 4.19: Sclerostin Expression in Cells with Inhibited Gap Junctional Intercellular Communication. Results indicated an increase in expression at most strains, except 8% and 10%.
Figure 4.20: Sclerostin Expression When Communication Was Inhibited. Results indicated an increase in communication at most strains, except for 8%. This was contrary to the sclerostin results in which higher strains led to expression near baseline levels.
Figure 4.21: Sclerostin Expression With Inhibited Communication Split by Region and Displacement. Top left showed the middle square, top right 3 squares away, middle left 6 squares away, middle right actuator displacement, and bottom left the location. Contrary to sclerostin results, 3.84 mm led to a level of expression near the control while 5.76 mm and 7.2 mm led to an increase in expression.
Table 4.9: Statistical Analysis of Sclerostin Intensity with Inhibited Communication. This was completed using the percentage results from equation 4.2 and figure 4.21. Results indicated no statistically significant difference between 34, 35, 54, and 60% strains. At lower strains, most comparisons were statistically significant.

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Table 4.10: Number of Cells Analyzed for Sclerostin with Inhibited Communication.

Similar to previous tables, 360 cells were analyzed per PDMS substrate.

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<td>6 Squares Away</td>
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<td>1080</td>
<td>4320</td>
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After confirming that strain impacted sclerostin expression both with and without intercellular communication, the two sets of data were plotted next to each other, figure 4.2. In addition, a Two-Way Analysis of Variance was ran. Table 4.1 contained a summary of the results.

![Figure 4.2: Results of Sclerostin Expression With and Without Inhibited Communication. At low strains (5% to 10%), the communication inhibition led to a significant reduction in expression. At moderate strains (15% to 35%), the communication inhibition increased expression significantly. At 54 and 60% strain, the inhibition still led to increased communication, but less prominently.](image-url)
A Mann-Whitney U test was ran on each set of conditions with and without intercellular communication. These results were found in table 4.11.

Table 4.11: Mann-Whitney U Tests of Sclerostin Expression With and Without Communication Inhibition. Results were significant at all conditions indicating a significant impact from the inhibition of communication.

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</tr>
<tr>
<td>8% strain</td>
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<td>3.84 mm displacement</td>
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<td>10% strain</td>
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<td>5.76 mm displacement</td>
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<tr>
<td>15% strain</td>
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<td>7.2 mm displacement</td>
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<td>Middle square</td>
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<td>All data</td>
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</table>

Following the pixel analysis a biphasic data pattern was observed. The LDH activity showed a significant increase at a displacement of 3.84 mm, corresponding to an increase in energy conversion, before having a value near the control and below the control at displacements of 5.76 mm or 7.2 mm, corresponding to mechanically-induced microdamage in the cells. The sclerostin expression was indicated as increased at the 3.84 mm displacement before staying near the control at 5.76 mm and 7.2 mm. The sclerostin with inhibited communication also had biphasic behavior with expression near the control at a displacement of 3.84 mm and above the control at a displacement
of 5.76 and 7.2 mm. This biphasic behavior was consistent across the data measured suggesting the cellular microdamage impacted the sclerostin expression.

Following the analysis of pixel intensity the concentration of a variety of cytokines was analyzed using a sandwich ELISA. While several factors of interest were below the detection threshold, such as sclerostin or receptor activator of nuclear factor k B (RANK), other factors were above the threshold at each condition. Factors of particular interest were angiopoietin 1 and galectin-3. Results from the sandwich ELISA were found in table 4.12.

Table 4.12: Angiopoietin 1 and Galectin-3 Analysis by Sandwich ELISA. All units are pg/mL. Results indicated a difference in expression between samples with and without gap junctional intercellular communication of both angiopoietin 1 and galectin-3. Results indicated an average angiopoietin 1 increase of 37% when communication was inhibited while galectin-3 increased by 28% when communication was inhibited.

<table>
<thead>
<tr>
<th>Factor</th>
<th>medium</th>
<th>Control and 3.84 mm</th>
<th>5.76 mm and 7.2 mm</th>
<th>Inhibited control and 3.84 mm</th>
<th>Inhibited 5.76 mm and 7.2 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiopoietin 1</td>
<td>622</td>
<td>574</td>
<td>590</td>
<td>762</td>
<td>829</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>0</td>
<td>1110</td>
<td>1081</td>
<td>1505</td>
<td>1298</td>
</tr>
</tbody>
</table>
CHAPTER V

DISCUSSION

The discussion is divided into three sections for organization purposes, similar to Chapters III and IV. First, the microloading platform and PDMS were characterized. Following, cellular loading experiments investigating the viability and sclerostin response to changes in strain were completed and analyzed. Finally, gap junctional intercellular communication was inhibited for additional sclerostin experiments.

5.1: Loading Platform and PDMS Substrates:

Initial mechanical loading platform characterization consisted of confirming the microactuator would offer the needed precision. For the characterization a gauge block of known thickness was compared to the actuator displacement needed to fit the gauge block between the actuator and a fixed surface. Results indicated correlation, figure 4.1. An ideal correlation would have a slope of 1 indicating a perfect relationship between the measured and theoretical data. In this case the slope was .9994, indicating an excellent correlation and enough precision for reliable experimentation using this microactuator. Additionally, the correlation extended across a range of gauge block thicknesses indicating the microactuator performed well across a range of values.
Following microactuator characterization finite element analysis was completed on the proposed PDMS well design, tables 4.2 and 4.3. By running a variety of displacements, including those exceeding the range needed, the study predicted the PDMS well would not fail during loading. This was confirmed by the maximum predicted stress (4.89 MPa) not exceeding the tensile strength of PDMS (10 MPa). Numerical results from the finite element also demonstrated an increase in maximum strain, as desired. Thus, the proposed well design was found to be sufficient for the displacements needed.

The finite element analysis also examined the strains and displacements throughout the well, figures 4.2 A-C and figure 4.3. The tenting load used caused very little predicted stress and strain in the center of the well, but caused the maximum displacement. Near the edge of the platen the stress and strain was at a maximum, as the displacement began decreasing. This demonstrated the nonuniform strains placed on the PDMS well. As a range of strains was desired, the center of the well, immediately above the center of the platen was chosen for later analysis due to the minimal strain. In addition, areas at the edge of the platen where strains were expected to reach a maximum were chosen. A midpoint between these two areas was also chosen, figure 4.5. This provided a range of surface stresses, strains, and displacements in one experiment, optimizing the use of the nonuniform design.
To confirm the results of the finite element analysis, digital image correlation work was completed to experimentally approximate the strains placed on the surface. A well structure was spray painted and loaded, with images taken at varying displacements. These images were run through a MATLAB algorithm that tracked the movement of particles of spray paint providing an estimate of the strains. The average strain measured along a vertical and horizontal line moving through the platen indicated a significant increase in strain as displacement increased, figure 4.3. At a displacement of 3.84 mm, the average of the strains along the horizontal and vertical lines through the platen was 9%. A displacement of 5.76 mm led to an average strain of 21%, and a 7.2 mm displacement led to an average strain of 47%. These values were less than the maximum strains predicted by the finite element, but increased significantly with increased displacement as expected. These results indicated a wide range of strains as desired for loading. The results also indicated that high displacements were necessary, as the strains did not increase linearly. As samples were recycled and consistently tore immediately at 7.68 mm, the displacements used for loading were chosen as 3.84 mm, 5.76 mm, and 7.2 mm. While a displacement of 7.2 mm did cause some substrates to tear during loading, large displacement and high strain was necessary to get a wide range of strains and recycling of the PDMS wells was kept to a minimum.

In addition to finding the average strain across the PDMS well, the digital image correlation algorithm was also used to estimate the local strains in the areas of the PDMS wells chosen for analysis in figure 4.4. The strains across the entirety of the well
were viewed at the displacements used for experimentation, and regions of interest were chosen, figure 4.6. Using the digital image correlation legend, the average strain in each area of interest was estimated. These results estimated surface strains to range from 5% to 60%. This range of strains demonstrated the displacements selected were sufficient for experimentation.

5.2: Response of Cells to Loading

LDH activity was quantified in initial cell loading studies. LDH is an enzyme heavily involved with cellular energy conversion, and was thus used to represent cellular activity and by extension viability. The study aimed to mechanically induce microdamage in the MLO-Y4 osteocyte-like cells, and LDH activity was used to determine if this damage occurred. In this assay, LDH activity catalyzed a color change. Cells with high levels of LDH activity stained dark purple while cells that were dead or not undergoing energy conversion did not undergo any color change. To quantify this pixel intensity was measured relative to a control without loading.

LDH work, figures 4.11-4.13 and table 4.5, indicated an increase in activity at low strains, and a decrease at higher strains. Surface strains of 5%, 8%, and 15% all showed mean intensities with statistical significance above the control. At a surface strain of 15%, average LDH staining intensity increased by 58% relative to the control. This represented a significant increase in cellular energy conversion, and thus activity.
As surface strain increased cellular activity decreased. At surface strains of 19%, 34%, and 54%, and 60%, LDH staining intensity decreased significantly relative to controls. This decrease indicated a reduction in cellular activity, consistent with what would be expected with mechanically-induced microdamage. The cells in these strains appeared to be microdamaged and undergoing cellular death based on the reduced LDH activity and staining intensity. This, in conjunction with cells exhibiting higher levels of activity at low strains, indicated that mechanical loading using the platform and PDMS well substrate led to a range of cellular activity that could be quantified, including cellular death. The lowest displacement, 3.84 mm, led to an increase in LDH expression by 15%, while the highest displacement, 7.2 mm, led to a 9% decrease in expression. Therefore, the displacements used indicated a range of cellular behavior as desired.

After the LDH work confirmed the displacements used induced a range of cellular behavior, immunocytochemistry was used to investigate sclerostin expression. Using the same loading conditions, figures 4.15-4.17 and table 4.7 indicated a statistically significant increase in sclerostin expression at strains of 5%, 8%, 10%, 15%, 54%, and 60%, and a statistically significant decrease in sclerostin expression at 34% strain. While this does show that sclerostin expression was modified by the mechanically-induced microdamage, interestingly, the expression increased.

Previous in vivo work found that unloading led to increases in expression, and mechanical loads led to decreased expression. Previous studies examined the effects of
long term load, such as 14 days of limb unloading or 2 days of limb loading [Lin et al., 2009; Robling et al., 2008]. Other studies used short term loads, such as 20 minutes of pulsed ultrasound, but used an extended incubation time to allow a cellular response [Liu and Ou, 2008]. As this experiment examined expression after 15 minutes of loading and 90 minutes of incubation, the reduction in sclerostin may require an extended loading or incubation time. As this work aimed to study the immediate effect of microdamage, extended times were not investigated. Alternatively, other techniques for measuring sclerostin expression may be ideal. Other studies have used western blotting to quantify expression or immunolocalization to study the percentage of osteocytes expressing sclerostin [Lin et al., 2009; Liu and Ou, 2008; Robling et al., 2008]. These techniques were not chosen for this work as the nonuniform strain profile necessitated a method to study individual areas, instead of an average response. Further work to investigate sclerostin expression may necessitate an alternative substrate or method for cell loading, thus allowing the use of western blotting or real-time polymerase chain reaction to study the average response of the cells.

In addition to alternative techniques, an improved in vitro model may also be used in further studies of sclerostin expression after mechanically-induced microdamage. Previous studies have demonstrated that integrins play a key role in the mechanotransduction process of osteocytes by mediating mechanosensitive signaling pathways [Litzenberger et al., 2010]. Additional studies have demonstrated that the osteocyte response to mechanical stimulation was dependent on the presence of
integrins. In this work, control MLO-Y4 cells were cultured and mechanically loaded on plates using the standard CTI coating while other cells were cultured using a coating that did not allow integrins to interact with the extracellular matrix. This change in interaction between the integrins and the extracellular matrix led to a significantly different mechanical response, as shown by extracellular signal-related kinases expression [Plotkin et al., 2005]. This demonstrated that the extracellular matrix plays a role in the mechanotransduction response of osteocytes. While collagen is the most abundant protein in the extracellular matrix, additional proteins and glycosamingoglycans may play a significant role in the mechanotransduction response of osteocytes. A more complete extracellular matrix may be necessary for the decrease in sclerostin expression seen in vivo at increased loads.

The first hypothesis, that levels of sclerostin and cell viability in MLO-Y4 cells would not be altered by mechanically-induced microdamage is rejected based on the LDH and sclerostin results. These two techniques demonstrated that the mechanical load did lead to mechanically-induced microdamage and did alter levels of cellular viability and sclerostin expression. While LDH activity and sclerostin expression did not have a statistically significant difference from the control at 19 and 35% strain, the remaining 7 conditions were significantly different. This demonstrates the ability of the platform to impact cell expression, and provides sufficient data to reject the null hypothesis. Thus, the level of sclerostin and cell viability in MLO-Y4 cells was altered by mechanically-induced microdamage.
5.3 Studying Impact of Gap Junctions

Following the confirmation that mechanically-induced microdamage was possible using the loading platform, the impact of gap junctional intercellular communication was studied. This began by confirming the inhibition of communication was possible and was followed by studying the sclerostin expression with inhibited communication. To confirm the effectiveness of the 18α-glycyrrhetinic acid, a parachute assay was run first.

The parachute assay used two fluorescent molecules, Calcein AM and Dil. Donor cells were incubated with a solution containing Calcein AM and Dil for 30 minutes. After pipetting these cells onto a plate of confluent acceptor cells and 2 hours of incubation, gap junctional intercellular communication was. Donor cells contained Dil allowing them to fluoresce orange and Calcein AM fluorescing green. After incubating, Calcein AM could move through gap junctions into nearby acceptor cells while Dil could not due to its larger size. After two hours of incubation fluorescence was quantified as the acceptor cells fluoresced green faintly, but could not fluoresce orange. In figure 4.18A several bright green and orange cells were fluorescing near the center of the image with faint green cells fluorescing nearby, indicating the presence of gap junctional intercellular communication. Figure 4.18 B, in which 18α-glycyrrhetinic acid was used to inhibit gap junctional intercellular communication revealed only the donor cells fluorescing, as no cells fluorescing faintly near the donors were observed. This
demonstrated that the 18α-glycyrrhetinic acid treatment was effective at inhibiting gap junctional intercellular communication.

After confirming the effectiveness of the 18α-glycyrrhetinic acid treatment, the sclerostin study was completed with the treatment. Figures 4.19-4.21 and table 4.9 displayed a statistically significant increase in sclerostin expression at strains of 5%, 15%, 19%, 34%, 54%, and 60%, and a statistically significant decrease at 8%. While a similar number of strains were expressing sclerostin above and below the control as was without the 18α-glycyrrhetinic acid treatment, low strains led to the decrease in the treated study. The Mann-Whitney U test results indicated a statistically significant difference in sclerostin expression when gap junctional intercellular communication was inhibited, table 4.11. Figure 4.22 further emphasized this, plotting sclerostin communication with and without gap junctional intercellular communication inhibition. The inhibition of communication led to a 38% decrease in sclerostin expression at a displacement of 3.84 mm, a 30% increase at 5.76 mm, and a 10% increase at 7.2 mm.

As the inhibition of gap junctional intercellular communication altered the sclerostin response to mechanically-induced microdamage relative to cells without inhibition, the second null hypothesis was rejected. This suggests that gap junctions do play a role in the sclerostin response of osteocytes to microdamage. While this work found gap junctional intercellular communication to play a role, later studies may benefit from additional time for mechanical load or incubation may be necessary, as
previously discussed. Future work investigating the impact of gap junctional intercellular communication may also benefit from a substrate with a uniform strain allowing the use of western blots or real time polymerase chain reaction assays to obtain the average response of MLO-Y4 cells to mechanically-induced microdamage.

Following the study of the impact of the inhibition of gap junctional intercellular communication on sclerostin expression, a sandwich ELISA was ran to study the concentration of a variety of cytokines. Several proteins of interest, such as sclerostin and receptor activator of nuclear factor κ B (RANK), were below the detection threshold of the technique. This technique studied the soluble factors found in cellular medium of loaded cells. Future work interested in studying sclerostin expression using this technique may benefit from the use of additional cells or additional loading time. Previous work studying sclerostin after mechanical loading of MLO-Y4 osteocytes used extended periods of loading and incubation, as well as a larger cell volumes [Lin and Ou, 2008; Yu et al., 2011]. This work aimed to study the initial impact of mechanically-induced microdamage on a small number of cells, thus preventing the use of longer times. Future studies into mechanically-induced microdamage and sclerostin may benefit from an alternative substrate and experiment design that allows for additional time and cell number. In this work intracellular sclerostin expression was measured using immunocytochemistry while soluble sclerostin expression was measured using the sandwich ELISA. As the sclerostin was below the detection threshold with the ELISA the experiment may have also been improved by examining the intracellular expression as
well. This work focused on the soluble expression, as future work aimed to study the impact of factors released into conditioned medium.

In addition to sclerostin and RANK, angiopoietin 1 and galectin-3 were analyzed. These were two factors associated with osteoblasts, as angiopoietin 1 overexpression in osteoblasts led to an increase in bone mass, and that chimeras of angiopoietin 1 led to osteoblast differentiation and bone formation [Jeong et al., 2010; Suzuki et al., 2007]. Galectin-3 has also been shown to play a role in bone development and bone formation [Fowlis et al., 1995; Ortega et al., 2005]. In this work, levels of these proteins were examined at a range of mechanical loads, both with and without gap junctional intercellular communication. The results found that both factors were increased with the inhibition of communication, table 4.12. Angiopoietin 1 increased by an average of 37% while galectin-3 increased by an average of 28%. This further suggests that gap junctional intercellular communication plays an important role in regulating bone remodeling. This study demonstrated that the inhibition of gap junctional intercellular communication led to increases in angiopoietin 1, galectin-3, and sclerostin in cells exposed to mechanically-induced microdamage. These factors have been associated with bone remodeling suggesting that gap junctional intercellular communication also has a role in regulating bone remodeling.
CHAPTER VI

CONCLUSION

In this thesis two hypotheses were investigated. The first hypothesis, that the level of sclerostin expression and cell viability in MLO-Y4 cells was not altered by mechanically-induced microdamage, was rejected. The second hypothesis, that the inhibition of gap junctional intercellular communication would not alter the sclerostin response to microdamage was also rejected. This suggested that gap junctional intercellular communication may play a role in the sclerostin response to mechanical load of MLO-Y4 osteocyte-like cells. While these results were statistically significant, the sclerostin expression interestingly increased, contrary to the published literature. Additional mechanical loading or incubation time may have been necessary to observe the complete cellular response, and the decrease in sclerostin expression. The study may also have been improved by the measuring of the average response to strain instead of a local response. While this would have required an alternative substrate design it would also have allowed the use of additional quantitative techniques such as western blotting and real time polymerase chain reaction. Future work, built upon the results of this project may be designed to utilize these alternative techniques.
This research laid important groundwork for future studies involving bone remodeling and the role of osteocytes and gap junctions. This work demonstrated that the loading platform used could lead to mechanically-induced microdamage *in vitro*, which was necessary for future work. Later work aims to build upon this work by building a multicellular model of osteocytes, osteoblasts, and osteoclasts. This three cell model was designed to study the impact of mechanically-induced microdamage on osteocytes, and how the rate of bone formation in osteoblasts and bone resorption in osteoclasts was impacted. The LDH and sclerostin results indicated that this platform could induce the needed damage, allowing future studies to continue as planned.

The design of the three cell model will allow a variety of additional experiments. This model could be used to further investigate the role of osteocytes in the cellular control of bone remodeling. The platform design allows the loading of osteocytes, and the use of the conditioned medium to feed osteoblasts and osteoclasts *in vitro* are currently ongoing in the lab. By using mechanically-induced microdamage in osteocytes, and the soluble factors released into the osteocyte medium, this multicellular model can quantify the impact of the microdamage on osteoblast and osteoclast activity, or bone formation and bone resorption. This provides a method to further investigate the role of osteocytes in the cellular control of the process. This multicellular model also allows the treatment of osteocytes with 18α-glycyrrhetinic acid, further investigating the impact of gap junctional intercellular communication on bone remodeling.


Burgers, TA; Williams, BO, Regulation of Wnt/β-Catenin Signaling Within and from Osteocytes. *Bone*. **2013**, 54(2), 244-249.


Cardoso, L; Herman, BC; Verborgt, O; Laudier D; Maeska, RJ; Schaffler, MB, Osteocyte Apoptosis Controls Activation of Intracortical Resorption in Response to Bone Fatigue. *Journal of Bone and Mineral Research*. **2009**, 24(4), 597-605.

Clarke, B, Normal Bone Anatomy and Physiology. *Clinical Journal of the American Society of Nephrology*. **2008**, 3 (Supplement 3), S131-S139.

Colopy, SA; Benz-Dean, J; Barrett, JG; Sample, SJ; Lu, Y; Danova, NA; Kalscheur, VL; Vanderby, R; Markel, MD; Muir, P, Response of the Osteocyte syncytium Adjacent to and Distant from Linear Microcracks During Adaptation to Cyclic Fatigue Loading. *Bone*. **2004**, 35(4), 881-891.


Fowlis, D; Colnot, C; Ripoche, MA; Poirier, F, Galectin-3 is Expressed in the Notochord, Developing Bones, and Skin of the Postimplantation Mouse Embryo. *Developmental Dynamics*. 1995, 203(2), 241-251.

Han, Y; Cowin, SC; Schaffler, MB; Weinbaum, S, Mechanotransduction and Strain Amplification in Osteocyte Cell Processes. *Proceedings of the National Academy of Sciences of the United States of America*. 2004, 101(47), 16689-16694.


Henriksen, K; Neutzsky-Wulff, AV; Bonewald, LF; Karsdal, MA, Local Communication on and Within Bone controls Bone Remodeling. *Bone*. 2009, 44(6), 1026-1033.

Huddleston, AL; Rockwell, D; Kulund, DN; Harrison, B, Bone Mass in Lifetime Tennis Athletes. *The Journal of the American Medical Association*. 1980, 244(10), 1107-1109.

Jeong, BC; Kim, HJ; Bae, IH; Lee, KN; Lee, KY; Oh, WM; Kim, SH; Kang, IC; Lee, SE; Koh, GY; Kim, KK; Koh, JT, COMP-Ang1, a Chimeric Form of Angiopoietin 1, enhances BMP2-Induced Osteoblast Differentiation and Bone Formation. *Bone*. 2010, 46(2), 479-486.

Jones, SJ; Gray, C; Sakamki, H; Arora, M; Boyde, A; Gouride, R; Green, C, The Incidence and Size of Gap Junctions Between the bone Cells in Rat Calvaria. *Anatomy and Embryology*. 1993, 187(4), 343-352.

Kamiya, N; Ye, L; Kobayashi, T; Mochida, Y; Yamauchi, M; Kronenberg, HM; Feng, JQ; Mishina, Y, BMP Signaling Negatively Regulates Bone Mass Through Sclerostin by Inhibiting the Canonical Wnt Pathway. *Development and Disease*. 2008, 135(1), 3801-3811.


Kramer, I; Halleux, C; Keller, H; Pegurri, M; Gooi, JH; Weber, PB; Feng, JQ; Bonewald, LF; Kneissel, M, Osteocyte Wnt/β-Catenin Signaling is Required for Normal Bone Homeostasis. *Molecular and Cellular Biology*. **2010**, 30(12), 3071-3085.


Li, X; Zhang, Y; Kang, H; Liu, W; Liu, P; Zhang, J; Harris, SE; Wu, D, Sclerostin Binds to LRP5/6 and Antagonizes Canonical Wnt Signaling. *The Journal of Biological Chemistry*. **2005**, 280(1), 19883-19887.

Lin, C; Jiang, X; Dai, Z; Guo, X; Weng, T; Wang, J; Li, Y; Feng, G; Gao, X; He, L, Sclerostin Mediates Bone Response to Mechanical Unloading Through Antagonizing Wnt/β-Catenin Signaling. *Journal of Bone and Mineral Research*. **2009**, 24(10), 1651-1661.


Mullen, CA; Haugh, MG; Schaffler, MB; Majeska, RJ; McNamara, LM, Osteocyte Differentiation is Regulated by Extracellular Matrix Stiffness and Intercellular Separation. *Journal of the Mechnical Behavior of Biomedical Materials*. **2013**, 28, 183-194.

Noble, BS, The Osteocyte Lineage. *Archives of biochemistry and Biophysics*. **2008**, 473(2), 106-111.


Robinson, JA; Chatterjee-Kishore, M; Yaworsky, PJ; Cullen, DM; Zhao, W; Li, C; Kharode, Y; Sauter, L; Babij, P; Brown, EL; Hill, AA; Akhter, MP; Johnson, ML; Recker, RR; Komm, BS; Bex, FJ, Wnt/β-Catenin Signaling Is a Normal Physiological Response to Mechanical Loading in Bone. *The Journal of Biological Chemistry*. **2006**, 281(1), 31720-31728.

Robling, AG; Niziolek, PJ; Baldridge, LA; Condon, KW; Allen, MR; Alam, I; Mantila, SM; Gluhak-Heinrich, J; Bellido, TM; Harris, SE; Turner, CH, Mechanical Stimulation of Bone in Vivo Reduces Osteocyte Expression of Sost/Sclerostin. *The Journal of Biological Chemistry*. **2008**, 283, 5866-5876.


Saunders, MM, Microdamage and Bone Remodeling. Retrieved from National Science Foundation Grant. Adapted with permission from SM Ott Graphic, American Society for Bone and Mineral Research. **2010**


Suzuki, T; Miyamoto, T; Fujita, N; Ninomiya, K; Iwasaki, R; Toyama, Y; Suda, T, Osteoblast-Specific Angiopoietin 1 Overexpression Increases Bone Mass. *Biochemical and Biophysical Research Communications*. **2007**, 362(4), 1019-1025.


Tu, X; Joeng, KS; Nakayama, KI; Nakayama, K; Rajagopal, J; Carroll, TJ; McMahon, AP; Long, F, Noncanonical Wnt Signaling through G Protein-Linked PKC Activation Promotes Bone Formation. *Developmental Cell*. **2007**, 12(1), 113-127.


Vezeridis, PS; Semeins, CM; Chen, Q; Klein-Nulend, J, Osteocytes Subjected to Pulsating Fluid Flow Regulate Osteoblast Proliferation and Differentiation. *Biochemical and Biophysical Research Communications*. **2006**, 348(3), 1082-1088.


Winkler, DG; Sutherland, MK; Geoghegan, JC; Yu, C; Skonier, JE; Shpektor, D; Jonas, M; Kovacevich, BR; Staehling-Hampton, K; Appleby, M; Brunkow, ME; Latham, JA, Osteocyte Control of Bone formation via sclerostin, a Novel BMP Antagonist. *The European Molecular Biology Organization*. **2003**, 22(23), 6267-6276.


Zaidi, M; Blair, HC; Moonga, BS; Abe, E; Huang, CLH, Osteoclastogenesis, Bone Resorption, and Osteoclast-Based Therapeutics. *Journal of Bone and Mineral Research*. **2003**, 18(4), 599-609.