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Impact of Gap Junctional Intercellular Communication on MLO-Y4 Sclerostin and Soluble Factor Expression

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Abstract

Bone remodeling is a continual process in which old bone is resorbed by osteoclasts and new bone is formed by osteoblasts, providing a mechanism for bones' ability to adapt to changes in its mechanical environment. While the role of osteoblasts and osteoclasts in bone remodeling is well understood, the cellular regulation of bone remodeling is unclear. One theory is that osteocytes, found within bone, play an important role in controlling the bone remodeling response. Osteocytes possess gap junctions, narrow channels that extend between nearby cells and allow communication between cells via the transfer of small molecules and ions. This work investigated the potential role of gap junctional intercellular communication in bone remodeling by exposing osteocyte-like MLO-Y4 cells to mechanical strains and quantifying the expression of soluble factors, including sclerostin, a protein closely associated with bone remodeling. The soluble factors and sclerostin expression were further examined after inhibiting gap junctional intercellular communication to study the impact of the communication. At supraphysiologic strains, the inhibition of gap junctional intercellular communication led to increases in sclerostin expression relative to cells in which communication was present, indicating that the communication may play a significant role in regulating bone remodeling.

Key Terms

Mechanotransduction; Osteocyte; In-vitro; Cell-Cell Communication; Soluble Factors

Introduction

Bone remodeling, in which osteoclasts resorb old bone and osteoblasts lay down new bone, is normally a paired process that maintains bone at equilibrium.²⁰ By altering the amount and rate of bone resorption or bone formation, bone can adapt to changes in its mechanical environment. Previous studies have demonstrated that increases in the mechanical load that

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Conflict of Interest

The authors have no conflicts of interest to disclose.

bone is exposed to leads to increases in bone mass and strength.⁸ The converse is also true, in that decreased usage of bone or a reduction in the mechanical load leads to a reduction in mass.¹³ While the role of osteoclasts and osteoblasts in bone homeostasis is well understood, the cellular regulation of bone remodeling has yet to be fully elucidated.^{3, 15, 16}

Current theory is that osteocytes, a cell residing within bone, play a role in the cellular regulation of the bone remodeling process.³ Osteocytes are terminally differentiated osteoblasts that have become entrapped in freshly deposited bone matrix.¹⁵ This location inside bone allows osteocytes to detect changes in bone's mechanical environment, suggesting that osteocytes are critical to the initiation of the bone remodeling process.³ Osteocytes also possess gap junctions, narrow channels that extend between nearby osteoblasts and osteocytes. This direct contact enables intercellular communication through the passage of small molecules including calcium ions and phosphates.⁶ Osteocytes may also play a role in bone remodeling due to their expression of soluble factors shown to have important roles in bone formation and resorption, such as sclerostin and receptor activator of nuclear factor kappa-B ligand (RANKL).^{16, 24} Previous studies have indicated that sclerostin inhibits bone formation in osteoblasts.³ The location of osteocytes, the presence of gap junctions, and the expression of soluble factors shown to have roles in bone formation and resorption support the theory that osteocytes may have a pivotal role in the cellular regulation of bone remodeling.³ While this theory is widely accepted, the exact mechanisms and pathways by which this occurs have yet to be fully elucidated.

In addition to sclerostin, a number of other soluble proteins have been shown to impact the rate of bone formation or bone resorption. Angiopoietin 1 and galectin-3 have been shown to play a role in increases in bone mass and bone formation.^{7, 11, 18, 23} Previous studies have also demonstrated that the inhibition of activin-a, a cytokine commonly found in bone, leads to increased bone formation.^{5, 19} Additionally, mutations in SERPINF1, a gene associated with bone, have been shown to lead to osteogenesis imperfecta, a disorder in which bone has significantly reduced mass and strength.^{2, 9} Von Willebrand factor has been shown to inhibit osteoclastogenesis, and thus the rate of bone resorption.¹ While sclerostin has been closely associated with osteocytes, many of these other soluble factors have not. Mechanotransduction studies offer an approach to investigate relationships between cells and mechanical loading.

Mechanotransduction studies aim to better understand the processes by which cells convert stimuli from mechanical loads into biophysical or cellular responses. As tissues throughout the body are exposed to a wide variety of forces, including gravity, movement, and pressure, it can be difficult to characterize these forces *in vivo*.¹⁰ Thus, mechanotransduction studies are often completed with cells cultured *in vitro* that are exposed to an isolated mechanical load using techniques such as fluid shear, stretching the cell-seeded substrate, and ultrasound.^{4, 17} *In vitro* studies are designed to replicate the proposed mechanical forces from the *in vivo* environment to improve the level of biomimicry in the study.

In this work, a mechanical loading platform was used to load osteocyte-like MLO-Y4 cells cultured on a gridded polydimethylsiloxane (PDMS) well structure. PDMS well substrates were tented from below using a platen, introducing a nonuniform load. This nonuniform

load allowed the study of osteocytes exposed to a range of strains on the same substrate and under identical culture conditions. Following load, sclerostin expression was analyzed as a function of strain and soluble factors in the conditioned medium were collected and analyzed. Experiments were conducted in communication-intact and –inhibited environments. This work aimed to investigate the potential role of mechanically-induced microdamage and gap junctional intercellular communication on the cellular regulation of bone remodeling.

Materials and Methods

Approach

To complete this work the following approach was used. A platform for loading osteocytes seeded on PDMS was fabricated. Digital image correlation was employed to approximate PDMS surface strains and a culturing system and alignment jigs were fabricated to enable manipulation of cell-seeded substrates and ensure reproducible placement of the substrates on the loading platform and microscope stage. Short term static out-of-plane distention loading was applied to the cell-seeded substrates at three loading ranges (physiologic, physiologic/supraphysiologic, supraphysiologic) and sclerostin activity as a function of strain was semi-quantified. In addition, conditioned medium was collected and subject to protein analysis. To address the importance of cell communication, these studies were conducted in intact and inhibited communication environments.

Loading Platform

This study used a mechanical loading platform developed in-house, Figure 1A and B. Briefly, the base of the platform was an aluminum plate with three leveling screws. This base plate was connected to a Plexiglass® top plate by four aluminum corner rods. The top plate had a center hole cut out, which provided the clearance for sample tenting from below using a linear actuator and aluminum platen. An aluminum mounting plate suspended below the top plate was connected by corner rods. This plate was used for mounting the linear microactuator (Zaber Technologies, T-NA08A25) to the platform.

The microactuator connected to a 250 gram load cell (Sensotec) to detect contact of a platen with the cell substrate. Fully retracted, the platen was 0.30 mm below the top plate. As the displacement increased, the end of the platen extended through the center hole in the Plexiglass® top plate, applying a load to the sample on the platform from below. This introduced a nonuniform reproducible strain field to the substrate.

In addition to the loading platform, a polycarbonate alignment jig and cell culture holding system were fabricated, Figure 1A and B. The alignment jig was designed to center samples on top of the mechanical loading platform through the use of centering legs that hung over the edge of the platform. The alignment jig contained a slot to accommodate the cell culture holding system and PDMS substrate. The cell culture holding system was designed to eliminate sample preloading, such as petri dish adhesion, while enabling physical manipulation during experimental setup. The alignment jig maintained positioning of the

PDMS substrate during loading and analysis. This enabled tracking strain as a function of substrate location.

PDMS Substrate

The PDMS well substrate was assembled in three layers, Figure 1C and 1D. The bottom layer consisted of a piece of PDMS 1.9 mm in thickness, with a 9.5 mm in diameter center hole to accommodate for platen loading from below. The middle layer was 200 μm thick and contained a positional reference grid consisting of alphanumerically-labeled squares 490 μm by 490 μm . This allowed comparison of the same areas before and after experimentation, as well as between experiments. This also improved quantification of experiments by enabling the effects of cell substrate strain as a function of loading to be studied. The top layer of PDMS was 3.5 mm thick and contained a center hole 12.7 mm in diameter, creating the side walls of a cell culture well which held medium for culture and testing.

PDMS was fabricated by thoroughly mixing the PDMS base and curing agent at a 10:1 ratio (Dow Corning, Sylgard 184). The solution was vacuum desiccated and poured onto a silicon wafer or into a 60 mm petri dish. The silicon wafer was used for preparing the central layer as it had a pattern for the positional reference grid. After pouring onto the silicon wafer, it was spun at 1500 RPM for 30 seconds, generating a 200 μm thick central layer of PDMS. After being poured onto the petri dish or silicon wafer, PDMS was cured for 2 hours at 70°C then cut to shape using a razor blade. A core borer was used to create center holes in the top and bottom PDMS pieces. The 3 PDMS layers were assembled by applying a thin layer of uncured PDMS between each layer of cured PDMS and curing for 15 minutes at 70°C.

After well fabrication, a digital image correlation algorithm was used to estimate the surface strains on the well. This was done by loading PDMS well substrates that had been spattered with paint. Images were taken every 480 μm (corresponding to every 1000 μm as reported by the microactuator controller). A freeware MATLAB algorithm was used to analyze the images by tracking the pixel movement between sequential images.¹² This provided an estimate of the strains placed on the surface of the PDMS well substrate for generation of a surface strain map. This was repeated for three loading ranges, low (physiologic), medium (physiologic/supraphysiologic) and high (supraphysiologic). These loading ranges were selected to subject osteocytes to a variety of strains ranging from stimulatory to damage inducing.

Cell Culture

Osteocyte-like MLO-Y4 cells (a gift from Dr. Lynda Bonewald), were cultured using a previously developed protocol.²² Minimum essential alpha medium (α -MEM, Gibco) was supplemented with 5% fetal bovine serum (Hyclone), 5% fetal calf serum (Hyclone), and 1% penicillin/streptomycin (Pen/Strep, Invitrogen) used for cell culture. The cells were maintained at 5% CO_2 and 37° C in 25 cm^2 flasks coated with rat tail collagen type I (BD Bioscience) at a concentration of 5 $\mu\text{g cm}^{-2}$ in .02 M acetic acid (Sigma). Cells for continuous culture were passaged between 70 and 80% confluency using .25% Trypsin-EDTA.

MLO-Y4 cells were seeded onto collagen type I coated PDMS well substrates at a density of 2×10^4 cells cm^{-2} . All cells for loading were cultured 4 days prior to experimentation to allow the MLO-Y4 cells to exhibit their characteristic protein expression and morphology.²⁵ Cells were maintained in .5 ml of medium and fed every other day. Cleaning wipes were dipped in sterile water and wrapped around the edge of the petri dish to increase the humidity and lessen medium evaporation.

Cell-Cell Communication

Functional quantification of communication and the effectiveness of its inhibition were quantified using a parachute assay.²⁸ A group of donor cells were dyed with two fluorescent particles before being pipetted onto acceptor cells. After 2 hours of incubation the acceptor cells were imaged with 2 Zeiss fluorescence filters, 1 for each dye. A cyanine 3 filter (with an excitation wavelength of 544 nm and an emission wavelength of 605 nm) was used to detect DiI, while a green fluorescent protein filter (excitation wavelength of 470 nm and emission wavelength of 525 nm) was used to detect calcein AM. The calcein AM could travel through gap junctions from the donor to acceptor cells, while the DiI was membrane bound and used to identify donor cells.

A dye containing 28 μl of .25% 1,1'-Diocadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) (Life Technologies) in dimethyl sulfoxide (Sigma Aldrich), .20 μl of 1% calcein AM (Life Technologies) in dimethyl sulfoxide, 2 ml of 2% bovine serum albumin in Hank's Buffered Salt Solution (Hyclone), and 100 μl of pluronic acid (Life Technologies) was prepared. This dye was added to a plate of donor cells and incubated for 30 minutes before being washed with HBSS, trypsinized, and centrifuged. The acceptor cells on a PDMS well substrate and the centrifuged donor cells were counted and the donor cells were resuspended such that the addition of 100 μl of medium containing cells to the PDMS well substrate would lead to a ratio of 1 donor cell to 500 acceptor cells on the PDMS well substrate. The substrate was incubated for 2 hours and then imaged using two fluorescence filters and overlaid. For inhibition studies, 18- α glycyrrhetic acid (Sigma Aldrich, G8503) was added. Previous studies indicated that this drug treatment did not alter the adhesion or viability of the MLO-Y4 cells.²⁵

Cellular Loading

Following 4 days in culture, PDMS well substrates were loaded using the mechanical loading platform. Samples in which gap junctional intercellular communication was inhibited were treated with 30 μM 18 α -glycyrrhetic acid in dimethyl sulfoxide two hours prior to loading. A short loading time of 15 minutes was used to study the initial impact of microdamage and a static load was employed. The actuator displacement was increased until contact was registered on the load cell. Once contact was made, the displacement was increased by 3840 microns (8000 microns on the controller). This displacement induced physiologic loads based on previous cell viability work.²⁶ Following the 15 minutes of static loading, cells were incubated for 90 minutes prior to the completion of immunocytochemistry or medium collection for an ELISA. Two additional loads were studied. A platen displacement of 5760 μm (12000 μm on the controller) induced a combination of physiologic/supraphysiologic loads as a function of substrate location while

a platen displacement of 7200 μm (15000 μm on controller) induced supraphysiologic loads consistent with microdamage.

Immunocytochemistry and ELISAS

Immunocytochemistry was performed to quantify sclerostin expression as a function of load and in the presence and absence of gap junctions. After 90 minutes of incubation, cells were washed with phosphate buffered saline (PBS) and fixed for 15 minutes at room temperature in 4% paraformaldehyde. Cells were permeabilized by a wash in .2% Triton-x100 in PBS, and nonspecific binding was blocked using a 10% goat serum solution in PBS. Additional PBS washes were completed prior to applying the primary sclerostin antibody (Sclerostin Antibody N-22 goat anti rabbit IgG FITC, Santa Cruz Biotechnology) in 1% bovine serum albumin for an overnight incubation. Following thorough washing in PBS, the secondary antibody in 1% BSA was added to the samples and incubated for 1 hour at room temperature. The samples were then washed and imaged using a Zeiss AxioVert fluorescence microscope.

For the immunocytochemistry, a rigorous microscopy protocol was developed to improve the quality of data obtained. All secondary antibody work was completed in the dark to maintain the fluorescence intensity. While imaging the fluorescence shutter was also shut off to reduce the time the cells were exposed to light whenever possible. At the beginning of each experiment, the exposure time for fluorescence imaging was established by taking images and adjusting the time until finding the longest exposure time that led to a black background. This time was used for the control and each of the experiments that day to improve repeatability. DAPI images were taken to insure the cells were in focus, and the time taken during imaging was minimized when possible. Following imaging, measurements were recorded and analyzed.

Fiji, a distribution of ImageJ, was used for analysis. For each PDMS well, 9 regions of interest were analyzed. These regions were the central positional reference grid square, the 4 grids that were 3 squares away from the middle in the 4 cardinal directions, and the 4 grids that were 6 squares away from the middle in the cardinal directions. In each of these squares, 40 cells were manually selected and the mean pixel intensity in each cell was recorded, yielding 360 mean pixel intensities in each PDMS well substrate. Ten background measurements were also taken in each region of interest to account for differences in the background intensity. Results were then compared to controls (osteocytes seeded on unloaded wells), generating results as a percentage of the sclerostin expression in an unloaded sample. This was done using the control well from the experiment run that day, insuring the data was obtained at the same exposure time and with cells exposed to the same conditions.

ELISAs were used to analyze the concentration of soluble factors in the medium after experimentation. Cytokine specific antibodies were bound to a glass slide and incubated with the medium being analyzed. 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 added in a third incubation. The Cy3 was detected using a laser scanner and compared to standards,

allowing the detection and quantification of cytokines. Sandwich ELISAS were also run in triplicate with 1.5 mL of medium collected. From this 1.5 mL, 1 mL was analyzed, generating an average response across the three samples.

Statistics

D'agostino & Pearson normality tests were run on all sclerostin data. As the results indicated that the sclerostin data from the experiments did not follow a normal distribution, a Mann Whitney U test was used for all nonparametric analyses, and a p value of .05 was used throughout the study.

Results

A digital image correlation algorithm was used to estimate the strains placed on the PDMS well substrate for a given actuator displacement, Figure 2A–C. As microactuator displacement was increased, the average strain seen across the PDMS substrate also increased substantially from 8% at the lowest displacement and reaching a maximum of 47%, figure 2A. This was expected, as a higher level of tenting from below lead to an increase in the strain placed on the PDMS. The digital image correlation algorithm was also used to estimate the strains throughout the PDMS well substrate, Figure 2B–C and Table 1. As indicated, strains consistently peaked near the edge of the platen, and were at their lowest directly over the middle of the platen. In this system, the substrate strains corresponding to the edge of the platen were approximately double the magnitude of strains centrally detected.

After obtaining the digital image correlation algorithm results, sections of the well were chosen for later study. Based on the results of Figure 2B–C and Table 1, sections of the well were chosen to study the range of strains observed, Figure 3. The middle grid in the PDMS well substrate was selected for study because it showed the lowest strains. That is, as shown in Table 1, middle grid strains ranged from 5% with a 3840 μm actuator extension to 35% with a 7200 μm extension. For a given extension, a variable strain field developed across the substrate surface. 4 grids that were 1.5 mm or 3 mm away from the center of the well in the 4 cardinal directions were selected for study. The 4 grids that were 1.5 mm away from the center were selected to represent an intermediate strain and the 4 grids that were 3 mm away were selected to represent a high strain. This is graphically represented in Figure 3.

Cell-Cell Communication

Osteocytes were mechanically loaded in communication intact and inhibited environments. When gap junctions were intact, 64% of osteocytes were in communication. This was significantly reduced in the presence of 18 α -glycyrrhetic acid treatment where only 13% of osteocytes were in communication.²⁵ Figure 4 A and B shows osteocytes in communication intact, while Figure 4 C and D display an inhibited environment.

Immunocytochemistry and ELISAS

Immunocytochemistry was used as a semi quantitative assessment tool for sclerostin expression throughout the PDMS well, figure 5A. As shown, sclerostin expression varied

significantly around the well when gap junctional intercellular communication was present, figure 5B. As the strain increased, the sclerostin expressed increased. Alternatively, when communication was inhibited, a significant increase was seen at physiologic strains before values closer to the control at the physiologic/supraphysiologic and supraphysiologic conditions, figure 5B–C. The inhibition of gap junctional intercellular communication significantly altered the sclerostin expression, Table 2. Physiologic strains at or below 10% led to a decrease in expression, while physiologic/supraphysiologic and supraphysiologic strains led to a significant increase in sclerostin expression. The number of cells analyzed at each condition can be found in Table 3.

Sandwich ELISAs were run to quantify a variety of factors, Table 4. The results indicated that gap junctional intercellular communication significantly altered the expression. Angiopoietin 1 and galectin-3, proteins associated with increased levels of bone formation, increased in expression significantly when communication was inhibited. Galectin-3 was of particular interest as the inhibition of communication led to a 28% increase in galectin-3 expression in unloaded cells, 52% in cells at the physiologic level, and 20% at the supraphysiologic level. SERPINF1 was associated with promoting bone resorption and decreased in expression when communication was inhibited. Activin- α and von Willebrand factor, previously shown to inhibit bone formation and resorption respectively, showed an interesting response when communication was inhibited. When communication was present, both factors showed a decrease in expression when cells stimulated at physiologic/supraphysiologic levels were compared to the cells stimulated at physiologic levels. When inhibited, both factors saw expression increase in cells stimulated at physiologic/supraphysiologic levels relative to cells stimulated at physiologic levels

Discussion

In this study, a mechanical loading platform was used to investigate the impact of mechanically load and gap junctional intercellular communication on sclerostin and other soluble factor expression in osteocyte-like MLO-Y4 cells. A digital image correlation algorithm was used to estimate the strains placed on the PDMS well substrate when samples were tented from below using a platen connected to a linear actuator. From these studies and previous viability studies, it was determined that 3840 micron displacements induced substrate strains no greater than 10%. Substrate strains on the order of 10% were believed to correspond to physiologic osteocyte stimulation which was supported by preliminary data in which conditioned medium from these osteocytes was added to osteoblasts and increased bone formation was observed. In contrast, 5760 and 7200 micron displacements induced substrate strains ranging from 15–60%. These substrate strains induced strains, particularly above 15%, that were assumed damaging. In addition, preliminary data from conditioned medium studies at these magnitudes resulted in decreased bone formation. As such, data was further separated for analysis as physiologic mechanical loads ($\leq 10\%$) and physiologic/supraphysiologic ($>10\%$). Immunocytochemistry studies were conducted as a function of strain, whereas ELISAs which represented a sample of conditioned medium from a range of strain for a given load were grouped as physiologic or physiologic/supraphysiologic.

Mechanical loading studies demonstrated that the sclerostin expression was significantly different in control and loaded osteocytes. These results indicated an increase in sclerostin expression at physiologic strains, below 15%. This aligns with previous *in vivo* results which indicated that mechanical unloading led to an increase in sclerostin expression.¹⁴ Supraphysiologic strains led to values near the control or a slight decrease in expression, consistent with previous *in vivo* studies that saw a decrease in sclerostin expression in mechanically stimulated bone.²¹

This study investigated the impact of gap junctional intercellular communication on sclerostin expression as a function of changes in mechanical strain. A significant decrease in sclerostin expression was observed when communication was inhibited at strains at or below 10% while a significant increase in sclerostin expression was observed at strains above 10%. Thus, the inhibition of gap junctional intercellular communication interestingly led to a switch of the expression pattern between physiologic and physiology/supraphysiologic strains, with an increase at physiologic strains and control levels with communication, and control levels at physiologic strains with an increase at physiologic/supraphysiologic strains when communication was inhibited. This suggests that gap junctional intercellular communication plays an important role in controlling the sclerostin response of MLO-Y4 cells to mechanical loads. This is consistent with a previous *in vivo* study examining the impact of the inhibition connexin 43, a protein that forms gap junctions. In that study, mice with osteocytes and osteoclasts without connexin 43 were exposed to mechanical loads and this led to increased levels of bone resorption.²⁷ The impact of gap junctional intercellular communication on bone remodeling is worth further study.

After examining the sclerostin expression in response to mechanical load and the inhibition of gap junctional intercellular communication, additional soluble proteins were studied using a sandwich ELISA. Factors of particular interest were activin-a, angiopoietin 1, galectin-3, SERPINF1, and von Willebrand Factor, as these factors had previously been associated with bone formation or resorption. The proteins angiopoietin 1 and galectin-3 were previously associated with promoting bone formation.^{7, 11, 18, 23} In this study, both factors increased significantly at all conditions when gap junctional intercellular communication was inhibited. Additionally, SERPINF1, connected to promoting bone resorption, decreased in expression at each of the conditions when communication was inhibited.^{2, 9} This suggested that the inhibition of gap junctional intercellular communication altered the balance of bone remodeling, leading to additional formation and decreased resorption. This also suggested that the presence of this communication led to decreased formation and increased resorption. Activin-a and von Willebrand factor, shown to inhibit bone formation and resorption respectively, showed an altered expression pattern when communication was inhibited.^{1, 5, 19} When present, cells exposed to strains at the physiologic/supraphysiologic level displayed a significantly lower level of expression than those exposed to physiologic strains. However, when communication was inhibited, both factors saw a higher expression in the physiologic/supraphysiologic conditions than they had in the physiologic condition. This suggests that gap junctional intercellular communication may also play a role in regulating the cellular response to microdamage.

In this work we investigated the effects of a range of mechanical strains on osteocyte response in communication-intact and –inhibited environments. This work used a mechanical loading platform that introduced a non-uniform strain field by applying a load using out of plane distension. This allowed the tracking of cellular activity as a function of location and strain using immunocytochemistry, while the conditioned medium could be collected to detect an averaged response. Previous work in the lab indicated that the lowest loading displacement, 3840 μm , induced a stimulatory effect on cellular metabolic activity. The results indicated that the 7200 μm displacement induced damage and decreased the level of metabolic activity, while the 5760 μm load led to an intermediate response. This allowed the study of a range of cellular behavior using this platform.

The use of a non-uniform strain field allowed the study of multiple strains in one experiment. To investigate the sclerostin expression, immunocytochemistry was used to measure sclerostin activity in various locations around the PDMS well substrate. While this allowed the study of different strains in one experiment, the study of a local response necessitated the use of a semiquantitative immunocytochemistry technique. To improve the reliability of the data, a rigorous microscopy protocol was used, and a large number of cells were analyzed in each location. While western blots and ELISAs offer a more quantitative and averaged approach, the study of a variety of locations necessitated the immunocytochemistry. Further experimentation using a further developed substrate may allow the further study of an averaged response.

In addition to the semiquantitative approach, this study could be improved by the development of a more biomimetic cell culture system. This initial study utilized a 2D system in which cells were cultured on a flat piece of PDMS in a well substrate. Current work in the lab aims to develop a structure in which the lacunae environment of osteocytes can be better replicated. Additionally, the platform could be improved by introducing osteoblasts and osteoclasts, the other bone cells. As osteocytes are theorized to play a role in bone the cellular regulation of bone remodeling, a multicellular model consisting of all three cell types provides the best mechanism for investigating the impact of mechanical load and gap junctional intercellular communication on bone formation and resorption. This model is currently under development in the lab, and this work provided an important first step in the platform.

Conclusion

In vivo, osteocytes are subjected to a variety of loads. It is accepted that osteocytes sense load and play a critical role in remodeling and there are a multitude of mechanisms by which this may occur. These include cell-cell interactions (communication), cell-matrix interactions and soluble factors. Our approach to studying these interactions has been to load osteocyte-seeded substrates and correlate mechanical strain with substrate location in an attempt to distinguish physiologic and supraphysiologic substrate loads. Differential osteocyte activity may then be correlated to load and communication extent. While measures of osteocyte activity are currently indirect, current work in the lab is being conducted to quantify the effects of conditioned medium from these cells on bone formation and bone resorption.

This study investigated the impact of mechanical strain and cell-cell communication on osteocyte-like MLO-Y4 sclerostin expression. The results indicated that supraphysiologic levels of strain significantly altered sclerostin expression, leading to an increase in expression at physiologic strains and a slight decrease at supraphysiologic strains, in agreement with previous *in vivo* studies. Additionally, the inhibition of gap junctional intercellular communication significantly altered the sclerostin expression, leading to values near the control at physiologic strains and an increase at supraphysiologic strains. Finally, the expression of soluble factors associated with bone formation and resorption was investigated. The results indicated that the inhibition of gap junctional intercellular communication may shift the balance in bone remodeling by increasing rates of bone formation and decreasing rates of bone resorption. Additional factors indicated that the inhibition of the communication may have also altered the response of MLO-Y4 cells to supraphysiologic strains.

This study indicated that the inhibition of gap junctional intercellular communication significantly altered the response of MLO-Y4 cells, and suggests the need for further study into the impact of this communication on rates of bone formation and bone remodeling. This may best be accomplished through the development of a multicellular model consisting of osteocytes, osteoblasts, and osteoclasts. A PDMS well substrate has been designed to allow the culture of all three cells on one platform. On this device, osteoblasts and osteoclasts can be exposed to conditioned medium from microdamaged osteocytes, including the soluble factors discussed in this paper. Osteoblasts and osteoclasts will be cultured on bone wafers, allowing the precise quantification of the impact of microdamage and gap junctional intercellular communication on the rates of bone formation and resorption.

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Figure 1.

A) Assembled mechanical loading platform. The linear actuator was connected to the load cell, and tented the substrate being loaded from below. The alignment jig sat on top of the platform and held the cell culture holding system in place. B) PDMS well substrate in position for loading. The well substrate sat inside the cell culture holding system, while the alignment jig positioned the well substrate over the platen. C) Detailed view of PDMS well substrate. The top circle corresponded to the well used for cell culture, while the bottom cutout circle provided platen clearance to load the PDMS from below. D) Scanning electron microscopy image of the grid pattern on the PDMS well substrate and MLO-Y4 cells.

Figure 2.

A) Average strain across the PDMS well substrate. Results indicated significant increases in strain as the displacement increased. B and C) Digital image correlation results across the PDMS well substrate at two strains. B corresponded to the combination physiologic/supraphysiologic loading condition, while C corresponded to the supraphysiologic loading condition. Dark colors corresponded to areas of low strains, while the brighter areas indicated higher surface strains.

Figure 3.

Mosaic of PDMS well substrate. The white square labeled M corresponded to the middle, hatched squares labeled 3 corresponded to 3 squares away, and the black square labeled 6 corresponded to 6 squares away from the center. The two white circles corresponded to 1500 μm away, and the black circles to 3000 μm away.

Figure 4.

Example parachute assay. A) Calcein AM expression in cells with gap junctional intercellular communication. B) DiI expression in cells with gap junctional intercellular communication. Images indicated the calcein AM moved through the gap junctions from the donor cells to acceptor cells. C) Calcein AM expression in cells without gap junctional intercellular communication. D) DiI expression in cells without gap junctional intercellular communication. Images indicated that the 18 α -glycyrrhetic acid effectively blocked communication, as calcein AM was expressed only in the donor cells.

Figure 5.
 A) Typical image of sclerostin immunocytochemistry. B) Sclerostin expression after mechanical load. Results indicated a significant increase relative to the controls in each of the three locations, and an increase as the strain increased from 5 to 8 to 10%. C) Sclerostin expression after gap junctional intercellular communication inhibition and mechanical load. Graph displayed a significant increase at the lowest strain, prior to a decrease relative to the control at 8% and a value near the control at 10% surface strain. The results differed significantly from when communication was present.

Table 1

Estimated surface strains on PDMS well substrate. Results indicated the highest strains were seen near the area of the substrate corresponding to the edge of the platen, or 6 squares away. The lowest strains across the PDMS well substrate were seen in the middle square and directly over the middle of the platen. 3 squares away was chosen to cover the midpoint between the two extremes.

Location	Middle	3 Squares Away	6 Squares Away
3.84 mm	5%	8%	10%
5.76 mm	15%	19%	34%
7.2 mm	35%	54%	60%

Table 2

Summary of statistical comparisons of sclerostin expression with communication present and inhibited. A D'Agostino & Pearson omnibus normality test was used to determine that not all samples followed a normal distribution, and a Mann-Whitney U test was used for non-parametric statistical comparisons.

Condition	P Value, Difference
5% Strain	*, 16% decrease
8% Strain	*, 41% decrease
10% Strain	*, 39% decrease
15% Strain	*, 24% increase
19% Strain	*, 40% increase
34% Strain	*, 21% increase
35% Strain	*, 25% increase
54% Strain	*, 10% increase
60% Strain	*, 7% increase
3840 micron Displacement	*, 38% decrease
5760 micron Displacement	*, 30% increase
7200 micron Displacement	*, 10% increase
Middle Square	*, 6% increase
3 Squares Away	NS, 5% decrease
6 Squares Away	*, 10% decrease
All Data	*, 6% decrease

NS represents a comparison that was not significant, while *'s indicate a significant difference, with a p value of .05.

Percentages indicated the change in expression when communication was inhibited, relative to when it was present. Results indicated that the inhibition of communication led to statistically significant decrease at the lowest strains, from 5 to 10% as well as at the lowest displacement. Strains above 15% led to an increase in sclerostin expression, and this was also seen at the physiologic/supraphysiologic and supraphysiologic conditions, at displacement of 5760 and 7200 microns. Comparisons at 6 squares away and of the overall data indicated a decrease, due to the significant decrease in expression at the lowest strains. Results suggested that the inhibition of communication would lead to a decrease in bone mass at supraphysiologic strains.

Number of cells in which sclerostin expression was quantified at each of the conditions used for experimentation. 40 cells were counted in each of the squares counted in an experiment, for a total of 360 cells per PDMS well substrate.

Table 3

Condition	5%	8%	10%	15%	19%	34%	35%	54%
Number of cells	360	1440	1440	360	1440	1440	360	1440
Condition	60%	3.84 mm	5.76 mm	7.2 mm	Middle Square	3 Squares Away	6 Squares Away	Total
Number of cells	1440	3240	3240	3240	1080	4320	4320	9720
Condition – Inhibited	5%	8%	10%	15%	19%	34%	35%	54%
Number of cells	320	1280	1280	320	1280	1280	440	1760
Condition - Inhibited	60%	3.84 mm	5.76 mm	7.2 mm	Middle Square	3 Squares Away	6 Squares Away	Total
Number of cells	1760	2880	2880	3960	1080	4320	4320	9720

Table 4

Soluble factor expression after experimentation, with and without inhibited communication. All numbers indicate the concentration, in pg mL⁻¹. Results indicated significant differences in expression when cells were exposed to mechanically-induced microdamage, as well as the inhibition of gap junctional intercellular communication. Increases in angiopoietin 1, galectin-3, and a decrease in SERPINF1 expression when communication was inhibited suggested that the inhibition of communication increased the rate of bone formation and decreased the rate of bone resorption. Of particular interest was galectin 3, in which the expression in controlled cells increased by 28%, the expression in predamage cells was increased by 52%, and the expression in postdamage cells was increased by 20% when gap junctional intercellular communication was inhibited. Changes in the pattern of expression in activating-a and von Willebrand factor indicated that the inhibition may have altered how the MLO-Y4 cells responded to microdamage.

Factor	Role in Bone Homeostasis	Control	Pre damage	Post damage	Control with Inhibited communication	Pre damage with Inhibited communication	Post damage with Inhibited communication
Activin-a	Inhibit Formation	654	829	488	1143	337	943
Angiopoietin 1	Promote Formation	587	560	590	896	628	829
Galectin-3	Promote Formation	1136	1085	1081	1457	1653	1298
SERPINF1	Promote Resorption	1928	1863	1130	1849	651	747
Von Willebrand factor	Inhibit Resorption	1200	883	652	884	184	570