OSTEOCYTIC LACUNAR-CANALICULAR SYSTEM AND PERICELLULAR MATRIX IN MECHANOSENSING

by

Xiaohan Lai

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Mechanical Engineering

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by

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DEDICATED TO MY BELOVED GRANDPARENTS.
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# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................................... x
LIST OF FIGURES .......................................................................................................................... xii
LIST OF ABBREVIATIONS .......................................................................................................... xvii
ABSTRACT ................................................................................................................................... xviii

Chapter

1 INTRODUCTION ......................................................................................................................... 1

   1.1 Bone Structure .................................................................................................................. 1
   1.2 Bone Cells ...................................................................................................................... 4
       1.2.1 Types of bone cells ............................................................................................... 4
       1.2.2 Interactions between bone cells ........................................................................... 6
   1.3 Bone Growth (Bone Modeling) and Bone Remodeling ........................................... 8
   1.4 Bone Mechanotransduction ......................................................................................... 13
   1.5 Osteocyte Pericellular Matrix ..................................................................................... 18
   1.6 Dynamic Method to Quantify the Osteocytic PCM Using FRAP Approach and Mathematical Model ........................................................ 22
       1.6.1 \textit{In Situ} FRAP measurements of solute diffusion in LCS ................. 22
       1.6.2 Two-compartment mathematical model to derive solute diffusion from FRAP experiments ...................................................... 23
       1.6.3 Three-compartment mathematical model in loaded bone .............. 25
       1.6.4 Real-Time measurement of solute transport in loaded bone ..... 26
       1.6.5 PCM ultrastructure derivation from solute transport ...................... 28
   1.7 Organization of the Dissertation ..................................................................................... 29

2 THE ROLE OF OSTEOCYTE PERICELLULAR MATRIX IN REGULATING SOLUTE TRANSPORT IN THE BONE LACUNAR-CANALICULAR SYSTEM ........................................... 33

   2.1 Abstract ........................................................................................................................... 33
   2.2 Introduction ....................................................................................................................... 34
   2.3 Methods ........................................................................................................................... 39
2.3.1 Mice ............................................................................................. 39
2.3.2 Tibial compliance measurements ............................................. 39
2.3.3 Quantification of the LCS anatomy ......................................... 40
2.3.4 FRAP specimen preparation ................................................... 41
2.3.5 FRAP tracer velocimetry ......................................................... 42
2.3.6 Cellular-level mechanical stimulations .................................... 46
2.3.7 In vivo tibial loading ................................................................. 47
2.3.8 Statistical analysis ................................................................. 48

2.4 Results .......................................................................................... 49
2.4.1 Tibial compliance ................................................................. 49
2.4.2 LCS anatomy ........................................................................ 49
2.4.3 Morphologies of lacunae subjected to FRAP ....................... 51
2.4.4 Tracer diffusivity ................................................................. 52
2.4.5 Transport enhancement ......................................................... 54
2.4.6 Solute velocities ................................................................. 56
2.4.7 Reflection coefficient in the PCM ......................................... 59
2.4.8 Osteocytic PCM ultrastructure in the canaliculi ................. 60
2.4.9 Cellular-level mechanical stimulations ............................. 62
2.4.10 Responses to in vivo loading .............................................. 64

2.5 Discussion .................................................................................. 67
2.5.1 Validation of the tracer velocimetry approach .................... 68
2.5.2 Plug-flow versus parabolic flow .......................................... 69
2.5.3 Shear stress versus fluid drag .............................................. 70
2.5.4 PCM fibers as the osteocyte’s “sensing antenna” ............... 72
2.5.5 PCM fiber density as an indicator of mechanosensitivity .... 73
2.5.6 Limitations of the present studies ...................................... 76

2.6 Conclusions ................................................................................. 77

3 THE INFLUENCE OF BONE COMPARTMENT, AGE, AND DISEASE
ON THE CYTO-ARCHITECTURE OF OSTEOCYTE LACUNAR-
CANALICULAR SYSTEM ................................................................. 79

3.1 Abstract ...................................................................................... 79
3.2 Introduction ................................................................................ 80
3.3 Materials and Methods ............................................................ 83
3.3.1 Experimental groups .......................................................... 83
3.3.2 Confocal imaging and analysis of 3D osteocyte LCS macrostructure ...................................................... 84
3.3.2.1 Plastic embedded processing of basic fuchsin stained specimens ......................................................... 84
3.3.2.2 Confocal imaging ................................................................. 84
3.3.2.3 Quantitative measures of 3D LCS macrostructure ...... 87

3.3.3 TEM analysis of osteocyte LCS ultrastructure............... 88
3.3.3.1 Processing for TEM imaging................................. 88
3.3.3.2 Transmission electron microscopy ......................... 89
3.3.3.3 Quantitative analysis of osteocyte LCS ultrastructure . 90

3.3.4 Statistical Analysis ................................................................. 92

3.4 Results ................................................................................................. 92
3.4.1 Confocal-Based Measurements ................................................... 92
3.4.2 TEM-Based Measurement ......................................................... 94

3.5 Discussion ........................................................................................... 103

4 REDUCED HEPARAN SULFATE PROTEOGLYCAN (HSPG) IN CALVARIA----A POTENTIAL MECHANISM FOR CALVARIA’S RESISTANCE TO DISUSE? .......................................................... 110

4.1 Abstract............................................................................................. 110
4.2 Introduction ......................................................................................... 111
4.3 Methods ............................................................................................ 113

4.3.1 Alcian Blue staining ................................................................. 113
4.3.1.1 Animals ............................................................................. 113
4.3.1.2 Sample preparation ............................................................ 113
4.3.1.3 Staining ............................................................................. 114
4.3.1.4 Data collection and analysis ............................................. 114

4.3.2 FRAP diffusion study ................................................................. 115
4.3.2.1 Specimen preparation .......................................................... 115
4.3.2.2 FRAP diffusion experiments .............................................. 116
4.3.2.3 Histological measurements of lacunar-canalicular microanatomy of calvaria ........................................ 116
4.3.2.4 Data analysis........................................................................ 117
4.4 Results ................................................................................................................................. 118
  4.4.1 Alcian Blue staining ................................................................................................. 118
  4.4.2 FRAP diffusion study .............................................................................................. 119
4.5 Discussion .................................................................................................................... 125
5 CONCLUSIONS AND FUTURE DIRECTIONS ................................................................. 129
  5.1 Significance .................................................................................................................. 129
  5.2 Future directions .......................................................................................................... 131
REFERENCES ....................................................................................................................... 133

Appendix
A IMAGING AND QUANTIFYING SOLUTE TRANSPORT ACROSS PERIOSTEUM: IMPLICATIONS FOR MUSCLE–BONE CROSSTALK . 151
B PERLECAN/HSPG2 DEFICIENCY ENHANCES THE DIFFERENTIATION AND MINERALIZATION OF MESENCHYMAL STROMAL CELLS BUT REDUCES THEIR INTRACELLULAR CALCIUM SIGNALING RESPONSES TO FLUID FLOW STIMULATION ................................................................................................................. 160
C UNIVERSITY OF DELAWARE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPLICATION TO USE ANIMALS IN RESEARCH AND TEACHING ................................................................................................. 162
D PUBLICATION INFORMATION ....................................................................................... 186
**LIST OF TABLES**

Table 1.1. Representative biological molecules in bone [34]........................................ 12

Table 2.1. Confocal and TEM imaging of the LCS transport features in the 8-9 month-old male control (CTL) and perlecan deficient (Hypo) mice........ 51

Table 2.2. Lacunar morphology in the 12-13 month-old male control (CTL) and perlecan deficient (Hypo) mice subjected to FRAP tests............... 52

Table 2.3. Load-induced tracer velocity in the bone canaliculi of the 12-13 month-old male control (CTL) and perlecan deficient (Hypo) .......... 59

Table 2.4. PCM’s sieving property measured in young adult, aged CTL and aged Hypo bones using the FRAP tracer velocimetry approach............. 60

Table 2.5. Fiber volume fraction estimated for young adult, aged CTL and aged Hypo bones.............................................................................................. 61

Table 2.6. Fiber spacing estimated for young adult, aged CTL and aged Hypo bones........................................................................................................ 61

Table 2.7. Comparisons of fluid shear stress and drag force between aged CTL and Hypo bones under 3-N tibial loading........................................ 63

Table 2.8. Results of µCT imaging, mechanical testing, and dynamic bone labeling analysis at the tibial mid-diaphysis of 3.5 month-old CTL and Hypo mice.................................................. 66

Table 3.1. Confocal-Based LCS Measurements—Compartmental Comparisons .... 97

Table 3.2. Confocal-Based LCS Measurements—Effects of Age............................... 98

Table 3.3. Confocal-Based LCS Measurements—Effects of Perlecan Deficiency. 98

Table 3.4. Confocal-Based LCS Measurements—Effects of Diabetes ................. 99

Table 3.5. TEM-Based LCS Measurements—Compartmental Comparisons ........ 100

Table 3.6. TEM-Based LCS Measurements—Effects of Age............................... 101
Table 3.7. TEM-Based LCS Measurements—Effects of Perlecan Deficiency ...... 101
Table 3.8. TEM-Based LCS Measurements—Effects of Diabetes ......................... 102
Table 4.1. Anatomical parameters and tracer diffusion coefficients measured in the FRAP experiments in murine calvaria ........................................... 121
Table 4.2. Anatomical parameters and tracer diffusion coefficients measured in the FRAP experiments in murine tibia ................................................. 123
LIST OF FIGURES

Figure 1.1. Anatomy of bone structure and the LCS. Bone matrix is a composite material with three levels of pores. Signaling molecules, nutrients and metabolites mainly transport through the tiny pores consisting of lacunae (diameter of 10-20 μm) and canaliculi (diameter of 0.5 μm) in LCS [7]. ................................................................. 4

Figure 1.2. The communication network between osteocytes and osteoblasts and osteoclasts [31]. ........................................................................................................ 8

Figure 1.3. Diagram showing a longitudinal section of bone remodeling unit. (A) Osteoclast radially enlarges a resorption cavity advancing from right to the left. (B) Precursor cells from blood. (C) Capillary. (D) Osteoblast progenitors. (E) Osteoblasts forming bone radially. (F) Flattened cells lining Haversian canal. Different stages of remodeling: (I) resorption, (II) reversal, (III) formation, (IV) completed Haversian system [33]. .................................................................................. 11

Figure 1.4. The existence of osteocyte PCM and the transverse elements were verified using TEM imaging. Perlecan was identified in osteocyte PCM using immune-gold labeling and TEM imaging. .............................. 20

Figure 1.5. FRAP measurement of solute diffusion. (a) The bone was injected with fluorescent tracer to allow the ellipse shaped lacunae and LCS to fill with soluble fluorescence-labeled molecules. (b) One lacuna was photobleached by high intensity laser. The photobleached area became darker than its surrounding. (c) As time passed, fluorescent intensity of the photobleached area recovered because the surrounding unphotobleached fluorescent molecules moved into the photobleached region. (d) The fluorescent changes of the photobleached lacuna before and after photobleaching were recorded using confocal microscopy. The time course of the fluorescent intensity changes was fitted into a mathematic model to quantitatively determine the diffusion coefficient of the fluorescent molecule [107]... 23
Figure 1.6. The tracer diffusion characteristic was analyzed using the two-compartment model. The sink represents the photobleached lacuna, while the source represents the neighboring unphotobleached lacunae. The channel connecting source and sink represents canaliculi. The diffusion coefficient ($D$) was calculated for each FRAP experiment, determined by the slope of the fitting line: $k = VrD/d^2$ [107].

Figure 1.7. A three compartment mathematical model was developed in loaded bone. This model consists of a central photobleached lacuna and its two neighboring lacunae as source reservoir through connecting canaliculi (six at each side). The tracer fluorescent recovery rate in the sink lacuna was calculated from the concentration profile [108].

Figure 1.8. The osteocyte PCM was approximated with that of an orthogonal fiber network where 1/3 of the fibers form a longitudinal fiber array and the rest of fibers (2/3) form the transverse fiber array. A periodic fluid unit (dash circle) was created to derive fluid flow field around the fibers [111].

Figure 2.1. Diffusivity of sodium fluorescein (Stokes radius 0.45 nm) and parvalbumin (Stokes radius 1.31 nm) in cortical bone LCS of the 12- to 13- month-old CTL and Hypo mice. Two-way ANOVA showed that genotype and tracer type had significant effects on the measurements. Perlecan deficiency increased diffusion for both small and large molecules. Bars denoted with different letters are significantly different in paired comparisons ($p<0.05$). The sample size (the number of lacunae subjected to FRAP tests) is indicated for each study group directly under the corresponding data.

Figure 2.2. Transport enhancement of sodium fluorescein and parvalbumin in loaded versus non-loaded tibial LCS of the 12- to 13-month-old Hypo and CTL mice. Two-way ANOVA showed that genotype and tracer type had significant effects on the measurements. Although no significant difference was detected for multiple comparison using Bonferroni post hoc tests, the larger parvalbumin moved significantly faster in the Hypo PCM relative to the CTL PCM as assessed by unpaired Student’s t test ($p = 0.03$). The $n$ values of paired FRAP tests per study group ranged from 10 to 20 lacunae.
Figure 2.3. Simulated transport enhancement as a function of solute velocity for the 12- to 13-month-old CTL and Hypo bone LCS, using their custom three-compartment transport models. The transport enhancement followed a power relationship with the solute velocity in both CTL (solid lines) and Hypo bones (dashed lines) and for sodium fluorescein (smaller circles) and parvalbumin (larger squares). Detailed relationships can be found in the text (Eq. [2.4–7]).

Figure 2.4. Canalicular flow profiles for the 12- to 13-month-old CTL and Hypo bones under 3-N peak load. For the case of fiber radius of 2 nm, the velocity profile between the cell process and the canalicular wall appeared to be more plug flow-like in the CTL bones (the fiber spacing of 13.4 nm) but more parabolic-like in the Hypo bones (the fiber spacing of 17.4 nm). Note that the fluid annulus in the Hypo bones had a gap of 78 nm, which was 10% smaller than that in the CTL bones (a gap of 87 nm) as reported in Table 2.1.

Figure 2.5. The 3.5-month-old Hypo mice did not respond to anabolic tibial loading (8.5-N peak load, 4 cycles/s, 5 minutes per session, five sessions over 10 days) as did the age-matched CTL mice in a preliminary experiment. (A) Ct.pMOI; (B) tibial stiffness; (C) Ps.MS/BS; and (D) Ps.MAR. Sample sizes: CTL (n=8) and Hypo (n=6) mice for µCT analysis; CTL (n=7) and Hypo (n=6) mice for mechanical testing and bone labeling analyses. Student’s t tests on the nonleaded versus loaded paired tibiae revealed significant changes (p<0.05, denoted by* symbols) in CTL mice but not in Hypo mice. A full data set with other histomorphometry parameters and detailed p values of statistical tests can be found in Table 2.8.

Figure 2.6. A working hypothesis depicting the osteocytic perlecan-rich PCM, which act as flow-sensing antenna, capturing flow-induced fluid drag, and triggering osteocyte’s mechanotransduction process. A denser PCM (as in normal CTL bone) would result in a higher fluid drag force on the transverse tethered fibers, which could trigger downstream signaling and/or gene expression through interactions with cell membrane via various channels membrane receptors, or the physically connected PCM-integrin/focal adhesion complex-cytoskeleton system. Conversely, a sparser PCM (as in perlecan-deficient bone) would result in a smaller fluid drag and the degree of downstream responses would be reduced in spite of the same mechanical loading at the whole bone level. PCM=pericellular matrix; CTL=control; Hypo=hypomorphic, perlecan-deficient.
Figure 3.1. A representative confocal preview image (A) of basic fuchsin stained sagittal section of a murine distal femur, showing the two ROIs located in metaphyseal cortical and cancellous bone compartments. (B&C) Due to larger number of lacunae in ROI1, a grid was overlaid on the image and 30-40 lacunae that fell on the intersections of the grid, as outlined by the yellow boundaries, were selected for a quick check of full-depth structures. Ten lacunae with intact 3D structures were chosen for high-resolution 3D imaging. For ROI2, ten lacunae were randomly chosen per animal without the grid. ................................. 86

Figure 3.2. Representative 3D renderings built from z-stack confocal images of lacunae and associated canaliculi were used to quantify (A) canalicular number in VOLOCITY® and (B) lacunar volume, surface area and major and minor radii in AMIRA®. ............................................................ 88

Figure 3.3. The ultrastructural measurements of osteocyte lacunae were obtained from TEM images by (A) tracing the lacunar wall and cell body in Photoshop® for (B) quantifying the shape of the lacuna (width/height), the cross-sectional areas of lacuna, cell body, and the pericellular annulus in Image J. (C) The mean thickness of the pericellular annular gap was measured using the “bubble” method implemented in the BoneJ plugin of Image J. ......................................................... 91

Figure 3.4. Measurements of osteocytic canaliculi were obtained from TEM images following the same procedure as the measurements of lacunae. (A) Traces of canalicular wall and cell process; (B) Quantification of areas of cell process, canalicular wall, and pericellular regions; (C) Measurements of pericellular thickness in canaliculi using the “bubble” method........................................................................................................ 91

Figure 3.5. Osteoblasts are differentiated into osteocytes, which form an orderly network through the connecting canaliculi. The number of canaliculi is determined mainly by the surface area of lacuna (N=S [μm²]/5) regardless of bone compartment, age, and disease conditions. In cortical bone, aging is associated with larger canalicular annulus area and lower fiber density, while perlecan (Pln) deficiency reduces both annulus area and fiber density compared with normal sample (Tables 4.6 & 4.7). ........................................................................................................ 107
Figure 4.1. Representative images of Alcian Blue staining in B6 calvaria (A) and PLN Hypo femur (C). Blowup images of the boxed areas in (A) and (C) are shown in (B) and (D), respectively. Scale bars = 500µm in (A, C); Scale bars = 50µm in (B, D). ............................................................ 114

Figure 4.2. HSPG content in calvaria and femurs in B6 (blue color) and PLN Hypo mice (red color). Significant difference was found between femur and calvaria regardless of genotype. No significant difference between PLN Hypo and B6 bones. ........................................................ 119

Figure 4.3. Representative curves for calvaria and tibia FRAP. $I_0$, pre-bleach intensity; $I_\infty$, recovered intensity at new equilibrium; $I_b$, intensity immediately after photobleaching. Bound fraction ($\partial$) is shown in the figure, and it is significantly higher in calvaria than in tibia. .......... 127
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>A</th>
<th>ATP</th>
<th>Adenosine triphosphate</th>
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<tbody>
<tr>
<td>B</td>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>D</td>
<td>DMP</td>
<td>Dentin matrix protein</td>
</tr>
<tr>
<td>E</td>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td></td>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>Electron microscope</td>
</tr>
<tr>
<td>F</td>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td></td>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td></td>
<td>FSS</td>
<td>Fluid shear stress</td>
</tr>
<tr>
<td>G</td>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>H</td>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>I</td>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>L</td>
<td>LCS</td>
<td>Lacunar-canalicul system</td>
</tr>
<tr>
<td>M</td>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O</td>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td></td>
<td>OSF-1</td>
<td>Osteoblast stimulating factor-1</td>
</tr>
<tr>
<td>P</td>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td></td>
<td>PCM</td>
<td>Pericellular matrix</td>
</tr>
<tr>
<td></td>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>R</td>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td></td>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td></td>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>S</td>
<td>SGP</td>
<td>Strain-generated potentials</td>
</tr>
<tr>
<td>T</td>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>V</td>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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ABSTRACT

Bone is an important component of the skeletal system to ensure locomotion and provides mechanical support to the body. Bone tissue is able to adapt its mass and three-dimensional structure to the prevailing mechanical usage and thus obtain a higher efficiency of load bearing. My studies aimed to better understand the cellular mechanisms responsible for this adaptation phenomenon of bone.

As the most abundant cells in bone, osteocytes form an extensive cellular network through numerous cell processes emanating from individual cell bodies. These cellular protrusions and cell bodies are housed with an extensive pore system, the lacunar-canalicular system (LCS), and buried within the mineralized matrix of bones. This cellular network allows osteocytes to obtain nutrients from the blood supply, sense external mechanical signals, and communicate among themselves and with other cells on bone surfaces. The osteocytic pericellular matrix (PCM), a fibrous thin coating surrounding osteocytes and their long “dendritic” processes, is believed to play a critical role in osteocyte nutrition, cell-to-cell signaling, and mechanotransduction through regulation of fluid shear stress on the cell membrane and drag forces on the PCM fibers. The study of the osteocyte PCM in situ, however, is challenging because it is thin (~100 nm) and enclosed in mineralized processes. To fill the knowledge gap, our laboratory recently developed a novel tracer velocimetry
approach that combined fluorescence recovery after photobleaching (FRAP) imaging with hydrodynamic modeling to quantify the structure of the osteocytic PCM in murine bone. In my first study, I applied the technique to mice with a deficiency in the expression of perlecan/HSPG2, a large heparan sulfate proteoglycan that is secreted in the osteocytic PCM of wild type mice. The aims of the study were to examine the effects of perlecan deficiency in the PCM on mechanosensing and test the sensitivity of the velocimetry approach. The results indicated increased diffusion and convection of tracer molecules and sparser osteocyte PCM in the perlecan-deficient bone. This result showed the usefulness of the new technique in studying osteocyte functions in vivo. Furthermore, the ultrastructure of the PCM and cellular stimulation forces were compared among young adult, aged and perlecan-deficient bones. Since the osteocyte LCS structure is an important parameter in the cellular stimulation, I adopted the TEM imaging in my second study to obtain the parameters such as canalicular density, canalicular annual gap and compared them between cortical and cancellous compartments, at different ages, and in two disease conditions. The LCS network showed both topological stability, in terms of the conservation of canalicular connectivity among osteocyte lacunae, and considerable variability in the pericellular annular fluid gap surrounding lacunae and canaliculi. This information may further help in estimating load-induced fluid flow in the LCS and the resultant cellular-level stimulation forces due to the interactions of the fluid flow and PCM within the LCS channels. My third study addressed the issue whether the PCM differs in different bone sites (long bone vs. flat bone) using Alcian Blue staining and FRAP diffusion
experiments. Murine flat bones showed lower Alcian blue staining intensity, suggesting reduced expression of HSPG than that of long bones. Also, tracer molecules diffused faster in the flat bones than in the long bones, indicating potential reduction of HSPG content in PCM of flat bone. The differences in PCM composition between long bone and calvaria may provide a potential explanation to calvaria’s surprising resistance to disuse bone loss. Taken together, the studies suggest the PCM fibers act as osteocyte’s sensing antennae, regulating the hydrodynamic forces experienced by the cell and thus bone’s sensitivity and *in vivo* adaptation to its mechanical environments. Quantification of the PCM fiber density or related characteristics could be a powerful tool to identify an individual’s sensitivity to loading and provide new targets to promote bone formation in osteoporotic patients.
Chapter 1

INTRODUCTION

In this chapter, I review the basics of bone structure, bone cells, bone growth, osteocyte pericellular matrix and experimental techniques like fluorescence recovery after photobleaching (FRAP) approach. These sections serve as introduction of my three projects, which are outlined here and detailed in the following chapters.

1.1 Bone Structure

Bone maintains the shape of the body, protects the soft tissue, transmit force during movement and serves as a reservoir for minerals. Also bone is a self-repairing structural material, able to adapt its mass, shape and properties to changes in mechanical environment. Bone consists of 65% mineral, 35% organic matrix, cells and water.

In terms of overall shape, bones are classified into four types: long bone (e.g., humerus, femur, and tibia), short bone (patella), flat bone (e.g., scapula and calvaria), and irregular bone (e.g., vertebrae). Long bone and flat bone are investigated in the dissertation. A typical long bone, the major subject of this dissertation, consists of a central cylindrical shaft (diaphysis) and two wider and rounded ends (epiphyses). Conical regions called the metaphysis connect the diaphysis with each epiphysis (Figure 1.1 left panel).
The adult human skeleton is composed of approximately 80% cortical bone and 20% trabecular bone. The diaphysis of a long bone is composed mainly of cortical bone, while the epiphysis and metaphysis contain mostly trabecular bone. Cortical bone is a dense, solid mass with the structural unit the osteon, or called Haversian systems, 200 ~ 250 µm in diameter. Osteon is made of circular rings of concentric lamellae (3 ~ 7 µm thick) surrounding a longitudinal vascular channel, called the Haversian canal (~ 50µm in diameter) (Figure 1.1 middle panel). Neighboring Haversian canals are connected by transverse vascular channels, called the Volkmann canal (Figure 1.1 middle panel). Trabecular bone, which is more porous than cortical bone, consists of large plates and rods known as the trabecula (50 µm thick and 1 mm long).

Bone tissue is a porous medium with hierarchical porosities. The three levels of bone porosities are vascular porosity (~ 10µm), lacunar-canalicular porosity (~ 100nm) and collagen-hydroxyapatite porosity (20 ~ 60nm) [1]. The lacunar-canalicular system (LCS) is composed of small ellipsoidal cavities (lacunae, major diameter ~ 20 µm, minor diameters, ~ 10 µm) and slender tubular canals (canaliculi, diameter ~ 260 nm, length ~ 30 µm) connecting neighboring lacunae [2] (Figure 1.1 right panel). Osteocytes, mature bone cells that reside in a fluid-filled mineralized bone matrix, are housed in lacunae with their cell processes contained in canaliculi. About 20 to > 100 canaliculi emanate from each lacuna, depending on species [3]. The LCS ensure the communication among osteocytes, osteocytes to bone lining cells on bone surface, as well as progenitors and vascular cells in bone
marrow and Haversian canals. Between the osteocytic processes and the canalicular wall, there is an annular space (50 ~ 100 nm wide), which is hypothesized to be filled with proteoglycan-like fibers [1, 4]. The gap between these fibers should be 6 ~ 12 nm based on previous perfusion studies [5, 6]. Mathematical models by Weinbaum and coworkers have shown that the fiber spacing is very critical to determine the magnitude of load- induced bone fluid flow. However, there are few measurements on PCM fiber spacing, which motivated my first study detailed in Chapter 2. Since the other parameters such as canalicular density, canalicular annual gap between the cell process membrane and canalicular wall are also important for bone fluid flow and nutrient supply and removal, my second TEM study aimed to elucidate the features of LCS at different bone compartments, age, and diseased conditions.
Figure 1.1. Anatomy of bone structure and the LCS. Bone matrix is a composite material with three levels of pores. Signaling molecules, nutrients and metabolites mainly transport through the tiny pores consisting of lacunae (diameter of 10-20 μm) and canaliculi (diameter of 0.5 μm) in LCS [7].

1.2 Bone Cells

1.2.1 Types of bone cells

The major cellular elements of bone consist of osteoclasts, osteoblasts, osteocytes, bone-lining cells, and the precursors of these specialized cells.

Osteoclasts, the bone resorbing cells, are multinucleated giant cells that contain from 1 to > 50 nuclei with a diameter from 20 to > 100 μm. Their role is to resorb bone, solubilize both the mineral and organic component of the bone matrix. The osteoclasts are derived from cells in the mononuclear/phagocytic lineage of the hematopoietic marrow, and their life span appears to be up to 7 weeks in vivo with a
half-life of 6 to 10 days. The osteoclast number and maturation are regulated by the receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin (OPG) ratio. Bisphosphonates, calcitonin, and estrogen are commonly used to inhibit resorption and are believed to act by inhibiting the formation and activity of osteoclasts and promoting osteoclast apoptosis [8, 9].

Osteoblasts, the bone forming cells, are cuboidal cells (that rarely undergo mitosis) with a large nucleus, cellular processes, gap junctions, abundant endoplasmic reticulum (ER), enlarged Golgi, and collagen-containing secretory vesicles. They occur as a layer of contiguous cells with thickness 15 ~ 30 µm. Their role is to form bone, synthesize and secrete the un-mineralized bone matrix or ground substance of bone. The osteoblasts are derived from mesenchymal progenitors, and their precursors are located near bone surfaces. The development and differentiation of osteoblasts are regulated by transcription factors (Runx2), cytokines (IFNs, TNFα, IL1, IL6), hormones (PTH) and growth factors (TGFβ, FGF, PDGF, VEGF) [10].

Bone-lining cells are flattened, elongated cells covering quiescent bone surface. The bone-lining cell has a distinct morphological phenotype with a thin, flat nuclear profile and attenuated (1 µm thick and up to 12 µm long) cytoplasm extending along the bone surface. Bone-lining cells are believed to be derived from osteoblasts that have become inactive, and they are able to become active osteoblasts [11], which are capable of forming bone, but their fate is not known.

Osteocytes are the most abundant cell type (90 ~ 95%) in mature bone with about ten times more osteocytes than osteoblasts in normal human bone. It is well
established that the origin of osteocytes is osteoblasts embedded in its own matrix. The embedded osteoblasts in lacunae differentiate into osteocytes by losing much of their organelles but obtaining long and slender processes. Osteocytes are important for bone health and bone quality. Osteocytes are thought to be the primary sensing cells responsible for bone’s adaptation to mechanical force, one of the most potent anabolic stimuli [10, 12-14]. They are dispersed throughout the bone matrix and connect with each other via slender cell processes, allowing direct cell-to-cell coupling through gap junctions. Previous experimental studies [12, 13, 15, 16] have demonstrated that osteocytes in intact bone change their metabolic activity rapidly after mechanical loading, indicating their function as mechanosensors. Ablation of osteocytes was found to produce bone fragility and defective mechanotransduction [17].

1.2.2 Interactions between bone cells

It has been well established that osteoblasts plays an important role in the differentiation and function of osteoclasts by producing several factors that directly bind to osteoclastic precursors [18]. Both osteoblasts and osteoclasts are derived from precursors originating in bone marrow and osteoblast differentiation is a prerequisite for osteoclast development. The importance of osteoblasts in osteoclastogenesis has been demonstrated by previous experimental research [19]. In addition, study has revealed that a paracrine factor secreted by osteoblasts, known as macrophage colony stimulating factor (M-CSF), would bind to its receptor, c-fms, on osteoclastic precursors [20]. The experiments showed the mice that lacked M-CSF had very few
osteoclasts. Furthermore, osteoblastic cells have been found to express osteoprotegerin (OPG), a crucial osteoclast inhibitory factor [21], and also receptor activator of nuclear factor-κB ligand (RANKL), important for osteoclast development [22]. RANKL-deficient mice could develop severe osteopetrosis with no osteoclasts in bone [23].

Besides the interaction between osteoblasts and osteoclasts, fundamental communication network was discovered between osteocytes and osteoblasts and osteoclasts (Figure 1.2). Evidence [24] was found that osteocytes may stimulate osteoblast recruitment and differentiation by expressing osteoblast stimulating factor-1 (OSF-1). The osteoblasts further differentiate to osteocytes being surrounded by the osteoid matrix that they produce, and they then may become a new source of OSF-1 for the next round of osteoblast recruitment. Osteocytes also act as inducers of osteoclast activation. Microcracks may induce osteocyte apoptosis [25], signaling the osteoclasts to resorb bone and remove damaged tissues.

There is increasing evidence that osteocytes sense mechanical loading through the interstitial fluid flow [1, 26, 27]. In response to fluid flow, osteocytes were found to release signaling molecules like nitric oxide (NO), adenosine triphosphate (ATP), sclerostin, prostaglandin E2 (PGE2) and osteoprotegerin/receptor activator for nuclear factor ligand (OPG/RANKL), which regulates osteoblastic bone formation as well as osteoclastic-targeted bone resorption (Figure 1.2) [27-30].
1.3 Bone Growth (Bone Modeling) and Bone Remodeling

Skeletal development begins as mesenchymal condensations that happen during embryogenesis. The condensations ossify to form membranous bones through intramembranous ossification and cartilage bones through endochondral ossification. Bone formation begins with an increase in the number of cells and fibers. The cells differentiate into osteoblasts, which lay down an unmineralized matrix, the osteoid that mineralizes almost immediately. The shape and manner of growth of the new bone are genetically determined.

Intramembranous ossification or membrane bone formation forms the bulk of the cortical bone shell. This mechanism forms most of the bones of the skull vault,
many of the bones of the sense organs and of the facial skeleton, and parts of the clavicle and mandible. The primary center of ossification of each bone appears as an increase in cells and fibers, and then bone is apposed. Trabeculae are formed by osteoblasts and interconnect to form the primary cancellous bone. Compact bone is formed when the spaces between the primary cancellous bones are filled in by the primary osteons or Haversian systems.

Endochondral ossification or cartilage bone formation forms the bulk of the future cancellous bone. It forms most of the bones at the base of the skull, the vertebral column, the pelvis, and the extremities. Chondrocytes proliferate and deposit matrix until a cartilage model of the future bone is formed. Chondrocytes mature, grow, and its matrix calcifies. Unresorbed calcified cartilage cores form the substrate in which osteoblasts appose woven bone to form the primary cancellous bone [11].

Genetic and circulating systemic factors control general growth, while systemic and regional factors, especially mechanical usage, can modulate it locally. Growth and modeling go hand in hand. Modeling involves resorption drifts and formation drifts that remove or add bone over wide regions of bone surface, lead to increasing the outside cortex and marrow cavity diameters, shaping the ends of long bones and drifting of trabeculae and cortices. Modeling allows not only the development of normal architecture during growth, but also the modulation of this architecture and mass when the mechanical condition changes. Modeling controls the growth, shape, size, strength, and anatomy of bones and joints.
Bone must replace or renew itself because the quality of adult bone deteriorates with time during repeated loading. The replacement of immature and old bone occurs by resorption, followed by formation of new lamellar bone, a process called remodeling, which occurs throughout life. The operational group of cells that accomplishes bone remodeling process is referred to as a basic multicellular unit (BMU) shown in Figure 1.3. The life cycle of the unit includes six consecutive stages of resting, activation, resorption (Figure 1.3 I), reversal (coupling, Figure 1.3 II), formation (Figure 1.3 III), mineralization, and back to resting. In large adult animals, about 80% of the cortical and trabecular bone surfaces and 95% of intra-cortical surface are resting. In response to local structural or biomechanical requirements, activation phase begins with the bone-lining cells digest the endosteal membrane and to retract, exposing the mineralized bone surface, which is chemotactic for osteoclast precursor cells. Then osteoclasts would come in contact with the bone surface and begin to erode the bone at a speed of 20 ~ 40 µm/day longitudinally and 5 ~ 10 µm/day radially. The resorption phase takes about 1 to 3 weeks. After a 1 ~ 2 week reversal interval between resorption and formation, osteoblasts begin to deposits a layer of bone matrix called the osteoid seam which will take 5 ~ 10 days to reach 70% of its final mineralization and about 3 ~ 6 months to complete mineralization in both cortical and trabecular bone. Remodeling rate is dominated by mechanical usage and modulated by parathyroid hormone, thyroxin, growth hormone and microdamage. Estrogen, calcitonin, and bisphosphonates decrease BMU creations and the existing remodeling spaces fill with bone [32].
Figure 1.3. Diagram showing a longitudinal section of bone remodeling unit. (A) Osteoclast radially enlarges a resorption cavity advancing from right to the left. (B) Precursor cells from blood. (C) Capillary. (D) Osteoblast progenitors. (E) Osteoblasts forming bone radially. (F) Flattened cells lining Haversian canal. Different stages of remodeling: (I) resorption, (II) reversal, (III) formation, (IV) completed Haversian system [33].

A spectrum of biological molecules can be found in bone, and selected molecules are listed in Table 1.1.
### Table 1.1. Representative biological molecules in bone [34]

<table>
<thead>
<tr>
<th>Molecule</th>
<th>MW (Da)</th>
<th>Shape</th>
<th>Category</th>
<th>Functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>30</td>
<td>Small molecule</td>
<td>Signaling</td>
<td>Osteocyte transient signaling molecule, osteoblast proliferation</td>
<td>[35, 36]</td>
</tr>
<tr>
<td>Glucose</td>
<td>180</td>
<td>Small molecule</td>
<td>Nutrition</td>
<td>metabolism</td>
<td></td>
</tr>
<tr>
<td>PGE2</td>
<td>352</td>
<td>Globular</td>
<td>Signaling</td>
<td>osteoblastic formation</td>
<td>[37, 38]</td>
</tr>
<tr>
<td>ATP</td>
<td>507</td>
<td>Globular</td>
<td>Nutrition/signal</td>
<td>metabolism/intracellular Ca^{2+}</td>
<td>[39]</td>
</tr>
<tr>
<td>calcitonin</td>
<td>3,500</td>
<td>Globular</td>
<td>hormone</td>
<td>calcium &amp; phosphorus metabolism, osteoclast marker</td>
<td>[40]</td>
</tr>
<tr>
<td>EGF</td>
<td>6,000</td>
<td>Globular</td>
<td>Growth factor</td>
<td>cellular proliferation, differentiation; osteoclast formation</td>
<td>[41, 42]</td>
</tr>
<tr>
<td>BMP-7</td>
<td>15,680</td>
<td>Globular</td>
<td>Growth factor</td>
<td>osteoblast proliferation, differentiation</td>
<td>[43]</td>
</tr>
<tr>
<td>BMP-2</td>
<td>18,000</td>
<td>Globular</td>
<td>Growth factor</td>
<td>formation of bone, osteoblast differentiation</td>
<td>[44]</td>
</tr>
<tr>
<td>Sclerostin</td>
<td>24,000</td>
<td>Globular</td>
<td>Signaling molecule</td>
<td>Inhibitor of osteoblastic bone formation</td>
<td>[45]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>25,000</td>
<td>Globular</td>
<td>Growth factor</td>
<td>cell proliferation and</td>
<td>[46]</td>
</tr>
<tr>
<td>RANKL</td>
<td>35,000</td>
<td>Globular</td>
<td>Signaling molecule</td>
<td>osteoclastogenesis and bone loss</td>
<td>[47, 48]</td>
</tr>
<tr>
<td>DMP-1</td>
<td>100,000</td>
<td>Globular</td>
<td>matrix protein</td>
<td>mineralization of bone and dentin</td>
<td>[49, 50]</td>
</tr>
<tr>
<td>OPG</td>
<td>120,000</td>
<td>Globular</td>
<td>Signaling molecule</td>
<td>osteoclast formation</td>
<td>[47, 48]</td>
</tr>
<tr>
<td>Glycosaminoglycan</td>
<td>variable with length</td>
<td>Linear</td>
<td>Regulatory factors</td>
<td>Components of ECM, regulating cellular activities</td>
<td>[51]</td>
</tr>
</tbody>
</table>

*ECM*: Extracellular matrix
1.4 Bone Mechano-wtansduction

Functional adaptation is used to describe the ability of organisms to increase their capacity to accomplish a specific function with increased demand and to decrease this capacity with lesser demand. Julius Wolff proposed that mechanical stress is responsible for determining the architecture of bone and that bone tissue is able to adapt its mass and three-dimensional structure to the prevailing mechanical usage to obtain a higher efficiency of load bearing [10]. Adaptation via matrix deposition and resorption could improve an individual’s bone quality and survival chance since bone is not only hard but also heavy and to a certain extent bendable. Too much of bone is probably as bad as too little, leading either to uneconomic energy consumption during movement (for too high bone mass) or to an enhanced fracture risk (for too low bone mass) [10].

Although it is well accepted that mechanical signals are crucial to maintain an adequate skeleton, the mechanism by which bone cells sense their mechanical environment and initiate the deposition or resorption of bone tissue has not been ascertained completely. Mechanosensation could be understood as a multi-scale process: mechanically the organ-level loading produces tissue-level stress and strain that produce stimuli to cells, while intracellularly molecular signaling leads to changes in cell metabolism, cell-cell signaling and tissue-level response [52]. In the past decades several potential cell stimuli have been proposed including changes in electric fields, direct cell strain, hydrostatic pressure and fluid flow [10]. When bone tissue is dynamically loaded, it produces dynamic electric fields. These potentials contribute to
the piezoelectric effect and streaming potentials because of convective ion transport [53, 54]. Electric fields influence cellular morphology, increase alkaline phosphatase activity, regulate differentiation and mineralization [10, 52]. However, it was found that the endogenous electric fields produced by loading may not elicit a significant cellular effect [52], which turned the focus to direct perturbation (strain) of the cells. The habitual strains in the mineralized bone tissue are quite small with a maximum reported strain during vigorous activity of approximately 2000 microstrain (0.2%) [55]. It is still unclear whether the strains in vivo are sufficient to induce cellular responses since in vitro studies showed that in order to induce any cellular response by direct mechanical deformation of bone cells, deformations need to be 5%–10%, which is one to two orders of magnitude larger than the bone tissue strains [56, 57]. In vitro studies have shown that hydrostatic pressure regulates changes in bone cell behavior [58, 59]. Dynamic hydrostatic pressure at 13 kPa applied to bone rudiments leads to increased mineralization [58] and a pressure of 4 MPa leads to increased cell-cell and cell-matrix adhesion and reorganization of the cytoskeleton [59].

Bone is a hydrated tissue, and mechanical loading could influence the distribution and fluid flow, which is contained in three fundamental spaces: the vascular space, the fluid component in the bone matrix, and the LCS network. Because of the large diameter of vessels and high permeability, tissue loading has little direct influence on vascular pressures and flow [60]. In addition, the fluid component of the mineralized bone tissue is too tightly bound to undergo significant flow changes with
loading [61]. Thus, the primary load-induced fluid flow influence appears to be through the LCS network. Piekarski & Munro (1977) [62] first proposed that the small deformations of bone due to mechanical loading cause fluid movement in the lacunar-canalicular porosity, which has been shown experimentally by tracer studies [63]. The flow of interstitial fluid through the bone canaliculi will have two effects: a mechanical one derived from the fluid shear stress (FSS) and an electrokinetic one derived from streaming potentials [64]. Weinbaum et al. (1994) [1] introduced the hypothesis that FSS acting on the osteocyte processes residing in the lacunar-canalicular porosity was the initiating signal for cellular excitation and developed a detailed hierarchical model predicting the flow through the PCM surrounding an osteocyte process. A central tenet of their hypothesis was that the pericellular space surrounding the osteocyte processes was filled with an extracellular matrix that sieved molecules the size of albumin (7-nm diameter) or larger. The flow in the porous PCM was described by a Brinkman equation, and the flow in the larger tissue space was described by Biot theory. The model remarkably predicted that, bulk bone strains of about 0.1% will produce a fluid shear stress in the canaliculi of roughly 1 Pa [1], enough to produce a rapid response in endothelial cells [65, 66]. Klein-Nulend et al. (1995) [67] were the first to demonstrate the sensitivity of osteocytes to FSS at the levels predicted by Weinbaum et al. (1994). Cowin et al. (1995) [68] proposed that the lacunar-canalicular porosity was the site of the strain-generated potentials (SGP), and this theory was subsequently combined with electrical cable theory for the ionic current through the gap junctions of the cell network to predict the voltage difference
across the membranes of the osteocyte processes [60, 69] and the possible opening of voltage-gated ion channels in the plasma membrane processes. T-type voltage-sensitive calcium channels (VSCC) has been found to play a key role in bone mechanosensing [70]. In vitro inhibition of T-VSCC significantly reduced the expression of mechanoresponsive genes in osteoblasts [70]. Recently, a new potential activating mechanical signal was proposed [71], with the hypothesis that the cell process was attached by tethering elements to adhesion proteins lining the canalicular wall. The tethering fibers were proposed to be proteoglycans that spanned the fluid annulus [4], and when the bone tissue was deformed, the fluid passing through the osteocyte PCM would create a hydrodynamic drag on the matrix. This model predicted a cellular-level strain amplification of 10 to 100 and showed that the drag on the PCM was 20 times greater than the FSS over a same unit process length.

Fluid flow affects cellular metabolism within the bone, and involve a response of both the osteoblasts and osteocytes as shown using fluid flow stimulation in culture [67, 72]. The first fluid-flow studies on bone cells in culture were performed by Reich et al. (1990) [72], who demonstrated that osteoblasts exposed to FSS elicited similar responses as endothelial cells at the same FSS levels. Recently, it was found that fluid force influences the OPN gene expression in osteoblasts, suggesting that fluid flow provides the physical signal for the osteogenic response of bone to mechanical loading [73]. Also it was recently found that ATP is rapidly released from MC3T3-E1 pre-osteoblastic cells within 1 min of the onset of fluid shear [74]. Experimental studies in vitro have demonstrated that osteocytes are indeed quite
sensitive to the FSS compared to osteoblasts and osteo-progenitor cells [27, 67, 75]. In response to fluid flow, osteocytes were found to release signaling molecules like Nitric Oxide, ATP, sclerostin, PGE2 and OPG/RANKL, which are known regulators of osteoblastic bone formation as well as osteoclastic-targeted bone resorption [27-30]. Currently, oscillations of intracellular calcium, a pivotal second messenger regulating many downstream cellular activities and also one of the earliest mechanotransduction events in bone cells [76, 77], was experimentally demonstrated in osteocytes in intact long bone under \textit{in vivo} dynamic loading [78]. This study provides direct evidence that osteocytes respond to load-induced fluid shear by intracellular calcium oscillations [78]. In addition, perturbations in osteocyte networks have been shown to drastically alter bone remodeling and/or modeling in a mechanical disuse model [17]. These results suggest that the combination of the cellular three-dimensional network of osteocytes and the accompanying porous network of lacunae and canaliculi acts as the mechanosensory organ of bone.

A critical unanswered question is which part of the osteocyte, the cell body or the cell process, acts as the mechanical sensor. The elastic modulus for the osteocyte process would be much stiffer than the cell body due to a highly organized central actin filament bundle that is cross-linked by fimbrin [79, 80], while the structure in the cell body is more loosely cross-linked by \(\alpha\)-actinin. Thus the soft cell body is more likely to be deformed by a uniform FSS than the stiffer cell process. However, previous models predict shear stress to be at least two orders of magnitude larger than on the cell body because the pericellular space in the lacuna is much wider.
than the narrow annulus surrounding the cell process in the canaliculus [1, 81], suggesting the cell processes are more likely to be stimulated.

1.5 Osteocyte Pericellular Matrix

Pericellular matrix (PCM) is a universal structure in living cells. Between the plasma membrane and the extracellular matrix lies the PCM. Intermingled in this transition zone are the ectodomains of integral membrane glycoproteins, proteoglycans, glycolipids, and a variety of extracellular glycoproteins and proteoglycans [82, 83]. Although up to several microns thick, the PCM is an elusive structure due to its invisibility with phase contrast or DIC microscopy [83].

The PCM has multiple important roles, from serving structural and mechanochemical functions, to the regulation of cell division and motility, as well as cancer progression and metastasis [84]. PCM plays a biomechanical role in articular cartilage, and changes in the properties of the PCM with osteoarthritis may alter the stress–strain and fluid flow environment of the chondrocytes [85]. For epithelium cell PCM, it can detect flow in the kidney. The endothelial PCM has multiple functions: it acts as a transport barrier, serves as a porous hydrodynamic interface in the motion of red and white cells in microvessels, and also acts as a mechanotransducer of fluid shearing stresses to the actin cortical cytoskeleton of the endothelial cell [86].

The osteocyte PCM is a thin structure (~50nm thick) filling the fluid annulus between the canalicular wall and cell process within the lacunar-canalicular system (LCS) [1, 4, 26] (Figure 1.4). A pressure gradient would be induced when a whole
bone is deformed due to the applied loading, causing fluid and solute to flow through the PCM and inducing shear stress on the entire cell process membrane and a drag force exerted on the transverse elements [1, 71]. The interaction between load-induced fluid flow inside the LCS and PCM is critical for the cell survival, intracellular signaling and mechanotransduction of osteocytes. Under fluid flow, the transverse fibers in the PCM are deformed, transferring external mechanical signals into the cellular interior, initiating downstream biochemical intracellular responses, and releasing regulatory molecules like sclerostin and RANKL to the extracellular space [14]. These signaling molecules can act on neighboring osteocytes, osteoblasts and osteoclasts and thus coordinate bone’s adaptation and remodeling processes. To reach their target, these molecules need to pass through the PCM via diffusive and/or convective mechanisms [87, 88]. PCM serves as a molecular sieve, modulating the transport of signaling molecules and nutrients in bone.

Despite its physiological importance, the structure and mechanotransduction effect of PCM remain poorly understood due to the difficulty of accessing this thin fragile structure. Previous studies [89-91] have demonstrated the existence of PCM using electron microscopy (Figure 1.4), while the structural details are still largely unknown. You et al. (2004) investigated the ultrastructure of PCM using ruthenium III hexamine trichloride, a cationic stain that has been successfully applied in preserving cartilage proteoglycans [92]. They verified the existence of PCM in mature bone and found transverse elements spanning the entire pericellular space with an average spacing of 40nm. Their study also suggested proteoglycans must be present
in the annular space. Recently, perlecan, a five domain heparan sulfate proteoglycan [93], was identified in osteocyte PCM using immune-gold labeling and TEM imaging [94] (Figure 1.4). The study found that mice with reduced perlecan expression in the PCM decreased in the pericellular area, canalicular density, and tethering elements per canaliculus compared with normal mice.

**Figure 1.4.** The existence of osteocyte PCM and the transverse elements were verified using TEM imaging. Perlecan was identified in osteocyte PCM using immune-gold labeling and TEM imaging.

PCM could be a potential target to treat osteoporosis. Bone mass decreases with aging, which results in an increased incidence of fractures in elderly people, a
condition designated age-related osteoporosis [95]. Approximately 50% of elderly women and 20% of elderly men will have a fragility fracture in their lifetime [96]. This age-related osteoporosis may be related with decreased osteoblasts density and defective osteoblast function with advancing age [97-101]. It may also related with increased bone resorption with aging [102]. While these factors may all contribute to the pathogenesis of age-related osteoporosis, researchers have also found dramatic reduction in the responsiveness of older bones to the applied mechanical loads [103, 104], indicating the defective mechanical sensing with aging could be another factor contributing to this bone disease. In vitro and in vivo studies have suggested that this defective sensing may be related with the altered osteocyte PCM structure [105, 106]. Reilly et al. found that the fluid-flow induced PGE$_2$ release was reduced by glycocalyx degradation [105]. Since PGE$_2$ is an important factor that influences the Wnt/β-catenin signaling pathway, which plays a crucial role in bone response to loading [14], the reduced PGE$_2$ release may lead to defective cell mechanosensing. In addition, Burra et al. found that osteocyte PCM was required for cell hemichannels opening [106]. Disruption of the glycocalyx diminished the dendrite’s ability to induce the opening of hemichannels, important in the propagation of cellular signals contributing to mechanotransduction in bone. From these studies, PCM may be considered as a potential clinical target to treat osteoporosis. If it is possible to regulate the PCM structure, we may alter bone’s sensitivity to mechanical loading and use it to treat osteoporosis.
1.6 Dynamic Method to Quantify the Osteocytic PCM Using FRAP Approach and Mathematical Model

To quantify the osteocytic PCM, a novel tracer velocimetry approach that combined fluorescence recovery after photobleaching (FRAP) imaging with hydrodynamic modeling was recently developed in my lab. The step-by-step development of this particular approach is introduced as below:

1.6.1 In Situ FRAP measurements of solute diffusion in LCS

The diffusion of a small molecule (sodium fluorescein, 376 Da) in the osteocyte LCS was recently measured using FRAP [107]. In a typical FRAP experiment (Figure 1.5), exogenously injected tracer molecules are allowed to reach an equilibrium in bone, before the tracers in an individual osteocyte lacuna are irreversibly photobleached under intense laser illumination. Tracer transport from neighboring lacunae leads to fluorescence recovery of the photobleached lacuna. The time-series of the fluorescent intensity of the targeted osteocyte lacuna during recovery phase was recorded and analyzed to derive the tracer diffusion characteristic (Figure 1.5d). It was found the diffusion of sodium fluorescein was reduced ~ 40% in bone compared with its free diffusion in aqueous solution [88].
Figure 1.5. FRAP measurement of solute diffusion. (a) The bone was injected with fluorescent tracer to allow the ellipse shaped lacunae and LCS to fill with soluble fluorescence-labeled molecules. (b) One lacuna was photobleached by high intensity laser. The photobleached area became darker than its surrounding. (c) As time passed, fluorescent intensity of the photobleached area recovered because the surrounding unphotobleached fluorescent molecules moved into the photobleached region. (d) The fluorescent changes of the photobleached lacuna before and after photobleaching were recorded using confocal microscopy. The time course of the fluorescent intensity changes was fitted into a mathematic model to quantitatively determine the diffusion coefficient of the fluorescent molecule [107].

1.6.2 Two-compartment mathematical model to derive solute diffusion from FRAP experiments

A two-compartment mathematical model was used based on FRAP experimental data and the lacunar-canalicular anatomy to determine the diffusion coefficients of the fluorescent molecules [107]. The model consists of one photobleached lacuna (a sink) and one of its neighboring lacuna (a source reservoir with a constant fluorescent tracer concentration) with the connecting canaliculi (Figure 1.6). Mathematically, the problem is reduced to one-dimensional diffusion along a channel connecting the two well-mixed compartments from Fick’s first law. The fluorescent intensity within the photobleached lacuna is given by:
\[
\ln(I_0 - I(t')) = -V t' D / d^2
\]

where \(I_0\) and \(I_b\) are fluorescent intensity prior to and post photobleaching, respectively, \(I(t')\) is the fluorescent intensity during recovery, \(t'\) is the time after photobleaching, \(V_r\) is the fluid volume ratio between the canaliculi and the sink lacuna, where \(N\) is the canalicular number contributing to the recovery, \(A_c\) is the annular fluid area of canalicular cross section, \(V_s\) is the volume of the photobleached lacuna, \(D\) is the tracer diffusion coefficient, and \(d\) is the mean distance between the photobleached lacuna and its neighbors [107]. The diffusion coefficient \((D)\) was calculated for each FRAP experiment, determined by the slope of the fitting line: \(k = V_r D / d^2\). The diffusion coefficient of sodium fluorescein was measured to be \(3.3 \pm 0.6 \times 10^{-6} \text{cm}^2/\text{sec}\) based on 21 FRAP measurements from seven mice [107] (Figure 1.6 right panel).
25

1.6. The tracer diffusion characteristic was analyzed using the two-compartment model. The sink represents the photobleached lacuna, while the source represents the neighboring unphotobleached lacunae. The channel connecting source and sink represents canaliculi. The diffusion coefficient \( D \) was calculated for each FRAP experiment, determined by the slope of the fitting line: 

\[
k = \frac{V_r D}{d^2} [107].
\]

1.6.3 Three-compartment mathematical model in loaded bone

A tissue level mechanic model was created previously to calculate the pressure distribution in the osteocyte LCS and fluid velocity from axial compression loading by solving the equation based on poroelasticity theory [108]. The average fluid velocity in the canalicular channels was found to be sinusoidal as time changed. The radial flows were neglected since they were smaller than the circumferential flows when periosteum was relatively impermeable. Thus a microscopic one-dimensional transport model was developed for the circumferential solute flows of the FRAP site. This mathematical model consists of three compartments: the central photobleached lacuna and its two neighboring lacunae as source reservoir connecting with two sets of six canalicular channels (Figure 1.7 left panel). A modified diffusion-convection
equation with its boundary and initial conditions were solved to obtain the tracer concentration profile from the input fluid velocity. The tracer fluorescent recovery rate in the sink lacuna, which is the slope magnitude of the curve of $\ln \left[ (C - C_0) / (C_b - C_0) \right]$ vs time, could be obtained from the tracer concentration profile (Figure 1.7 right panel). The three-compartment model considers PCM’s sieving effect by applying the tracer reflection coefficient to the solute movement. The simulation results showed that the recovery rate increases for decreasing tracer size, which can be expected since small tracers move faster than large tracers in the same osteocyte LCS microstructure.

Figure 1.7. A three compartment mathematical model was developed in loaded bone. This model consists of a central photobleached lacuna and its two neighboring lacunae as source reservoir through connecting canaliculi (six at each side). The tracer fluorescent recovery rate in the sink lacuna was calculated from the concentration profile [108].

1.6.4 Real-Time measurement of solute transport in loaded bone

Previous study has demonstrated the load-induced fluid flow by synchronization of FRAP imaging and mechanical loading, in which the FRAP imaging sequence was synchronized with a rest-inserted loading protocol [109]. Paired
loaded and non-loaded FRAP experiments were conducted on the same targeted lacuna, and the diffusive transport rate \((k_{\text{Diff}})\) and the convective transport rate \((k_{\text{Load}})\) of sodium fluorescein were obtained. The diffusion coefficient \((D)\) was obtained from \(k_{\text{Diff}}\) measured in the non-loaded FRAP. Using diffusion coefficient \((D)\) as an input, the relationship between fluid velocity \((u_f)\) and transport enhancement \((k_{\text{Load}}/k_{\text{Diff}})\) was established by simulation in a range of fluid velocities \((0 \sim 120 \mu m/s)\) using the three-compartment model. The relationship was found to be: 

\[
k_{\text{Load}}/k_{\text{Diff}} = 1 + 0.00037 \times u_f \mu m/s^{1.65} (R^2 = 0.99).
\]

Thus the fluid velocity was obtained from the experimental measurement of transport enhancement, which was found to be \(\sim 60 \mu m/s\).

The FRAP approach in loaded bone has been expanded to a larger tracer (parvalbumin, 12.3kDa), which is comparable in size to some signaling proteins expressed by osteocytes such as growth factors BMP-2 and BMP-7 [87, 88]. By simulating for a range of solute velocities using the three-compartment model, the relationship between solute velocity \((u_s)\) and transport enhancement \((k_{\text{Load}}/k_{\text{Diff}})\) was obtained: 

\[
k_{\text{Load}}/k_{\text{Diff}} = 1 + 0.000164 \times u_s \mu m/s^{1.986} (R^2 = 0.99).
\]

Thus the solute velocity was obtained from the experimental measurement of transport enhancement \(k_{\text{Load}}/k_{\text{Diff}}\). The results showed that fluid flow velocity and solute flow velocity were \(60 \mu m/s\) and \(55.5 \mu m/s\), which yield the reflection coefficient of parvalbumin in the osteocyte PCM to be \(\sigma = 1 - u_s/u_f = 0.075\) [110].
1.6.5 PCM ultrastructure derivation from solute transport

The relationship between solute reflection coefficient of PCM and fiber ultrastructure has been investigated currently using a hydrodynamic model [111]. In this model, the sieving effect from the apparently random fibers in the PCM was approximated with that of an orthogonal fiber network where 1/3 of the fibers form a longitudinal fiber array and the rest of fibers (2/3) form the transverse fiber array (Figure 1.8 left panel). The reflection coefficient of PCM was thus a weighted average of the two fiber arrays. The fibers were modeled as a single species of cylinders with uniform radius \( r_f \). For both of the fiber arrays, the flow velocity field was determined first, then the reflection coefficient was determined by calculating the solute flux and fluid flux using steric exclusion [112, 113]. The relationship was found to be:

\[
\alpha = \frac{(\alpha + \beta)^2 \ln\left(1 + \frac{\alpha}{\beta}\right)}{\beta^2 - \frac{\beta^4}{4} \ln(\beta) - \frac{\beta^2}{4}} + \frac{1}{1 - \beta^2 + \frac{3}{2} \ln(\beta)} \left\{ \alpha \left(1 + \frac{\beta}{\alpha + \beta}\right) \right\} - 2(\alpha + \beta) \ln(1 + \frac{\alpha}{\beta}) \right\}.
\]  

(1.2)

Where \( \alpha = r_s/R \), \( \beta = r_f/R \), \( r_s \) was the solute radius, and \( R \) was the radius of the periodic fluid unit surrounding a fiber (Figure 1.8 right panel), which was approximately half of the center-to-center distance between two adjacent fibers. In the PCM model, the fibers in the longitudinal array were arranged in a square array and the fiber spacing \( (\Delta) \) could be estimated from the fiber volume fraction of the longitudinal fibers \( (k_{vf}/3) \), i.e., \( \Delta = 2R = 2r_f(k_{vf}/3)^{-0.5} \). Since the species and identity of the fibers in the osteocyte PCM were not known, the fiber size was estimated for several possible
candidates for the fibers, including the glycosaminoglycan side chain (0.5nm radius) [1], globular core proteins of aggrecan and hyaluronic acid (1-2nm radius) [114], as well as the repeated endothelial glycocalyx structure seen in the cryo-electron microscopy (4-6nm radius) [115, 116]. Thus the relationship between PCM sieving effect and its ultrastructure was established.

Figure 1.8. The osteocyte PCM was approximated with that of an orthogonal fiber network where 1/3 of the fibers form a longitudinal fiber array and the rest of fibers (2/3) form the transverse fiber array. A periodic fluid unit (dash circle) was created to derive fluid flow field around the fibers [111].

1.7 Organization of the Dissertation

The main body of my dissertation consists of three chapters (2-4), in which three related projects are presented. In Chapter 2, the sensitivity of the tracer velocimetry approach in detecting the PCM alterations was investigated. Tracer transport rates, PCM ultrastructure and cellular-level mechanical stimulations were
obtained in the altered PCM mice model, the perlecan deficient mice. The role of the PCM in osteocyte mechanosensing was dissected. In Chapter 3, the anatomy of osteocyte LCS was investigated using confocal and TEM imaging, since the ultrastructure of LCS is a critical parameter in calculation of the cellular stimulations. Different bone compartments, ages and diseased conditions were tested and compared. In Chapter 4, the problem whether PCM differs in different bone sites (long bone vs. flat bone) were addressed using Alcian Blue staining and FRAP diffusion experiments. The data obtained in the study are important to further investigate the cellular-level forces in different bone sites, and may provide a potential explanation of calvaria’s surprising resistance to disuse bone loss.

The Chapter 2 is adapted from my journal paper ‘Perlecan-containing pericellular matrix regulates solute transport and mechanosensing within the osteocyte lacunar-canalicular system’ published in Journal of Bone and Mineral Research, 2014, 29: 878-891. Using FRAP imaging and hydrodynamic modeling, solute transport and PCM mechanosensing were investigated in older mice expressing or deficient for perlecan. Overall, solute transport increases in the perlecan deficient mice compared with control mice. PCM fiber density decreases with aging and perlecan deficiency. Osteocytes in the perlecan deficient bones were predicted to experience higher shear stress, but decreased fluid drag force under 3-N peak tibial loading. When subjected to tibial loading in a preliminary in vivo experiment, the perlecan deficient mice did not respond to the anabolic stimuli as the CTL mice did. The study supports the
hypothesis that the PCM fibers act as osteocyte’s sensing antennae, regulating load-induced cellular stimulations and thus bone’s sensitivity and in vivo bone adaptation.

The Chapter 3 is adapted from my journal paper ‘The dependences of osteocyte network on bone compartment, age, and disease’ published on Bone Research, (2015) 3, 15009; doi: 10.1038. The LCS network features play important roles in osteocyte signaling and regulation of bone growth and adaptation. In this study, key features of the LCS network including the topological parameter and the detailed structure of individual connections and their variations in cortical and cancellous compartments, in bones from young and old mice, as well as in two diseased conditions (perlecan deficiency and diabetes) were examined. Overall, age, in the range of our study (15-32 weeks), affected only the pericellular fluid annulus in cortical bone but not in cancellous bone. Diabetes impacted the spacing of the lacunae, while the perlecan deficiency had a profound influence on the pericellular fluid annulus.

The Chapter 4 is addressing the issue whether PCM differs in different bone sites (long bone vs. flat bone) using Alcian Blue staining and FRAP diffusion experiments. The data confirmed that murine flat bones express significant lower content of HSPG than long bone, an indicator for smaller cellular-stimulation forces, which may provide a potential explanation of calvaria’s surprising resistance to disuse bone loss.

General conclusions and future directions were discussed in Chapter 5.
In Appendix, two of my other projects: ‘Imaging and quantifying solute transport across periosteum: implications for muscle–bone crosstalk’ and ‘Perlecan/HSPG2 deficiency enhances the differentiation and mineralization of mesenchymal stromal cells but reduces their intracellular calcium signaling responses to fluid flow stimulation’ were presented.
2.1 Abstract

The pericellular matrix (PCM), a thin coating surrounding nearly all mammalian cells, plays a critical role in many cell-surface phenomena. In osteocytes, the PCM is believed to control both “outside-in” (mechanosensing) and “inside-out” (signaling molecule transport) processes. However, the osteocytic PCM is challenging to study in situ because it is thin (~100 nm) and enclosed in mineralized matrix. To this end, we recently developed a novel tracer velocimetry approach that combined fluorescence recovery after photobleaching (FRAP) imaging with hydrodynamic modeling to quantify the osteocytic PCM in young murine bone. In this study, we applied the technique to older mice expressing or deficient for perlecan/HSPG2, a large heparansulfate proteoglycan normally secreted in osteocytic PCM. The objectives were: (1) to characterize transport within an altered PCM; (2) to test the sensitivity of our approach in detecting the PCM alterations; and (3) to dissect the roles of the PCM in osteocyte mechanosensing. We found that: (1) solute transport increases in the perlecan-deficient (hypomorphic [Hypo]) mice compared with control mice; (2) PCM fiber density decreases with aging and perlecan deficiency; (3)
osteocytes in the Hypo bones are predicted to experience higher shear stress (+ 34%), but decreased fluid drag force (− 35%) under 3-N peak tibial loading; and (4) when subjected to tibial loading in a preliminary in vivo experiment, the Hypo mice did not respond to the anabolic stimuli as the CTL mice did. These findings support the hypothesis that the PCM fibers act as osteocyte’s sensing antennae, regulating load-induced cellular stimulations and thus bone’s sensitivity and in vivo bone adaptation. If this hypothesis is further confirmed, osteocytic PCM could be new targets to develop osteoporosis treatments by modulating bone’s intrinsic sensitivity to mechanical loading and be used to design patient-specific exercise regimens to promote bone formation.

2.2 Introduction

Osteocytes, the most numerous cells in bone, play a central role in maintaining tissue homeostasis [117] and sensing the mechanical stimuli that drive bone adaptation [14]. A fibrous, nonmineralized pericellular matrix (PCM) has been found to surround osteocytes and their long “dendritic” processes within the lacunar-canalicular system (LCS) [4]. This thin cellular coating, also termed the glycocalyx, is a universal structure, found in nearly all mammalian cells such as red blood cells [118], endothelial cells [119], epithelial cells [120], and chondrocytes [121]. As an interface between the cell membrane and the extracellular space, the PCM is essential for cell surface phenomena such as cell-cell, cell-ligand, and cell-extracellular matrix (ECM) interactions [118, 119]. The PCM’s mechanosensitive
function is well established in endothelial cells [86, 119] and chondrocytes [122, 123]. Increasing evidence supports similar roles of osteocytic PCM in bone’s mechanosensing.

Being strategically positioned between the cell and its immediate external environment, the PCM is critical during both the “outside-in” and “inside-out” signaling processes that occur in and among osteocytes. Under dynamic loading, the porous bone matrix is deformed and the interstitial fluid is driven to flow through the LCS pores [62, 109], where the PCM fibers fill the annular space between the canalicular wall and the cell membrane [1, 4]. The magnitude of this load-induced fluid flow and its relaxation time constant are highly dependent on the hydraulic permeability of the LCS pores, which scales approximately to the square of the fiber spacing [1]. The PCM fibers, therefore, help regulate the outside-in process, whereby the tissue-level mechanical loads are converted into cellular stimulations. Among several proposed mechanisms responsible for osteocyte mechanosensing [124], two commonly accepted mechanisms involve fluid-fiber interactions. One is through the action of fluid shear stress on the cell membrane, in which the PCM fibers control the fluid velocity profiles in the canaliculi and determine the magnitude of the fluid shear stress [1]; the other is through the direct drag forces on the PCM fibers [71]. Although both loading signals result from fluid flow, shear stress describes the interactions between the flow and cell membrane surface (unit: Pa) and the fluid drag force indicates the normal force that fluid flow impacts on the transverse PCM fibers (unit: N). Both physical signals could be transmitted to the cell’s interior via apparatuses
such as focal adhesion complexes [91, 125], stretch-activated membrane channels [126], voltage-sensitive channels [127], or the cytoskeleton [71]. Once activated by mechanical stimuli, osteocytes can alter the expression of various signaling molecules and orchestrate the activities of osteoblasts, osteoclasts, and other functional cells, fostering bone’s adaptation to the mechanical environment [14]. During this “inside-out” signaling process, the osteocytic PCM serves as an important molecular sieve, controlling the passage and final presentation of signaling molecules within bone [6, 87, 88]. Therefore, alterations in PCM structure and composition are expected to impact both the osteocyte’s sensing and responses to mechanical loading at multiple levels.

Despite its potential significance in bone physiology, our current knowledge of the osteocytic PCM and its alterations in vivo remains limited because of the lack of quantitative measurement tools. The existence of fibrous PCM in adult bone has been demonstrated in transmission electron microscopy (TEM) since the 1990s [90, 128]. Using fixatives containing ruthenium III hexamine trichloride, a cationic dye previously used to stain cartilage proteoglycan [92] and the endothelial glycocalyx [115], You and colleagues [4] identified transverse tethering fibers spanning the entire annular fluid space, the essential force-transferring element for a proposed strain amplification mechanism [71]. In our recent study, perlecan/heparan sulfate proteoglycan 2 (HSPG2), a large secreted heparan sulfate proteoglycan, was discovered within the osteocytic PCM under immunofluorescence and TEM Immunogold staining methods [94]. The importance of perlecan in the osteocytic
PCM was further confirmed by TEM imaging of the osteocytic LCS from C1532Yneo mice [94], a transgenic model developed to recapitulate the reduced perlecan expression associated with Schwartz-Jampel Syndrome (SJS) [129] and derived by us on a C57BL/6J genetic background. These perlecan-deficient (hypomorphic [Hypo]) mice exhibited a significant decrease in perlecan secretion [129], and a decreased number of tethering elements per canaliculus (~35.8%) under TEM [94]. In nonmineralized tissues such as the kidney, decreased expression of heparan sulfate was reported to be associated with marked edema and proteinuria, suggesting an elevated glomerular permeability to solutes [130]. However, it is not known if hydraulic permeability and load-induced solute transport are altered within the bone LCS of the perlecan-deficient mice.

Although the TEM technique is useful to study morphology and composition of the osteocytic PCM, quantitative measurements of PCM fiber density based on TEM remain a challenge because (1) the PCM fibers are fragile and easily collapsible [119]; (2) the TEM histological procedures are tedious and prone to artifacts [115]. To this end, we recently developed a novel in situ approach to quantify PCM fiber density based on tracer velocimetry, which combines confocal fluorescence recovery after photobleaching (FRAP) imaging and hydrodynamic modeling [110]. Using this technique we successfully measured, for the first time, the osteocytic PCM fiber density of bone from young adult mice in situ [110]. It was not clear from that study if the tracer velocimetry approach would be sensitive enough to detect in vivo PCM alterations. The goal of the present investigation was fourfold. First, we
aimed to quantify changes in molecular diffusion and convection in the cortical bone of the perlecan-deficient mice relative to controls. We hypothesized that the perlecan deficiency would increase solute transport in the bone LCS due to reduced hydraulic resistance from the PCM. Second, using older perlecan-deficient and age-matched perlecan-expressing control mice, we tested the sensitivity of our tracer velocimetric FRAP approach in detecting PCM alterations associated with aging and perlecan deficiency. Third, the cellular-level stimulating forces (shear stress versus fluid drag) were estimated from the PCM structure using the Brinkman equation describing fluid flow in a porous medium [1], and fourth, we correlated mechanical stimulations at the cellular level with bone’s responses to in vivo tibial loading. Our preliminary results suggest that the fluid drag force acting on the PCM fibers is the primary physical signal driving bone adaptation and lead us to hypothesize that the proteoglycan perlecan in PCM, acting as flow-sensing antenna on osteocytes, regulates bone’s sensitivity to mechanical loading. If this hypothesis is further confirmed in vivo, quantification of the PCM fiber density or related characteristics could be a powerful tool to identify an individual’s sensitivity to loading, to design patient-specific exercise regimens, or to provide new targets to promote bone formation in osteoporotic patients.
2.3 Methods

2.3.1 Mice

Mice with perlecan deficiency, a generous gift from Dr. Kathryn Rodgers, were used in this study. The generation and characterization of this murine model of human SJS [131] have been reported [129]. Disruption of the functional perlecan gene expression was achieved by retention of a neomycin selection cassette (C1532Yneo) on intron 16 located between exons encoding for perlecan, resulting in a decreased perlecan secretion in homozygous mutants [129]. Increasing evidence supports similar roles of osteocytic PCM in bone’s mechanosensing [132]. Wild-type animals and those heterozygous for the C1532Yneo mutation were undistinguishable phenotypically, and both were used as controls (CTL) [129]. All animal studies were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Delaware.

2.3.2 Tibial compliance measurements

Prior to the tracer velocimetry FRAP tests, which required application of comparable surface strains to the Hypo and CTL tibiae, the compliances of the tibiae were quantified in 8- to 9-month-old male Hypo (n = 7) and CTL (n = 4) tibiae using an optical strain measurement method [133]. The tibiae were painted with fluorescent microspheres and subjected to incrementally increased loads, while axial strain was quantified at the anterior-medial surface (25% to 50% distal of the proximal end) using a digital image correlation algorithm. The mean compliance of the tibiae
was obtained by linearly fitting the strain versus load curves. Because no significant
difference was detected in the tibial compliance between the two genotypes, a 3-N
peak load was applied to both genotypes during the FRAP tests described later.

2.3.3 Quantification of the LCS anatomy

In a separate set of 8- to 9-month-old male Hypo and CTL mice (n = 3
mice/genotype), right tibiae were harvested and immediately immersed in chemical
fixatives containing ruthenium III hexamine trichloride in preparation for TEM
imaging [4]. High resolution TEM images were analyzed using the protocol described
[94]. The total canalicular area, cell process area, and the pericellular fluid area were
obtained from the TEM images of the canalicular cross-sections (n = 506 and 475
canalici) for the CTL and Hypo groups, respectively. In addition, the gap in the
perilacunar fluid area between the cell membrane and lacunar wall was measured in 12
and 16 lacunae for the CTL and Hypo cortical bones, respectively. The left tibiae were
fixed, bulk stained with basic fuchsin and embedded in methyl methacrylate, followed
by sagittal sectioning and polishing to a thickness of 100µm [107]. A total of 30 intact
lacunae per genotype (10 lacunae per mouse) were randomly selected from the tibial
cortical bone compartment and imaged using a Zeiss LSM510 confocal microscope.
Z-stack images were obtained with a z-step of 0.2µm using an oil-immersion lens
(40×). Three-dimensional reconstructions were performed using the Volocity software
package (PerkinElmer, Waltham, MA, USA) and the number of canaliculi emanating
from each lacuna was counted. The lacunar bodies were segmented using the Amira
software package (Visualization Sciences Group, Burlington, MA, USA) and their surface areas and volumes were calculated. An axial correction factor (0.803), which was determined optically using calibrated bone sections with known thicknesses, was applied to correct the axial stretching in the images [134]. The canalicular number density per unit surface area was determined for each genotype. These anatomical measurements were used in calculating tracer diffusivity and customizing genotype-specific LCS models to derive solute velocities in the section of FRAP tracer velocimetry (Step 2). More detailed description about the quantification of the LCS anatomy could be found in Chapter 4.

2.3.4 FRAP specimen preparation

Male Hypo (n = 7) and CTL (n = 5) mice aged 12 to 13 months were used in this study. These relatively older mice were chosen (1) to match our previous TEM studies where the LCS anatomical parameters were characterized in 8- to 9-month-old male mice, and (2) to allow comparison between aged and young adult bones, which were investigated in our previous PCM studies [110]. The mice were injected via the tail vein with 0.5 mL of phosphate buffered saline (PBS) containing either 5mg sodium fluorescein (Sigma-Aldrich, St. Louis, MO, USA) or 2mg parvalbumin conjugated with Alexa Fluor 488 (Molecular Probes/Invitrogen Corp., Carlsbad, CA, USA), as described [110]. Out of a total of 12 mice, 2 CTL and 5 Hypo mice received sodium fluorescein and 3 CTL and 2 Hypo mice received parvalbumin. We used both tibiae and imaged multiple lacunae per animal to increase our detection power. The
two tracers (molecular weights: 376 Da and 12.3 kDa) were chosen because they represented a small and a relatively large molecule in the broad spectrum of nutrients, metabolites, and signaling molecules involved in osteocyte function [14, 88]. The tracers were allowed to circulate for 0.5 hours and 2 hours in alert and mobile mice, respectively, prior to euthanasia [88]. The left tibia then was harvested, cleansed of soft/adherent tissues, and tested within 0.5 to 3 hours postmortem. The right contralateral tibiae were immediately frozen, stored, and then thawed prior to testing at a later date.

2.3.5 FRAP tracer velocimetry

To obtain measures of the osteocytic PCM’s fiber density, such as the fiber volume fraction and the fiber spacing, a three-step procedure was developed [110], and detailed below.

Step 1: Quantify solute transport using FRAP tests

As described [109], our experimental setup consisted of an electromagnetically actuated loading device (Electroforce LM1 TestBench; Bose Corporation, Eden Prairie, MN, USA) integrated with an inverted confocal laser-scanning microscope (Zeiss LSM 510; Carl Zeiss Inc., Thornwood, NY, USA). A 40 ×, 0.8 numerical aperture water dipping lens attached to an objective inverter was used to capture images of the tibia, which was held in a PBS bath maintained at 37°C. A typical FRAP procedure consisted of three phases of imaging (pre-bleach, photobleaching,
and recovery) [107, 109] of fluorescently labeled lacunae approximately 25 to 40 μm below the tibial periosteal surface on the anterior-medial surface ~25% to 50% distal from the proximal tibial plateau. The imaging settings include 488nm excitation, 505 to 530nm emission, 512-pixel × 512-pixel images, scanning speed of ~ 1 second/frame, and a pinhole of ~ 4.2 to 6.4 Airy unit. We subjected the same lacuna to two sequential FRAP trials: the first was a convection test under cyclic loads (3.0-N peak load at 0.5 Hz) and the second was a diffusion test under the tare load (0.2 N). A 4-second resting period was inserted between two adjacent loading cycles during the convection test to minimize motion artifacts during imaging [109].

The outcomes included the transport rates of the two tracers during diffusion and convection tests, as well as the diffusivity and the transport enhancement for each tracer. The specific methods in obtaining these measures from the FRAP and anatomical data have been published [107, 109]. The transport rate, the reciprocal of the characteristic time constant of the exponential recovery of the fluorescence, was obtained directly from the FRAP image series, from which tracer diffusivity was then derived [107]. In parallel, the transport enhancements \( \frac{k}{k_0} \), the ratio of the transport rate under loading over the rate under static condition, were obtained from paired FRAP tests [109, 110].
Step 2: Quantify tracer velocity using LCS transport simulations

Because the anatomical features of the LCS transport pathway are known, the flow velocity in individual canaliculus could be readily back calculated from the transport enhancement data by simulating the diffusion and convection during FRAP tests [109, 110]. Average anatomical parameters for the studied lacunae (summarized in the Results section) were used to customize a three-compartment LCS transport model for each genotype [109, 110]. The model consisted of three compartments representing the photobleached lacuna (sink) and two neighboring reservoirs that served as alternating upstream and downstream source to the transport sink during cyclic loading (Figure 1.6 left panel). Model parameters such as canalicular length, lacunar major and minor radii, and calculated lacunar surface area were obtained from the pre-bleach FRAP images [107]; the contributing canalicular number, canalicular annular fluid area, and the extracellular fluid area around lacunae were obtained in Quantification of the LCS anatomy [107]. Using the mean diffusivities of sodium fluorescein and parvalbumin in the LCS of the Hypo and CTL bones obtained in step 1, the temporal concentration profiles within the photobleached lacuna were simulated computationally for any given peak solute velocity (0–80 µm/s) [108], from which a relationship between solute convection and transport enhancement ($k/k_0$) was established [109, 110]. Thus, the solute velocities corresponding to the observed transport enhancements, $v_s$, were obtained.

The outcomes included the reflection coefficients of parvalbumin in the Hypo and CTL bones. The reflection coefficient ($\sigma = 1 - v_s/v_f$) characterized the
hindrance of the velocity of parvalbumin ($v_p$) relative to that of fluid ($v_f$), which was due to the steric and hydrodynamic interactions between parvalbumin and the PCM fibers in the LCS. The fluid flow velocity $v_f$ in loaded bone was measured using sodium fluorescein, which has a small Stokes radius ($\approx 0.45$ nm) and a negligible reflection coefficient [110]. These reflection coefficients were measured in aged (12- to 13-month-old) bone in this study and compared with that of younger (4- to 5-month-old) bone measured previously [110].

**Step 3: Quantify osteocytic PCM fiber density using hydrodynamic sieving modeling**

PCM configurations, such as the fiber volume fraction and the fiber edge-to-edge spacing in the CTL (12- to 13-month-old), Hypo (12- to 13-month-old), and young adult CTL (4- to 5-month-old) bone, were obtained using our newly developed PCM hydrodynamic sieving model [110]. Because the radius of individual fibers in the PCM is unknown, we parametrically varied the fiber radius from 0.5 nm (radius of glycosaminoglycan [GAG] side chains [1]), 1 to 2 nm (radius of perlecan core protein [135]), and 4 nm (repeated features of the endothelial glycocalyx) [115]. We determined the fiber volume and edge-to-edge fiber spacing of these fibers that accounted for the observed reflection coefficients in the three groups. Because the TEM images showed that within canaliculi fibers were dominantly arranged in the radial transverse direction [4, 94], a radial square fiber array was assumed in this study. In order to permit comparisons with previous tracer perfusion results, the fiber
spacing reported in this work was the edge-to-edge measure. The relationship between the fiber volume fraction \( (k_{vf}) \) and the edge-to-edge fiber spacing \( (\Delta) \):

\[
k_{vf} = \frac{\pi r_f^2}{(\Delta + 2r_f)^2}
\]

(2.1)

The outcomes included the fiber volume fraction and the fiber edge-to-edge spacing for aged Hypo, aged CTL, and young adult CTL bones.

2.3.6 Cellular-level mechanical stimulations

The peak fluid velocity and the fiber spacing, both measured using the above FRAP tracer velocimetry, were used to obtain the detailed spatial velocity profile inside the canaliculi, from which the shear stress on the cell process membrane and the fluid drag force experienced by the PCM transverse fibers were calculated. For fluid flow through a porous media (i.e., the PCM fibers inside the canaliculi), Weinbaum and colleagues [1] solved the Brinkman equation with non-slip boundary conditions and derived a formula of the fluid velocity as a function of the pressure gradient and the hydraulic permeability of the PCM fibers, which scales approximately to the square of the fiber spacing. We derived the formula for the fluid flux in one canaliculus by integrating the fluid velocity over the entire fluid annulus, with a single unknown factor (the pressure gradient). To resolve the pressure gradients in our loaded bones, we compared the measured fluid flux (the product of fluid velocity and the canalicular fluid annular cross-sectional area) with the predicted fluid
flux formula. The detailed fluid velocity profile, shearing force on the cell membrane [1], and the fluid drag force acting on the transverse PCM fibers per unit cell process length (1 μm) were therefore obtained as reported [71].

2.3.7 In vivo tibial loading

The right tibiae of 3.5-month-old Hypo mice (n=6) and age-matched CTL male mice (n=8) were subjected to compressive uniaxial-tibial loading using a published protocol [136] with a peak load of 8.5 N at 4 Hz (i.e., 0.075-second ramp up, 0.075-second ramp down, and 0.1-second dwell time), 5 minutes per session, and five sessions over 10 days. The peak load magnitude was found to induce similar surface strains (~1300 με) at the FRAP imaging sites in a separate set of Hypo and CTL tibiae (n=4). The left tibiae served as non-loaded control. The mice received intraperitoneal injections of calcium-binding calcein (30 mg/kg) on day 1 and day 11 and were euthanized on day 15. The 3-mm midshafts of harvested tibiae were first scanned in a micro-computed tomography (μCT) system (μCT35; Scanco Medical AG, Bassersdorf, Switzerland) with an isotropic voxel size of 6μm [137], subjected to a three-point bending test along the anterior-posterior direction with a lower support span of 4.5mm and a loading rate of 0.05 mm/s using a TA RSA G2 mechanical analyzer (TA Instruments, New Castle, DE, USA), and then fixed and embedded in methyl methacrylate for dynamic histomorphometry analysis using the OsteoMeasure package (OsteoMetrics, Inc, Decatur, GA, USA). The μCT-based microstructural parameters were obtained through three-dimensional (3D)
reconstruction and segmentation (using a Gaussian filter and a global threshold of 4311 Hounsfield units) in the manufacturer-provided software. The dynamic bone labeling analysis was performed on two mid-diaphyseal cross-sections per bone sample and their average values were used. These parameters were compared between loaded and non-loaded tibiae and between Hypo and CTL mice.

2.3.8 Statistical analysis

All statistical analyses and regressions were performed using the Prism software package (GraphPad Software, La Jolla, CA, USA). The optically measured strains at various load magnitudes were linearly regressed for the Hypo and CTL groups and the difference between the slopes of the two regression lines was detected. The anatomical measures of the studied lacunae, as well as the confocal 3D and TEM imaging data were analyzed using unpaired, two-tailed Student’s $t$ tests between the Hypo and CTL groups. The diffusivity and transport enhancement data were analyzed with two-way ANOVA (genotype and tracer type) and Bonferroni’s multiple comparison post hoc tests. Unpaired, two-tailed Student’s $t$ tests also were performed when comparing Hypo versus CTL groups. For the in vivo loading data, paired and unpaired two-tailed Student’s $t$ tests were used for comparing loaded versus non-loaded tibiae and Hypo versus CTL, respectively. The significance level was set at $p<0.05$ for all statistical tests.
2.4 Results

2.4.1 Tibial compliance

To ensure the induction of similar mechanical strains on loaded Hypo and CTL tibiae during the FRAP tests, the compliance of the tibiae under axial compression was measured optically for each group. The following strain-load relationships were found by linear regression:

- CTL tibiae: Strain ($\mu \epsilon$) = 101.9 $\times$ Load (N) ($R^2 = 0.93$, n = 7) (2.2)
- Hypo tibiae: Strain ($\mu \epsilon$) = 96.1 $\times$ Load (N) ($R^2 = 0.93$, n = 7) (2.3)

Due to intersample variation, there was no significant difference in the average compliance between CTL and Hypo groups (101.9 versus 96.1$\mu \epsilon$/N). Therefore, a 3-N peak cyclic compressive load (i.e., 2.8 N dynamic magnitude relative to the 0.2-N tare load) was applied to all tibiae during the convection FRAP trials.

2.4.2 LCS anatomy

To quantify solute diffusivity and to construct the three-compartment transport model, the number of canaliculi contributing to tracer recovery during the FRAP tests needed to be quantified, as well as the volume of the canalicular channels connecting to the photobleached lacuna. Using 3D confocal and TEM imaging, we first quantified the number density of the canaliculi emanating from each lacuna as well as the LCS annular fluid cross-sectional area (Table 2.1). The canalicular number
density per unit lacunar surface area was 0.21 ± 0.05 per µm² and 0.19 ± 0.02 per µm² for CTL and Hypo lacunae (n = 30 for both groups), respectively. The CTL value was not different from that of 4- to 5-month-old B6 mice (0.21 ± 0.05 per µm²) reported in our earlier studies [34], but was significantly higher than that of the Hypo group (p<0.05). Both cross-sectional areas for the canaliculi (bound by the canalicular walls) and the cell processes were significantly reduced in the Hypo bones compared with those in the CTL bones (p < 0.0001 and p = 0.0002, respectively), resulting in a decreased canalicular fluid annular area for the Hypo canaliculi (0.053 ± 0.026 µm², n = 475) versus the CTL ones (0.065 ± 0.034 µm², n = 506). The typical perilacunar fluid gap, the space between the cell membrane and lacunar wall, was measured to be 0.47 ± 0.27 µm (median 0.40 µm, n = 12 lacunae) and 0.38 ± 0.17 µm (median 0.35 µm, n = 16 lacunae) for CTL and Hypo bone, respectively. Because there was no significance between the two groups (p = 0.32) the data were pooled and a median value of 0.36 µm was used in this study. The genotype-specific canalicular values are listed in Table 2.1.
Table 2.1. Confocal and TEM imaging of the LCS transport features in the 8-9 month-old male control (CTL) and perlecan deficient (Hypo) mice

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Control Bone (CTL)</th>
<th>Perlecan Deficient Bone (Hypo)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (±SD)</td>
<td>Sample Size</td>
<td>Mean (±SD)</td>
</tr>
<tr>
<td>Canalicu lar number density (1/µm²)</td>
<td>0.21 (±0.05)</td>
<td>30 lacunae</td>
<td>0.19 (±0.02)</td>
</tr>
<tr>
<td>Canalic u lar wall cross-sectional area (µm²)</td>
<td>0.081 (±0.001)</td>
<td>506 canaliculi</td>
<td>0.066 (±0.007)</td>
</tr>
<tr>
<td>Cell process cross-sectional area (µm²)</td>
<td>0.017 (±0.002)</td>
<td>506 canaliculi</td>
<td>0.014 (±0.002)</td>
</tr>
<tr>
<td>Canalic u lar fluid annular area (µm²)</td>
<td>0.065 (±0.034)</td>
<td>506 canaliculi</td>
<td>0.053 (±0.026)</td>
</tr>
<tr>
<td>Lacunar fluid gap (µm)</td>
<td>0.47 (±0.27)</td>
<td>12 lacunae</td>
<td>0.38 (±0.17)</td>
</tr>
</tbody>
</table>

Student’s unpaired, two-tailed t tests were performed for comparisons.

2.4.3 Morphologies of lacunae subjected to FRAP

For both Hypo and CTL bones, 79 lacunae were subjected to FRAP tests (Table 2.2). The means and SDs of their projection area (A), calculated lacunar volume (LacVol) and surface area (LacSurf), contributing canalicular number (n) and canalicular length (d), as well as the relative volume ratio between the sink lacuna and the contributing canaliculi Vr (CanVol/LacVol) are listed in Table 2.2. The coefficients of variation for these measures were typically between 10% and 30%, but some parameters (LacVol) showed the coefficient of variation as high as 50%, indicating
significant variations among tested lacunae. Although no significant difference between the two genotypes ($p>0.05$) was detected in the projected area, lacunar volume, and surface area, significant differences were found in contributing canalicular number, canalicular length, and the relative volume ratio between the CTL and Hypo groups ($p<0.003$). These parameters measured in the FRAP tests (Table 2.2) in combination with the TEM data (Table 2.1) were used to construct the genotype-specific three-compartment LCS transport models.

### Table 2.2. Lacunar morphology in the 12-13 month-old male control (CTL) and perlecan deficient (Hypo) mice subjected to FRAP tests

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Control Bone (CTL, n=79 lacunae)</th>
<th>PCM Deficient Bone (Hypo, n=79 lacunae)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lacunar projected area ($A, \mu m^2$)</td>
<td>97 (±22)</td>
<td>96 (±20)</td>
<td>0.7</td>
</tr>
<tr>
<td>Lacunar volume ($LacVol, \mu m^3$)</td>
<td>497 (±178)</td>
<td>492 (±166)</td>
<td>0.8</td>
</tr>
<tr>
<td>Lacunar surface area ($LacSurf, \mu m^2$)</td>
<td>329 (±75)</td>
<td>325 (±68)</td>
<td>0.7</td>
</tr>
<tr>
<td>Contributing canalicular number ($n$)</td>
<td>15.2 (±3.5)</td>
<td>13.6 (±2.8)</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Canalicular length ($d, \mu m$)</td>
<td>26.7 (±4.2)</td>
<td>30.3 (±5.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$V_r (=CanVol/LacVol)*$</td>
<td>0.056 (±0.01)</td>
<td>0.046 (±0.009)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Student’s unpaired, two-tailed $t$ tests were performed for comparisons. 

* $V_r$ is the fluid volume ratio between the canaliculi and the sink lacuna ($CanVol/LacVol$); $CanVol$ is the product of the contributing canalicular number $n$, the canalicular annular area measured in TEM (Table 2.1), and the canalicular length $d$.

### 2.4.4 Tracer diffusivity

Taking into account each individual lacuna’s morphology and its connectivity to surrounding lacunae [34], tracer diffusivity in the LCS demonstrated a clear
dependency on the tracer type and genotype using two-way ANOVA analysis  
(genotype F\(_{1,92}=16.4, \ p<0.0001\) and tracer type F\(_{1,92}=25.31, \ p<0.0001\)) (Figure 2.1).  
The diffusivity of sodium fluorescein increased +33% in the Hypo bones (402±126 \(\mu\text{m}^2/\text{s}, \ n=38\)) compared with the CTL bones (302±72 \(\mu\text{m}^2/\text{s}, \ n=33, \ p<0.05\)), whereas  
that of parvalbumin increased +40% in the Hypo bones (280±61 \(\mu\text{m}^2/\text{s}, \ n=11\)) compared with the CTL bones (200±55 \(\mu\text{m}^2/\text{s}, \ n=14, \ p<0.05\)). Between the two  
tracers, the diffusivity of the larger parvalbumin decreased significantly (−34% and  
−30% in the CTL and Hypo bones, respectively), compared with that of the  
smaller tracer sodium fluorescein in bones of the same genotypes (\(p<0.05\); compare  
the hatched and solid bars in Figure 2.1).
Figure 2.1. Diffusivity of sodium fluorescein (Stokes radius 0.45 nm) and parvalbumin (Stokes radius 1.31 nm) in cortical bone LCS of the 12- to 13- month-old CTL and Hypo mice. Two-way ANOVA showed that genotype and tracer type had significant effects on the measurements. Perlecan deficiency increased diffusion for both small and large molecules. Bars denoted with different letters are significantly different in paired comparisons ($p<0.05$). The sample size (the number of lacunae subjected to FRAP tests) is indicated for each study group directly under the corresponding data.

2.4.5 Transport enhancement

Paired convection/diffusion FRAP tests performed on the same lacunae under both loaded and static conditions allowed measurements of transport enhancement ($k/k_0$) for both CTL and Hypo bones (Figure 2.2). Two-way ANOVA showed that genotype ($F_{1,56} = 4.44, p = 0.04$) and tracer type ($F_{1,56} = 8.08, p = 0.006$) had significant effects on the transport enhancement. Due to the small sample size, the Bonferroni multiple comparison tests did not detect any significant difference between
the group means. However, by Student’s $t$ test the transport enhancement of sodium fluorescein increased marginally ($+6\%, p = 0.17$) in the Hypo bones ($1.26\pm0.14, n = 16$) compared with the CTL bones ($1.19\pm0.15, n = 20$) whereas that of parvalbumin increased significantly ($+9\%, p = 0.04$) in the Hypo bones ($1.39\pm0.16, n = 10$) compared with the CTL bones ($1.29\pm0.15, n = 14$). Comparing the two tracers within the same animal group using Student’s $t$ tests, the transport enhancement of the larger tracer, parvalbumin, was higher than that of sodium fluorescein in both genotypes (CTL $+8\%, p = 0.08$; Hypo $+10\%, p = 0.04$; Figure 2.2).
Figure 2.2. Transport enhancement of sodium fluorescein and parvalbumin in loaded versus non-loaded tibial LCS of the 12- to 13-month-old Hypo and CTL mice. Two-way ANOVA showed that genotype and tracer type had significant effects on the measurements. Although no significant difference was detected for multiple comparison using Bonferroni post hoc tests, the larger parvalbumin moved significantly faster in the Hypo PCM relative to the CTL PCM as assessed by unpaired Student’s t test ($p = 0.03$). The $n$ values of paired FRAP tests per study group ranged from 10 to 20 lacunae.

2.4.6 Solute velocities

Using the genotype-specific three-compartment LCS models constructed for the Hypo and CTL bones, the transport enhancements ($k/k_0$) for sodium fluorescein and parvalbumin were obtained for various solute velocities $v_s (0–80.6 \, \mu m/s)$ through computer simulations (Figure 2.3). The results for the four experimental groups (two tracer types and two genotypes) fit well with power relationships:
Sodium fluorescein in the CTL bone:

\[ \frac{k}{k_0} = 1 + 1.514 \times 10^{-4} \times v_s^{1.814} \quad (R^2 = 0.99) \]  

(2.4)

Parvalbumin in the CTL bone:

\[ \frac{k}{k_0} = 1 + 2.888 \times 10^{-4} \times v_s^{1.784} \quad (R^2 = 0.99) \]  

(2.5)

Sodium fluorescein in the Hypo bone:

\[ \frac{k}{k_0} = 1 + 0.985 \times 10^{-4} \times v_s^{1.847} \quad (R^2 = 0.99) \]  

(2.6)

Parvalbumin in the Hypo bone:

\[ \frac{k}{k_0} = 1 + 1.820 \times 10^{-4} \times v_s^{1.815} \quad (R^2 = 0.99) \]  

(2.7)

For a given solute velocity, the transport enhancement is inversely related to the solute diffusivity. The magnitudes of transport enhancement are thus ordered (from the least to highest) as sodium fluorescein in Hypo LCS, sodium fluorescein in CTL LCS, parvalbumin in Hypo LCS, and parvalbumin in CTL LCS (Figure 2.3). From these relationships, the solute velocities giving rise to the mean transport enhancement measured within the four groups, as well as the range of velocities corresponding to 1SD above and below the mean transport enhancement were readily obtained (Table 2.3). On average, the current loading condition (3-N peak loads at 0.5 Hz with 4-second resting periods) resulted in a peak velocity of 51.1 µm/s and 48.2 µm/s in the canaliculi of the CTL bones for sodium fluorescein and parvalbumin, respectively. However, in Hypo LCS, the same loading conditions resulted in a +39%
and +42% increase in the peak velocity for sodium fluorescein (71.2 \( \mu m/s \)) and parvalbumin (68.4 \( \mu m/s \)), respectively.

Figure 2.3. Simulated transport enhancement as a function of solute velocity for the 12- to 13-month-old CTL and Hypo bone LCS, using their custom three-compartment transport models. The transport enhancement followed a power relationship with the solute velocity in both CTL (solid lines) and Hypo bones (dashed lines) and for sodium fluorescein (smaller circles) and parvalbumin (larger squares). Detailed relationships can be found in the text (Eq. [2.4–7]).
Table 2.3. **Load-induced tracer velocity in the bone canaliculi of the 12-13 month-old male control (CTL) and perlecan deficient (Hypo)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (Months)</th>
<th>Molecule</th>
<th>Loading Peak (N)</th>
<th>Solute velocity for mean TE (µm/s)</th>
<th>Velocity range for TE-SD to TE+SD (µm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL 12-13</td>
<td>Sodium Fluorescein</td>
<td>3</td>
<td>51.1</td>
<td>21.6 – 70.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parvalbumin</td>
<td>3</td>
<td>48.2</td>
<td>32.0 – 60.8</td>
<td></td>
</tr>
<tr>
<td>Hypo 12-13</td>
<td>Sodium Fluorescein</td>
<td>3</td>
<td>71.2</td>
<td>46.8 – 89.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parvalbumin</td>
<td>3</td>
<td>68.4</td>
<td>51.2 – 82.7</td>
<td></td>
</tr>
</tbody>
</table>

TE = transport enhancement

2.4.7 **Reflection coefficient in the PCM**

Due to its small size and negligible reflection coefficient (~0.5%) within the osteocytic PCM [30], the velocity of sodium fluorescein was assumed to be that of the fluid velocity ($v_f$). The reflection coefficient of parvalbumin ($\sigma_f = 1 - v_s/v_f$) through the osteocytic PCM in the CTL and Hypo bones was found to be 5.7% and 3.9%, respectively (Table 2.4), demonstrating an aging-related decrease in the aged CTL (~32.1%) and Hypo (~31.6%) mice, compared with that of young CTL mice measured previously ($\sigma_f = 8.4\%$) [74].
Table 2.4. **PCM’s sieving property measured in young adult, aged CTL and aged Hypo bones using the FRAP tracer velocimetry approach**

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>Young bone</th>
<th>Aged CTL bone</th>
<th>Aged Hypo bone</th>
<th>Relative change (Aged vs. Young)</th>
<th>Relative change (Hypo vs. CTL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reflection Coefficient</td>
<td>8.4%*</td>
<td>5.7%</td>
<td>3.9%</td>
<td>-32.1%</td>
<td>-31.6%</td>
</tr>
</tbody>
</table>

*Measured in our previous study [74].

### 2.4.8 Osteocytic PCM ultrastructure in the canaliculi

Using our newly published PCM sieving model [74], possible configurations of PCM fiber matrix that could account for the observed reflection coefficients were estimated (Tables 2.5 and 2.6). Depending on the fiber radius (0.5–4 nm), the fiber volume fraction varied in the ranges of 0.4% to 17.2%, 0.2% to 13.0%, and 0.1% to 9.5% for the young CTL bones, aged CTL bones, and aged Hypo bones, respectively (Table 2.5). For all the fiber sizes considered, a decrease in the fiber volume fraction ranging from −50% to −24% was clearly seen in the aged versus young bones. A similar degree of decrease in the fiber volume fraction (−50% to −27%) was observed in the Hypo versus age-matched CTL bones (Table 2.5). For an idealized square array of fibers, the effective fiber edge-to-edge spacing varied with fiber radius and among groups (Table 2.6). For the fiber radii considered (0.5–4 nm), the fiber spacing varied from 12.9 to 9.1 nm, 17.3 to 11.7 nm, and 23.1 to 15.0 nm for the young CTL, aged CTL, and aged-matched Hypo bones, respectively. Regardless of the radius assumed for the PCM fibers, the fiber spacing was consistently larger in the aged...
bones relative to the young bones (+34% to +29%) and in the Hypo bones relative to the CTL bones (+34% to +28%; Table 2.6).

<table>
<thead>
<tr>
<th>Fiber radius* (nm)</th>
<th>Young bone</th>
<th>Aged CTL bone</th>
<th>Aged Hypo bone</th>
<th>Relative change (Aged vs. Young)</th>
<th>Relative change (Hypo vs. CTL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.4%</td>
<td>0.2%</td>
<td>0.1%</td>
<td>-50%</td>
<td>-50%</td>
</tr>
<tr>
<td>1</td>
<td>1.7%</td>
<td>1.1%</td>
<td>0.6%</td>
<td>-35%</td>
<td>-45%</td>
</tr>
<tr>
<td>2</td>
<td>6.1%</td>
<td>4.1%</td>
<td>2.7%</td>
<td>-33%</td>
<td>-34%</td>
</tr>
<tr>
<td>4</td>
<td>17.2%</td>
<td>13.0%</td>
<td>9.5%</td>
<td>-24%</td>
<td>-27%</td>
</tr>
</tbody>
</table>

* Fiber radius was parametrically varied from 0.5nm (size of GAG), 1-2nm (size of proteoglycan core proteins), to 4nm (repeated features in endothelial glycocalyx).

<table>
<thead>
<tr>
<th>Fiber radius (nm)</th>
<th>Young bone (nm)</th>
<th>Aged CTL bone (nm)</th>
<th>Aged Hypo bone (nm)</th>
<th>Relative change (Aged vs. Young)</th>
<th>Relative change (Hypo vs. CTL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>12.9</td>
<td>17.3</td>
<td>23.1</td>
<td>34%</td>
<td>34%</td>
</tr>
<tr>
<td>1</td>
<td>11.6</td>
<td>15.3</td>
<td>20.2</td>
<td>32%</td>
<td>32%</td>
</tr>
<tr>
<td>2</td>
<td>10.3</td>
<td>13.4</td>
<td>17.4</td>
<td>30%</td>
<td>30%</td>
</tr>
<tr>
<td>4</td>
<td>9.1</td>
<td>11.7</td>
<td>15.0</td>
<td>29%</td>
<td>28%</td>
</tr>
</tbody>
</table>

Table 2.5. Fiber volume fraction estimated for young adult, aged CTL and aged Hypo bones

Table 2.6. Fiber spacing estimated for young adult, aged CTL and aged Hypo bones

61
2.4.9 Cellular-level mechanical stimulations

Knowledge of the PCM fiber spacing in the canaliculi allowed us to derive the detailed spatial profiles for the fluid flow in the CTL and Hypo bones under 3-N peak load (Figure 2.4). For the case of fiber radius of 2 nm, the spatial velocity profile across the radial gap between the cell process and the canicular wall followed a plug flow-like pattern in the CTL bones, in which the fiber spacing was 13.4nm (Table 2.6). However, the flow profile became more parabolic-like in the Hypo bones, in which the fiber spacing was increased to 17.4nm (Table 2.6). The peak fluid velocity was higher in the Hypo bones (99.7 µm/s) than that predicted in the CTL bones (67.8 µm/s). For the Hypo bones, the increased peak velocity and the narrower fluid annular gap (78nm versus 87 nm) resulted in a higher shear stress (as shown by a steeper slope) at the locations of the cell process membranes (CTL: radius (r)=74 nm; Hypo: radius (r)=67 nm). The fluid shear stress on the cell process membrane and the shearing force per unit length of the cell process were +34% and +24% increased, respectively, in the Hypo bones relative to CTL bones (Table 2.7). However, due to the reduced fiber density, the fluid drag force and the ratio of the fluid drag over the shearing force were reduced 35% and 48%, respectively, in the Hypo bones compared to the CTL bones (Table 2.7). Similar findings were found with three other fiber radii (0.5, 1, and 4 nm) (data not shown).
Figure 2.4. Canalicular flow profiles for the 12- to 13-month-old CTL and Hypo bones under 3-N peak load. For the case of fiber radius of 2 nm, the velocity profile between the cell process and the canalicular wall appeared to be more plug flow-like in the CTL bones (the fiber spacing of 13.4 nm) but more parabolic-like in the Hypo bones (the fiber spacing of 17.4 nm). Note that the fluid annulus in the Hypo bones had a gap of 78 nm, which was 10% smaller than that in the CTL bones (a gap of 87 nm) as reported in Table 2.1.

<table>
<thead>
<tr>
<th></th>
<th>CTL Bone</th>
<th>Hypo Bone</th>
<th>Relative change (Hypo vs. CTL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear stress</td>
<td>5.9</td>
<td>7.9</td>
<td>+34%</td>
</tr>
<tr>
<td>Shearing force</td>
<td>2.72</td>
<td>3.38</td>
<td>+24%</td>
</tr>
<tr>
<td>Fluid drag force</td>
<td>18.4</td>
<td>12.0</td>
<td>-35%</td>
</tr>
<tr>
<td>Ratio of fluid</td>
<td>6.8</td>
<td>3.5</td>
<td>-48%</td>
</tr>
</tbody>
</table>

Both bones were loaded at 3-N peak load at 0.5Hz; the fiber spacing was 13.4nm (CTL) and 17.4nm (Hypo), respectively (see Table 2.6, fiber radius = 2nm).
2.4.10 Responses to in vivo loading

The non-loaded tibiae did not show significant differences in cortical µCT parameters between CTL and Hypo mice, except for a slightly higher tissue mineral density (Ct.TMD) in the Hypo mice (Table 2.8). Loading resulted in an increase in cortical bone polar moment inertia (Ct.pMOI, +6.5%, \( p=0.02 \); Figure 2.5A) and a reduction in the cortical porosity (−3.1%, \( p=0.04 \); Table 2.8) in CTL mice, whereas no such anabolic effects were seen in the Hypo mice. Loading also significantly increased tibial stiffness in CTL mice (\( p=0.01 \)), but did not increase the stiffness of Hypo tibiae (\( p=0.19 \); Figure 2.5B). Dynamic bone labeling analysis revealed no difference in the mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) in the non-loaded tibiae at either periosteal or endosteal surfaces (Table 2.8). Loading did not significantly increase the Ps.MS/BS (Figure 2.5C) but significantly increased Ps.MAR (+75%, \( p=0.03 \); Figure 2.5D), resulting in an increase in Ps.BFR/BS (+141%, \( p=0.02 \); Table 2.8) in CTL mice, whereas no such effects were detected, perhaps due to relatively larger data variability, in the Hypo mice. Loading did not affect any of the endosteal measures (Table 2.8). Overall, the preliminary data suggested diminished anabolic response to mechanical loading in the Hypo mice compared with the CTL mice.
Figure 2.5. The 3.5-month-old Hypo mice did not respond to anabolic tibial loading (8.5-N peak load, 4 cycles/s, 5 minutes per session, five sessions over 10 days) as did the age-matched CTL mice in a preliminary experiment. (A) Ct.pMOI; (B) tibial stiffness; (C) Ps.MS/BS; and (D) Ps.MAR. Sample sizes: CTL (n=8) and Hypo (n=6) mice for µCT analysis; CTL (n=7) and Hypo (n=6) mice for mechanical testing and bone labeling analyses. Student’s t tests on the nonleaded versus loaded tibiae revealed significant changes (p<0.05, denoted by *symbols) in CTL mice but not in Hypo mice. A full data set with other histomorphometry parameters and detailed p values of statistical tests can be found in Table 2.8.
Table 2.8. Results of µCT imaging, mechanical testing, and dynamic bone labeling analysis at the tibial mid-diaphysis of 3.5 month-old CTL and Hypo mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>µCT parameters</th>
<th>Mechanical testing parameters</th>
<th>Dynamic bone labeling parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ct.Ar (mm²)</td>
<td></td>
<td>Ps.MS/BS (%)</td>
</tr>
<tr>
<td></td>
<td>0.64±0.08</td>
<td></td>
<td>5.1±4.6</td>
</tr>
<tr>
<td></td>
<td>0.66±0.06</td>
<td></td>
<td>10.5±9.1</td>
</tr>
<tr>
<td>CTL (N=8/7)</td>
<td>0.67±0.08</td>
<td></td>
<td>5.7±6.7</td>
</tr>
<tr>
<td>non-loaded</td>
<td>0.67±0.06</td>
<td></td>
<td>13.0±8.9</td>
</tr>
<tr>
<td>loaded</td>
<td>Non-loaded</td>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td>CTL vs. Hypo</td>
<td>loaded</td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td>loaded vs. non-loaded</td>
<td></td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>Non-loaded CTL vs. Hypo</td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>loaded vs. non-loaded</td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>CTL loaded vs. non-loaded</td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>Hypo (N=6)</td>
<td></td>
<td></td>
<td>0.94</td>
</tr>
</tbody>
</table>

Note: The sample sizes were N=8 CTL and N=6 Hypo mice for the micro-CT analysis. The right tibia of one CTL mouse was lost and thus the sample sizes were reduced to N=7 CTL for mechanical testing and dynamic bone labeling analyses. Unpaired two-tail Student t tests were performed for the comparisons on the non-loaded tibiae between CTL and Hypo groups; and the rest comparisons were performed using paired two-tail Student t tests. P values less than 0.05 were shown in **bold**.
2.5 Discussion

Although the osteocytic PCM, the critical interface between outer cell membrane and canalicular wall, is believed to play a key role in osteocyte nutrition, cell-to-cell signaling, and mechanosensing [1, 71, 107, 108], there have been few quantitative studies characterizing its functions due to its small dimensions, its inaccessibility, and a lack of proper investigative tools. Using our recently developed tracer velocimetric approach [110], which combined FRAP-based confocal imaging and a hydrodynamic sieving model, we were able to study the effects of alterations in the osteocytic PCM, particularly loss of perlecan/HSPG2, on solute transport and fluid flow. We found increased diffusion and convection of both small and large molecules in cortical bone when the osteocytic PCM became sparser (Figures 2.1 and 2.2). The perlecan-deficient mice used in this study represent a well-established model [94], in which the normal expression of perlecan, a large proteoglycan normally found in the osteocytic LCS, is genetically altered, similar to that in human SJS, with profound musculoskeletal impairments [94, 129]. We found that a moderate mechanical loading (~ 300 µε) resulted in a significant fluid flow in normal bone (51.1 µm/s), which was further elevated in the perlecan-deficient bone (71.2 µm/s; Table 2.3). More importantly, we successfully detected changes in the sieving properties and the ultrastructure of the osteocytic PCM among young adult, aged, and perlecan-deficient bones (Tables 2.4-6), which allowed us to obtain detailed velocity profiles within their canalicular channels (Figure 2.4), and the levels of mechanical stimulation forces such as the shear stress and fluid drag experienced by osteocytes in situ (Table 2.7). This
study demonstrated that our FRAP-based approach is sensitive enough to detect alterations in the osteocytic PCM density associated with aging and changes in specific PCM components. This study provides a solid foundation and a powerful tool for better characterizing the osteocytic PCM and dissecting its functional roles in bone physiology and pathology.

2.5.1 Validation of the tracer velocimetry approach

As discussed in our previous work [110], the novelty of the approach lies on its capability of quantifying the sieving and structural properties (i.e., reflection coefficient, fiber volume fraction, and fiber spacing) of the osteocytic PCM fibers, which are beyond the diffraction limited resolution of light microscopy (~ 0.2 µm). This is accomplished by measuring the fiber-tracer interactions, which become measurable due to the collective draining of the flows inside the 50 to 100 discrete PCM-containing canalicular channels into the central photobleached lacuna [110]. To the best of our knowledge there are no studies directly measuring the PCM fiber volume fraction and few data on fiber spacing with which to directly compare our findings. Alternatively, we first checked the consistency of our results with previously published tracer perfusion data, followed by analysis of solute transport behaviors in the transgenic perlecan hypomorphic mouse, a model with known alterations in the PCM composition [94, 129]. Various-sized tracers have been perfused in living bone and their spatial distributions observed in histological sections [5, 6, 87]. It was observed that molecules less than 6 nm in diameter (such as procion red, horseradish...
peroxidase, and 10 kDa dextran) could penetrate into the LCS, whereas larger molecules such as ferritin (12 nm) and 60 kDa dextran were excluded from the LCS [5, 6, 87]. These studies suggest that the effective pore size of adult osteocyte PCM is between 6 and 12 nm. In this study, we found that the fiber spacing in young adult bone ranged from 9.1 to 12.9 nm, depending on the individual fiber radius (Table 2.6). This result agreed with previous perfusion results very well. Second, our results demonstrated increased diffusion (Figure 2.1) and convection (Table 2.3) of both small and large tracers in the perlecan-deficient bone, consistent with the prediction of a sparser PCM, which have been implied in this mouse model due to a reduction in perlecan protein expression and decreased number of tethering elements per canaliculus relative to the controls [94, 129]. Specifically, we identified an approximately 30% increase in the fiber spacing in the perlecan-deficient PCM compared with the age-matched wide-type PCM. These agreements support the fidelity of our current approach in quantifying the osteocytic PCM.

2.5.2 Plug-flow versus parabolic flow

Quantification of the fiber spacing values in various bones also allowed us to obtain the spatial velocity profiles of the load-induced canalicular fluid flows. Weinbaum and colleagues [1] predicted a Darcy-like (plug) flow through GAG-filled canaliculi assuming a fiber spacing of 7 nm. This plug-flow pattern has been widely used in later studies [138]. The fiber spacing measured in the 12- to 13-month-old CTL and Hypo bones was in the range of 11.7 to 23 nm. Fitting our measured fluid
velocity and the fiber spacing values (13.4 nm and 17.4 nm in the case of 2-nm fiber radius) into the Brinkman equation, we found that the flow velocity profile approximated a Darcy-like plug-flow in the CTL canaliculus, especially in the central lumen region, whereas flow shifted to a more parabolic-like waveform in the Hypo canaliculus due to an increased fiber spacing (Figure 2.4). This result demonstrated that the local fluid velocity field in the PCM is quite sensitive to the density of the PCM fibers.

2.5.3 Shear stress versus fluid drag

One fundamental question regarding osteocyte mechanotransduction is which physical signal(s) are sensed by osteocytes in situ and eventually lead to in vivo bone adaptation processes [139]. Both fluid shear stress acting on the cell membrane and fluid drag force acting on the tethering PCM fibers have been proposed to be the triggering signals for osteocytes [1, 71]. Osteocytes subjected to fluid shear stress in parallel-plate chambers have demonstrated both short-term responses (eg, intracellular calcium signaling, and ATP, nitric oxide, and prostaglandin E2 [PGE2] release) and long-term responses (eg, expression of sclerostin, receptor activator of NF-kB ligand [RANKL]/osteoprotegerin [OPG], as well as apoptosis) [14, 54, 126]. However, very few experiments have been performed to investigate fluid drag via PCM. In one example, the PCM surrounding the MLO-Y4 cells was disrupted with hyaluronidase treatment and the flow-induced PGE2 release was found to be completely abolished [105]. In a recent study, MLOY4 cell processes were allowed to penetrate into
microscopic channels inside a filter membrane to establish a semi-3D contact between cell process and surrounding matrix. Similarly, hyaluronidase treatment was found to block mechanically activated opening of connexin43 hemichannels [106], a critical step in the release of ATP and PGE2 in response to mechanical stimulation [140]. These *in vitro* systems, although provide valuable insights on the PCM’s role in osteocyte mechanotransduction, are not ideal for assessing relative contributions of the fluid shear stress versus fluid drag force to osteocyte mechanotransduction, largely owing to their lack of 3D cell-PCM interactions and LCS pore system observed *in vivo*. The analysis from the present study (Table 2.7) clearly demonstrates that we can dissect the roles of shear stress and fluid drag *in vivo* by using the perlecan-deficient model. Comparing the Hypo and CTL bones under the same mechanical loading (3 N), osteocytes in the Hypo bones are anticipated to experience larger shearing force (+24%), but smaller fluid drag force (−35%) than those in the CTL bones (Table 2.7). The ratio between the drag force and the fluid shearing force is predicted to decrease by 48% in the Hypo bones compared with CTL bones. Therefore, the perlecan Hypo mouse can be used to test the relative contribution of shear stress and fluid drag during the *in vivo* bone adaptation process because the behaviors of shear stress and fluid drag are diverging under mechanical stimulation. If the shear stress is the primary signal that triggers osteocyte mechanotransduction, the Hypo mice are expected to respond to mechanical loading more rigorously than the CTL mice. On the other hand, if the fluid drag force is the triggering signal, the CTL mice are expected to be more responsive to loading. The latter case was supported by our preliminary in vivo
loading data [141]. We showed that tibial axial compressive loading (8.5N peak load at 4 Hz, 5 minutes per session, five sessions over a total of 10 days) significantly increased the stiffness and polar moment inertia in the loaded tibiae in the CTL mice by elevating the mineral apposition rate and bone formation rate (Figure 2.5, Table 2.8); in contrast, no significant anabolic changes were detected in the perlecan-deficient mice. These results suggest that bone adaptation is likely driven by fluid drag acting on the PCM fibers, but not shear stress acting on the osteocyte cell process membrane. Noting the relatively large variability in the Hypo data, we propose to use larger sample sizes and/or greater mechanical stimulations in future studies.

2.5.4 PCM fibers as the osteocyte’s “sensing antenna”

Our current data support the strain amplification hypothesis originally proposed by You and colleagues [71], and later refined by Han and colleagues [79], and Wang and colleagues [125]. In contrast to Weinbaum and colleagues’ [1] shear stress model, the strain amplification model assumed that the osteocytic PCM fibers tethering the canalicular wall and cell process are deformed by the fluid drag from the load-induced fluid flow. Tethering fibers have been visualized in TEM studies and the spacing of the tethering fiber was measured to be ~ 40 nm [4], which was approximately three to four times larger than the fiber spacing (9–13 nm) reported here for young adult bone (Table 2.6). It was likely that some tethering fibers were collapsed or lost during the TEM processing procedures. The chemical composition and mechanical strength of these tethering elements remain largely unknown, except
for the identification of extracellular perlecan inside the canaliculi in our recent study [94]. From structural and mechanical points of view, perlecan is a highly viable candidate for mechanosensitive tethers, forming stable associations both with the cell membrane and the bone matrix lining the canalaricular wall (Figure 2.6). It is well established that perlecan, with five independently functioning domains [93], interacts with numerous extracellular matrix (ECM) proteins (including those found in bone matrix) and binds many growth factors, cytokines, and various transmembrane proteins (including integrins) at the cell surface [93, 142]. It is also possible that some PCM fibers are anchored only at the cell process membrane, with the other end possibly just touching the canalaricular wall without being rigidly fixed to the wall. In this configuration, the PCM fibers could still capture fluid drag, similar to the way that endothelial glycocalyx interacts with blood flow. Conceptually, the single-end anchored PCM fibers, acting as sensing antenna, may be more compatible with motile osteocytic cell processes observed for newly embedded osteocytes [143]. We are currently investigating perlecan’s interactions with molecules associated with cell membrane and bone matrix.

2.5.5 PCM fiber density as an indicator of mechanosensitivity

Because the load-induced cellular stimulation forces are sensitive to the PCM fiber density (Table 2.7), we speculate that the PCM fiber density serves as an indicator/regulator of osteocyte mechanosensitivity. Two lines of experiments support this hypothesis. First, in our in vivo loading study, perlecan-deficient mice (with a
sparser PCM) failed to respond to mechanical loading that induced anabolic bone formation in control mice (with a denser PCM) (Figure 2.5, Table 2.8), demonstrating an association between the PCM fiber density and bone’s mechanosensitivity. Second, we detected a 29% to 34% decrease in PCM fiber density in the 12- to 13-month-old CTL bones compared with the 4- to 5-month-old CTL bones (Table 2.6). Data from the literature have demonstrated a diminishing mechanosensitivity in bone with aging [103, 144], again supporting a potential association between the PCM fiber density and bone’s mechanosensitivity. Because the synthesis of heparan sulfate proteoglycans does not change with aging [145], this age-related loss of PCM fibers is likely due to increased PCM shedding caused by accumulated oxidative stress in aging cells, as has been shown for the endothelial glycocalyx [146, 147]. Taken together, the existing evidence leads us to hypothesize: (1) that the PCM fibers act as the osteocyte’s sensing antenna, capturing flow-induced fluid drag and transmitting this mechanical signal to the intracellular domain, and (2) that PCM fiber density influences bone’s mechanosensitivity and adaptation to loading (Figure 2.6). Within the range of the PCM fiber spacing (9–23 nm) found herein, a denser PCM would result in a higher fluid drag force on the transverse fibers, which could trigger downstream signaling and/or gene expression processes. This could occur through actions that either modulate the opening or closing of transmembrane ion channels and hemichannels (for signaling with small secondary messengers such as Ca\(^{2+}\), PGE2, and ATP), or directly disturb the physically interconnected system consisting of PCM-integrin/focal adhesion complexes-cytoskeleton. Conversely, a sparser PCM would
result in a reduction in fluid drag and a decrease in the degree of downstream responses. Rigorous testing of this hypothesis calls for studies at the tissue, cellular, and molecular levels.

Figure 2.6. A working hypothesis depicting the osteocytic perlecan-rich PCM, which act as flow-sensing antenna, capturing flow-induced fluid drag, and triggering osteocyte’s mechanotransduction process. A denser PCM (as in normal CTL bone) would result in a higher fluid drag force on the transverse tethered fibers, which could trigger downstream signaling and/or gene expression through interactions with cell membrane via various channels membrane receptors, or the physically connected PCM-integrin/focal adhesion complex-cytoskeleton system. Conversely, a sparser PCM (as in perlecan-deficient bone) would result in a smaller fluid drag and the degree of downstream responses would be reduced in spite of the same mechanical loading at the whole bone level. PCM=pericellular matrix; CTL=control; Hypo=hypomorphic, perlecan-deficient.
2.5.6 Limitations of the present studies

The present investigation adopted the one-colored FRAP velocimetry approach [110] and thus suffered similar limitations. The main drawback was that the two fluid/solute tracking tracers were injected into separate sets of mice, introducing errors associated with intersample and intertest variability. Because of this limitation, the reflection coefficient reported herein was obtained using the mean transport and anatomical values for a given genotype. To address this drawback, I am currently developing a dual-color imaging approach in which both probes (with distinct emission wavelengths) are injected into a single mouse to simultaneously measure fluid and solute velocities [148]. Second, our hydrodynamic sieving model was limited to highly idealized fiber orientations; a single fiber species; and rigid, stationary fibers. These idealizations were made to obtain a closed-form solution to the problem. The more frequently observed radial transverse fibers within the canaliculi justified the assumption of the fiber arrays chosen in this model. As shown in our results (Table 2.6), the model-predicted edge-to-edge fiber spacing remains relatively constant regardless of the fiber radius, suggesting that this simple model succeeds in capturing the physics of the sieving properties of the PCM, which depends mainly on the effective fiber spacing. Third, because our previous TEM characterization of LCS anatomical parameters was performed in relatively old perlecan mice (8–9 months), the present study utilized 12- to 13-month-old perlecan-deficient mice and the age-matched normal controls to validate the tracer velocimetry approach. Although they are excellent models for aged osteoporosis, we are aware that most in vivo loading
studies including ours used younger animals; we plan to map the changes of the PCM as a function of age in order to better correlate with bone adaptation studies. Last, the perlecan deficiency in our Hypo mice affects not only bone but also other systems. It would be great to use transgenic models with bone-specific PCM alterations and/or on-demand initiation of PCM alteration. Because the perlecan/HSPG2 gene is a complex gene involved in many developmental processes [93, 149], knockout mutation is lethal [150] and conditional bone-specific perlecan knockout models have yet to be developed. The current model is by far the best available model and the results are relevant to SJS patients [129, 131, 150, 151].

2.6 Conclusions

We discovered that the FRAP tracer velocimetry approach was sensitive enough to detect (1) the increases of fluid (+39%) and solute (+42%) convection through the LCS due to perlecan deficiency in 12- to 13-month-old murine tibiae under 3-N and 0.5-Hz loading, and (2) the decreases in PCM fiber density associated with aging (from −29% to −34%) and perlecan deficiency (from −28% to −34%). PCM fiber spacing was found to be 9.1 to 12.9 nm, 11.7 to 17.3 nm, and 15.0 to 23.1 nm, for young (4- to 5-month-old) CTL tibiae, 12- to 13-month-old CTL tibiae, and 12- to 13-month-old Hypo tibiae, respectively. This new knowledge allowed us, for the first time, to obtain the velocity profiles of the load-induced flow through the LCS and to predict the magnitude of flow-induced cell stimulation forces, such as shear stress and fluid drag. Decreased perlecan and PCM fiber density in the Hypo bone was found to
increase shear stress (+34%) on the cell process membrane, but decreased fluid drag force per unit length of canaliculi (−35%), and a much reduced ratio of fluid drag force over shear force (−48%). When subjected to tibial axial loading (8.5 N, 4 Hz, 5 minutes/session, five sessions over 10 days) in a preliminary in vivo experiment, 3.5-month-old Hypo mice did not respond to the anabolic stimuli as did normal CTL mice, supporting the idea that fibers in the PCM act as the osteocyte’s sensing antennae and that bone’s mechanosensitivity depends on the PCM fiber density. If proven true in future studies, the PCM fiber density could be used to provide new targets to treat osteoporosis by modulating bone’s intrinsic sensitivity to mechanical loading and to guide designs of patient-specific exercise regimens to promote bone formation.
Chapter 3

THE INFLUENCE OF BONE COMPARTMENT, AGE, AND DISEASE ON THE CYTO-ARCHITECTURE OF OSTEOCYTE LACUNAR-CANALICULAR SYSTEM

3.1 Abstract

Osteocytes, the most abundant bone cells, form an interconnected network in the lacunar canalicular pore system (LCS) buried within the mineralized matrix, which allows osteocytes to obtain nutrients from the blood supply, sense external mechanical signals, and communicate among themselves and with other cells on bone surfaces. In this study, we examined key features of the LCS network including the topological parameter and the detailed structure of individual connections and their variations in cortical and cancellous compartments, at different ages, and in two disease conditions with altered mechanosensing (perlecan deficiency and diabetes). LCS network showed both topological stability, in terms of conservation of connectivity among osteocyte lacunae (similar to the “nodes” in a computer network), and considerable variability the pericellular annular fluid gap surrounding lacunae and canaliculi (similar to the “bandwidth” of individual links in a computer network). Age, in the range of our study (15-32 weeks), affected only the pericellular fluid annulus in cortical bone but not in cancellous bone. Diabetes impacted the spacing of the lacunae, while the perlecan deficiency had a profound influence on the pericellular fluid
annulus. The LCS network features play important roles in osteocyte signaling and regulation of bone growth and adaptation.

3.2 Introduction

As the most abundant cells in bone, osteocytes form an extensive cellular network through numerous cell processes emanating from individual cell bodies. These cellular protrusions and cell bodies are housed with an extensive pore system, the lacunar-canalicular system (LCS), and buried within the bones mineralized matrix. This cellular network allows osteocytes to obtain nutrients from the blood supply, sense external mechanical signals, and communicate among themselves and with other cells on bone surfaces [11]. Previous experimental studies [13, 15, 16, 152] have demonstrated that osteocytes in intact bone change their metabolic activity rapidly after mechanical loading, indicating their function as mechanosensors. There is increasing evidence that osteocytes sense mechanical loading through the interstitial fluid flow around osteocyte cell membranes in the LCS [109, 153]. The spatial and temporal profiles of load induced flow depend not only on the loading parameters but also the architecture of LCS. Alterations to the LCS structures are expected to impact how osteocytes perceive external mechanical stimulation during the “outside-in” mechinosensing processes [1, 26, 27] by modulating the levels of stimulatory forces, such as fluid shear stresses [1] and drag forces on the pericellular tethering fibers of osteocytes [154]. In response to these cellular stimulations, osteocytes release many signaling molecules like nitric oxide (NO), adenosine triphosphate (ATP), sclerostin,
prostaglandin E2 (PGE2) and osteoprotegerin/receptor activator for nuclear factor ligand (OPG/RANKL), which regulate osteoblastic bone formation as well as osteoclastic-targeted bone resorption [27, 28, 127, 155, 156]. Because the principle intracellular transport mechanisms that enable these molecules to reach their target cells are diffusion and/or convection through the LCS, the LCS structure also plays an important role in osteocytes’ “inside-out” signaling process [87, 88, 110]. Using a mathematical model [108], we previously demonstrated that solute transport can be altered with varied LCS parameters [157]. Furthermore, the surface area encasing the fluid-filled pericellular space in the LCS represents a significant interface for the regulation of mineral homeostasis. It is not surprising that LCS morphology has been recently shown to correlate with tissue mineralization [158, 159].

Because of its importance in bone physiology, the LCS morphology has been studied extensively using imaging tools with varied resolution (20μm-1nm) and 2D or 3D imaging capability (see a recent comprehensive review [160]). These studies provided quantitative assessments of the overall size, shape, volume fraction (porosity), and distribution density of the vascular channels, osteocyte lacunae and canaliculi in different bones from many species [160]. For example, lacunae were on the order of 290-455 μm³ (volume) and distributed at a number density of 26-90 lacunae per mm³ and lacunar separation of 21-40μm from mouse to human bones. Canaliculi ranged from 95 to 553 nm in diameter and were distributed at 41 to 387 per lacuna for different species, with a mean matrix distribution of ~0.55-0.85 per μm². Significant variations reported among these measures may be due to the different
methodologies and subjects used in the studies, but they also likely reflect the dynamic nature of the LCS structure in normal and diseased conditions.

These data lead one to ask what the invariant and variant features of the osteocyte network are present in the adult skeleton of normal and diseased subjects. Similar to telecommunications or computer networks, the osteocyte network consists of multiple nodes (lacunae) and interconnecting links (canaliculi) of potentially varying bandwidth (i.e., capacities of signal transfer) among the nodes. As the telecommunication or computer networks allows digital signals to pass among nodes, LCS network’s primary function is to allow osteocytes to obtain nutrients, sense mechanical loading through fluid flow, and communicate with other cells through molecular signaling. In this study, we focused on examining selective features of the osteocyte network in terms of the overall topology (such as the number density of canaliculi emanating from the lacunae) and detailed link bandwidth (such as the pericellular fluid annular gap). We quantified these network features in both cortical and cancellous compartments in long bones from younger and older adult mice as well as from mice with deficiency in the expression of the heparan sulfate proteoglycan perlecan and in mice with diabetes, both of which exhibited altered responses to mechanical loading [154, 161]. Because the central role of osteocyte LCS in fluid flow and bone mechanosensing, their changes, if any, would be important to understand the pathology associated with two disease conditions (perlecan deficiency and diabetes).

The objective of the present study was to identify the invariant and variant features of the osteocyte LCS network. This information will help understand the mechanisms for
intercellular communication among bone cells as well as the roles of bone fluid flow in bone’s response to mechanical forces, a potent anabolic factor regulating bone growth and adaptation.

3.3 Materials and Methods

3.3.1 Experimental groups

Five groups of male mice (N=3 mice/group) of various ages and genotypes were used. Group 1 included adult, 15-week old wild-type C57BL/6J mice (Jackson Laboratory), which served as younger controls to Group 2. Group 2 included 32-week-old C57BL/6J mice (older adults), which also served as wild-type controls to the perlecan-deficient mice in Group 3. Group 3 consisted of 32-week-old C57BL/6J mice with homozygous C1532Yneo mutations in the PLN/HSPG2 gene. Initially developed to model human Schwartz-Jampel Syndrome, these mice (termed “Hypo” herein) exhibited a significant deficiency in perlecan expression [129] and were bred back to C57BL/6J mice background in-house. Bones from Hypo mice were found to have altered canalicular structure [94] and attenuated bone formation in response to tibial uniaxial loading [154] in our previous studies. Group 4 consisted of 20-week-old Akita mice with heterozygous Ins2Akita mutation, a spontaneous type 1 diabetes model (Jackson Laboratory). Akita mice developed severe hyperglycemia from 5 weeks of age. Our previous work has shown that they failed to respond to anabolic ulnar loading when compared to age-matched wild-type mice [162]. Group 5 included age-matched wild-type controls to Group 4 (20-week-old C57BL/6J). The University
of Delaware Institutional Animal Care and Use Committee approved the handling and use of all animals in this study.

3.3.2 Confocal imaging and analysis of 3D osteocyte LCS macrostructure

3.3.2.1 Plastic embedded processing of basic fuchsin stained specimens

Immediately after sacrifice and dissection the right femora were prepared for basic fuchsin staining and plastic embedding following previously established protocols [163]. Briefly, intact right femora were fixed in 10% neutral buffered formalin for 24 hours at 4°C, repeatedly rinsed in PBS, and bulk stained in 1% basic fuchsin dissolved in ethanol solutions with ascending concentrations (70%, 70%, 90%, 90%, 100%, 100% ethanol v/v) with solution changes every 2-3 days. After clearance and embedding in methyl methacrylate, the distal 1/3rd of the femora were isolated and cut sagittally into ~0.20-mm thick slices using a low speed saw (Isomet, Buehler) and a diamond blade (Buehler). All sections were then polished to ~0.15-mm in thickness using graded sandpapers of decreasing grit size (600, 800, 120 grit; Buehler) and polishing liquids (6 & 1-µm; Buehler) to achieve a scratch free finished surface. The polished sections were then mounted on glass slides using Eukitt’s mounting media and #1.5 glass coverglass.

3.3.2.2 Confocal imaging

Two regions of interests (ROI) were selected for study, including i) femoral cortex and ii) cancellous bone that were 1.5-2.15 mm, and 0.25-1mm below the
epiphyseal growth plate, respectively (Figure 3.1A). Ten lacunae were randomly selected for each ROI per animal. In the cortical ROI, a regularly spaced grid was placed atop a preview image (Figures 3.1B&3.1C) captured via an inverted confocal laser-scanning microscope (Zeiss LSM510, Carl Zeiss, Inc. NA., Thornwood, NY). Ten lacunae that fell on the grid intersections and showed intact full-depth structures under a quick z-stack imaging were selected for subsequent high resolution 3D imaging. For the cancellous ROI, due to a smaller number of available lacunae, a selection grid was not utilized. Instead, ten lacunae with intact full-depth structures were randomly chosen per animal from the region of cancellous bone. To quantify individual lacunae measurements, such as lacunar size and canalicular number density, Z-stacks of high-resolution, 2048x2048-pixel, images were captured for each of the selected lacunae and their canaliculi, utilizing the following optical parameters:

- excitation wavelength = 561nm, emission wavelength = 650nm, objective = 40x (1.2NA) water lens (C-Apochromat W Korr, Carl Zeiss, Inc. NA), pinhole = 1 AU (optical slice thickness = 1.0μm), zoom = 5, pixel =0.022μm, scan speed = 62s/frame, frame averaging = 8, z-step = 200nm. Detector gains and offsets were adjusted to maximize the dynamic range within a given image stack and a linear gain/offset compensation protocol was employed to insure consistent image signal/quality as individual z-stack scans imaged deeper into the tissue blocks. For measurements of lacuna density, individual 2048x2048-pixel frames were randomly captured throughout the tissue regions using a 10X (0.20NA) objective and similar imaging
settings as described above (0.44μm/pixel). For groups 4 (Akita mice) and 5 (WT controls), only metaphyseal cortices were examined.

Figure 3.1. A representative confocal preview image (A) of basic fuchsin stained sagittal section of a murine distal femur, showing the two ROIs located in metaphyseal cortical and cancellous bone compartments. (B&C) Due to larger number of lacunae in ROI1, a grid was overlaid on the image and 30-40 lacunae that fell on the inter- sections of the grid, as outlined by the yellow boundaries, were selected for a quick check of full-depth structures. Ten lacunae with intact 3D structures were chosen for high-resolution 3D imaging. For ROI2, ten lacunae were randomly chosen per animal without the grid.
3.3.2.3 Quantitative measures of 3D LCS macrostructure

Three-dimensional renderings of individual lacuna and their associated canaliculi were reconstructed from confocal z-stacks using the VOLOCITY software package (PerkinElmer). Using VOLOCITY, an initial selection and cropping of a region of interest containing a single individual osteocyte lacuna and the emanating canaliculi was performed. Osteocyte lacunae were oriented such that the major/long axis of the osteocyte was aligned with the transverse (xy) plane of the image stack. The image stack was then filtered using an edge preserving smoothing filter to reduce the presence of noise in the image while maintaining structural integrity. 3D volume renderings of individual lacunae and their associated canaliculi were then generated (Figure 3.2A). Using these 3D renderings, the number of canaliculi emanating from the surface of each lacuna was counted directly. In order to calculate lacunae dimensions, individual lacunae were segmented from their associated canaliculi by outlining the lacunar body in each frame using the AMIRA software package (Visage Imaging, Inc., Figure 3.2B). Individual osteocyte lacunar volume, surface area, and the lengths of the major and minor axes of each ellipsoid-shaped lacuna were measured in AMIRA. To correct for the distortion (stretching) of images in the axial (z) direction that is inherent in confocal microscopy, an axial correction factor of 0.803 was applied to the quantitative measures of rendered surfaces and volumes, which was experimentally measured for mineralized, basic fuchsin-stained cortical bone using an established protocol [134]. The number density of canaliculi emanating from an individual lacuna was calculated as the ratio of the total number of canaliculi and the
lacunar surface area, and expressed as number/mm². To calculate the number density of osteocyte lacuna in each bone region the number of lacunae within each 10x confocal image were counted and divided by the traced area of bone tissue in the same image and expressed as number/mm².

**Figure 3.2.** Representative 3D renderings built from z-stack confocal images of lacunae and associated canaliculi were used to quantify (A) canalicular number in VOLOCITY® and (B) lacunar volume, surface area and major and minor radii in AMIRA®.

3.3.3 TEM analysis of osteocyte LCS ultrastructure

3.3.3.1 Processing for TEM imaging

Immediately after harvesting left metaphyseal femora were cut into small segments, each ~1-mm in height, using a low-speed saw (Isomet, Buehler) and a diamond wafering blade. In order to preserve the cellular and pericellular structure of the LCS, femoral bone segments were immersion fixed in cold (4°C) fixative containing 4% paraformaldehyde, 2% glutaraldehyde, 0.7% ruthenium III hexamine
trichloride (RHT) in 0.05M cacodylate buffer (pH 7.5) for 24-hours, as described previously [4]. Bone samples were then decalcified in 10% EDTA containing 1% paraformaldehyde in 0.1M Tris-HCl buffer (pH 7.4) for 14-days. Decalcification was performed at 4°C; the decalcification solution was refreshed every two days. After decalcification the samples were repeatedly washed in 0.1M sodium cacodylate buffer, post fixed for 3 hours with 1% osmium tetroxide in 0.1 cacodylate buffer, and washed. The samples were dehydrated in an ascending series of acetones and infiltrated with ascending Quetol 651-NSA/n-butyl glycidyl ether mixtures before being embedded in 100% Quetol 651-NSA resin and polymerized at 60°C, as described previously [94]. After polymerization, resin blocks containing the bones/bone segments of interest were isolated using a low speed saw and faced for thin sectioning. Most sample blocks containing femoral metaphyseal bone segments were faced for sagittal sectioning, except for samples of groups 4 and 5, which were prepared using cross-sections. Ultrathin sections (60-70 nm/section) were then cut on an ultramicrotome (Ultracut E; Reichert-Jung) and collected onto 200-mesh formvar/carbon coated copper grids for imaging. Prior to imaging sections were post-stained with a saturated solution of uranyl acetate in methanol, followed by Reynolds’ lead citrate [94].

3.3.3.2 Transmission electron microscopy

Bone sections were imaged using a Libra 120 TEM (Carl Zeiss, Inc. NA) operated at 120kV, and images were collected with a Gatan Ultrascan 1000 CCD camera [94]. Low magnification images (100X, 0.1μm/pixel) were acquired to identify
trabecular and cortical bone and to prevent re-imaging of osteocytes on separate sections. High resolution images of osteocyte canaliculi/processes and lacuna/cell bodies were acquired at 6,300X magnification (1.70nm/pixel). For imaging lacunae, which were much larger than canaliculi, multiple adjacent, non-overlapping, TEM images were captured using the Gatan Digital Micrograph software and stitched together to generate high-resolution montage images. All images were exported as uncompressed TIFF files.

3.3.3.3 Quantitative analysis of osteocyte LCS ultrastructure

LCS ultrastructure was quantified by manually tracing lacunae, osteocyte cell bodies, canaliculi, and osteocyte cell process boundaries using Adobe Photoshop (Photoshop CS4; Adobe) and a digitizing pen display (Wacom Version 6.1.4). The lacunar wall and cell membrane as well as canalicular wall and cell process membrane were clearly delineated in the images (Figure 3.3A). These traces were batch exported as TIFF images to the BoneJ plugin in the ImageJ software and the dimensions of lacunae and the pericellular fluid spaces between the mineralized matrix walls and the cell membranes were obtained using a local thickness method, alternatively known as a “bubble method”, and the mean thickness of the spaces was reported (Figures 3.3B-3C). Similar analysis was applied to transversely oriented canaliculi, defined as having a shape factor (length/width) less than 2.5, in order to avoid measurement of oblate sections that were indicative of obliquely or longitudinally oriented canaliculi (Figure 4.4).
Figure 3.3. The ultrastructural measurements of osteocyte lacunae were obtained from TEM images by (A) tracing the lacunar wall and cell body in Photoshop® for (B) quantifying the shape of the lacuna (width/height), the cross-sectional areas of lacuna, cell body, and the pericellular annulus in Image J. (C) The mean thickness of the pericellular annular gap was measured using the “bubble” method implemented in the BoneJ plugin of Image J.

Figure 3.4. Measurements of osteocytic canaliculi were obtained from TEM images following the same procedure as the measurements of lacunae. (A) Traces of canalicular wall and cell process; (B) Quantification of areas of cell process, canalicular wall, and pericellular regions; (C) Measurements of pericellular thickness in canaliculi using the “bubble” method.
3.3.4 Statistical Analysis

Descriptive data are presented as mean ± standard deviation. Measurements from individual animals within the same groups were pooled because no differences were found among individual animals using ANOVA (not shown). The total numbers of lacunae and canaliculi that were assessed for either confocal imaging or TEM analysis were reported. Unpaired Student t-tests were performed to determine the differences between cortical and trabecular LCS parameters within each group. To determine the effects of age, perlecan deficiency, and diabetes on the LCS measurement outputs, unpaired two-sided Student t-tests were performed between groups 1 and 2 (15 weeks vs. 32 weeks), between groups 3 and 2 (perlecan deficient bones vs. wild-type), and between groups 4 and 5 (diabetic bones vs. wild-type), respectively. All tests were performed in GraphPad (San Diego, CA) with a significant level set at p<0.05.

3.4 Results

3.4.1 Confocal-Based Measurements

The lacunar volume, surface area, major axis diameter, and density as well as canalicular number and number density were measured and compared.

- *Compartmental comparisons (Cancellous vs. Cortical):* For the three groups of animals (C57BL/6J-15wks, C57BL/6J-32wks, and Hypo-32 wks), there was a general trend that most measurements of lacunae and canalicular numbers were
smaller than those in cortical bone (Table 3.1). However, only a few measures such as the major axis diameter of lacuna, lacunar density, and canalicul number reached statistical significance (p<0.05, Table 3.1).

- **Effects of age (32 wks vs. 15 wks):** Older adult mice (32 wks) had significantly reduced (8.1%, p=0.03) lacunar density in cortical bone, whereas many other LCS measurements were not significantly different. It is noted that aging tended to decrease lacunar volume, lacunar surface area, lacunar density, canalicul number, and canalicul density in cancellous bone; yet, these changes did not reach significance (Table 3.2).

- **Effects of perlecan deficiency (Hypo vs. WT):** All the LCS measurements showed varied levels of decrease in the Hypo animals relative to the WT controls (Table 3.3). Canalicul number and canalicul number density were significantly reduced by 15.7% and 9.1% in the Hypo cortical bone compartment and lacunar volume and canalicul number showed 23.6% and 11.9% reduction in Hypo cancellous bone compartment (Table 3.3).

- **Effects of diabetes (Akita vs. WT):** Although the LCS measures tended to be lower in diabetic Akita mice, only the lacunar density showed a significant decrease (10.1%) relative to the non-diabetic WT controls (Table 3.4).
Summary of the results: A few compartment-, age-, and disease-associated changes in confocal-based LCS measures such as lacunar size, spacing, and their associated canaliculi were observed (Tables 3.1-3.4). Notably, perlecan deficiency and diabetes affected the LCS to different degrees. While perlecan deficiency resulted in significant decreases in more parameters (such as lacunar volume and the canalicular number density), diabetes impacted only the lacunar density. However, among all the examined parameters, the canalicular number density was highly consistent among all groups with mean values in the range of 0.190-0.210 per µm² and coefficients of variation in the range of 11%-24%. In comparison, the variations seen in the size of lacunae were much higher (coefficients of variation in volume: 30%-49% and in surface area: 21%-40%).

3.4.2 TEM-Based Measurement

The pericellular area and thickness in lacunae, the cross-sectional areas of canalicular wall and cell process, as well as the pericellular area and thickness in canaliculi were measured and compared using TEM-based approaches. Please note that the numbers of lacunae and canaliculi examined under TEM may vary among groups. Although the numbers of animals, bone segments, and sections were the same for all the groups, we did not control the total bone areas and imaged all the available lacunae and canaliculi with good imaging quality.
• **Compartmental comparisons (Cancellous vs. Cortical):** In contrast with the confocal-based measures, significant decreases were observed in the mean values of most measurements (with the exception of cell process cross-sectional area) in the cancellous compartment compared with cortical bone for the three groups of animals (C57BL/6J-15wks, C57BL/6J-32wks, and Hypo-32wks) (Table 3.5). Cell process cross-sectional area was consistently greater in cancellous bone, and only canalicular wall area remained unchanged in 15wk WT and 32wk Hypo mice (p>0.05, Table 3.5).

• **Effects of age (32 wks vs. 15 wks):** Again, in contrast with the confocal-based measures, all parameters (with the exception of lacunar pericellular thickness) showed significant changes in cortical bone from 32-wk old mice. In particular, the cortical canalicular measures were significantly larger at 32 wks, while these measures remained unchanged in the cancellous bone compartment (Table 3.6).

• **Effects of perlecan deficiency (Hypo vs. WT):** Perlecan deficiency reduced all the TEM-based measures with high statistical significance, except for the cell process area in cancellous bone (Table 3.7). The impact was broad and independent of the bone compartment.

• **Effects of diabetes (Akita vs. WT):** Diabetes did not significantly alter the pericellular space in lacunae and only marginally within the canaliculi, while the canalicular wall area and cell process area were significantly reduced in diabetic
cortical bone (Table 3.8). However, the alterations seen in diabetes were less dramatic than those seen in perlecan deficiency (Table 3.7).

- Summary of the results: In comparison with the confocal-based measures, more extensive changes in the TEM-based measures were observed with different compartments, ages, and disease conditions. Among the factors examined herein, tissue compartment and perlecan deficiency showed greater effects on variations of the pericellular space than age and diabetes (Tables 3.5-3.8).
### Table 3.1. Confocal-Based LCS Measurements—Compartmental Comparisons

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>C57BL/6J Adult Mice (15wks)</th>
<th>C57BL/6J Aged Mice (32wks)</th>
<th>C1532Yneo Aged Perlecan Hypomorph Mice (32wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Compartment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cortical</td>
<td>Cancellous</td>
<td>Difference Between Compartments</td>
</tr>
<tr>
<td># of Lacuna</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Lacunar Volume (μm³)</td>
<td>449.7 ± 173.7</td>
<td>400.9 ± 133.1</td>
<td>-10.8% p=0.23</td>
</tr>
<tr>
<td></td>
<td>447.2 ± 209.4</td>
<td>399.7 ± 141.1</td>
<td>-10.7% p=0.31</td>
</tr>
<tr>
<td></td>
<td>368.6 ± 168.7</td>
<td>305.4 ± 150.4</td>
<td>-17.1% p=0.13</td>
</tr>
<tr>
<td>Lacunar Surface Area (μm²)</td>
<td>410.0 ± 108.0</td>
<td>384.7 ± 95.3</td>
<td>-6.3% p=0.34</td>
</tr>
<tr>
<td></td>
<td>390.8 ± 120.4</td>
<td>350.4 ± 88.0</td>
<td>-10.3% p=0.14</td>
</tr>
<tr>
<td></td>
<td>347.7 ± 7.4</td>
<td>316.5 ± 126.3</td>
<td>-8.9% p=0.29</td>
</tr>
<tr>
<td>Lac. Major Axis (μm)</td>
<td>15.8 ± 2.9</td>
<td>12.8 ± 2.5</td>
<td>-18.9% p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>16.5 ± 3.7</td>
<td>13.6 ± 3.6</td>
<td>-17.6% p=0.003</td>
</tr>
<tr>
<td></td>
<td>15.4 ± 3.7</td>
<td>12.3 ± 2.8</td>
<td>-20.1% p=0.0005</td>
</tr>
<tr>
<td>Lacunar Density (/μm²)</td>
<td>1200.7 ± 149.0</td>
<td>1078.2 ± 213.9</td>
<td>-10.2% p=0.01</td>
</tr>
<tr>
<td></td>
<td>1103.9 ± 187.1</td>
<td>1049.9 ± 239.0</td>
<td>-4.9% p=0.33</td>
</tr>
<tr>
<td></td>
<td>1075.5 ± 185.3</td>
<td>1010.5 ± 253.7</td>
<td>-6.0% p=0.26</td>
</tr>
<tr>
<td>Canalicular Number</td>
<td>73.8 ± 20.1</td>
<td>68.3 ± 13.0</td>
<td>-7.5% p=0.21</td>
</tr>
<tr>
<td></td>
<td>77.5 ± 12.0</td>
<td>67.3 ± 8.8</td>
<td>-13.2% p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>65.3 ± 15.2</td>
<td>59.3 ± 17.2</td>
<td>-9.2% p=0.16</td>
</tr>
<tr>
<td>Canalicular Number Density (/μm²)</td>
<td>0.209 ± 0.048</td>
<td>0.210 ± 0.050</td>
<td>0.5% p=0.94</td>
</tr>
<tr>
<td></td>
<td>0.209 ± 0.043</td>
<td>0.200 ± 0.039</td>
<td>-4.3% p=0.40</td>
</tr>
<tr>
<td></td>
<td>0.190 ± 0.020</td>
<td>0.190 ± 0.020</td>
<td>0 p&gt;0.99</td>
</tr>
</tbody>
</table>

Note: Difference between compartments was calculated as (Cancellous-Cortical)/Cortical
### Table 3.2. Confocal-Based LCS Measurements—Effects of Age

<table>
<thead>
<tr>
<th>Difference (%)</th>
<th>Cortical</th>
<th>Cancellous</th>
</tr>
</thead>
<tbody>
<tr>
<td>(32 wks-15 wks)/15wks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Compartment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacunar Volume ($\mu m^3$)</td>
<td>-0.6% p=0.96</td>
<td>-0.3% p=0.97</td>
</tr>
<tr>
<td>Lacunar Surface Area ($\mu m^2$)</td>
<td>-4.9% p=0.52</td>
<td>-8.9% p=0.15</td>
</tr>
<tr>
<td>Lac. Major Axis ($\mu m$)</td>
<td>4.2% p=0.42</td>
<td>6.3% p=0.32</td>
</tr>
<tr>
<td>Lacunar Density (/$\mu m^2$)</td>
<td>-8.1% p=0.03</td>
<td>-2.7% p=0.63</td>
</tr>
<tr>
<td>Canalicul Number</td>
<td>5.0% p=0.39</td>
<td>-1.5% p=0.73</td>
</tr>
<tr>
<td>Canalicul Number Density (/$\mu m^2$)</td>
<td>0 p=0.99</td>
<td>-4.8% p=0.39</td>
</tr>
</tbody>
</table>

Note: Comparisons were made between 15 wks and 32 wks WT male mice.

### Table 3.3. Confocal-Based LCS Measurements—Effects of Perlecan Deficiency

<table>
<thead>
<tr>
<th>Difference (%)</th>
<th>Cortical</th>
<th>Cancellous</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Hypo-WT)/WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Compartment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacunar Volume ($\mu m^3$)</td>
<td>-17.6% p=0.11</td>
<td>-23.6% p=0.02</td>
</tr>
<tr>
<td>Lacunar Surface Area ($\mu m^2$)</td>
<td>-11.0% p=0.13</td>
<td>-9.7% p=0.23</td>
</tr>
<tr>
<td>Lac. Major Axis ($\mu m$)</td>
<td>-6.7% p=0.25</td>
<td>-9.6% p=0.12</td>
</tr>
<tr>
<td>Lacunar Density (/$\mu m^2$)</td>
<td>-2.5% p=0.56</td>
<td>-3.8% p=0.54</td>
</tr>
<tr>
<td>Canalicul Number</td>
<td>-15.7% p=0.001</td>
<td>-11.9% p=0.03</td>
</tr>
<tr>
<td>Canalicul Number Density (/$\mu m^2$)</td>
<td>-9.1% p=0.03</td>
<td>-5.0% p=0.22</td>
</tr>
</tbody>
</table>

Note: Comparisons were made between 32 wks-old WT and perlecan deficient (Hypo) male mice.
### Table 3.4. Confocal-Based LCS Measurements—Effects of Diabetes

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>WT</th>
<th>Akita</th>
<th>Difference (%) (Akita-WT)/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Compartment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of Lacuna</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Lacunar Volume (μm³)</td>
<td>462.4 ± 177.7</td>
<td>403.4 ± 120.0</td>
<td>-12.7% p=0.14</td>
</tr>
<tr>
<td>Lacunar Surface Area (μm²)</td>
<td>449.4 ± 129.5</td>
<td>420.3 ± 87.0</td>
<td>-6.5% p=0.31</td>
</tr>
<tr>
<td>Lac. Major Axis (μm)</td>
<td>18.3 ± 3.8</td>
<td>18.2 ± 2.8</td>
<td>-0.55% p=0.91</td>
</tr>
<tr>
<td>Lacunar Density (/μm²)</td>
<td>1073.3 ± 167.8</td>
<td>965.1 ± 132.6</td>
<td>-10.1% p=0.008</td>
</tr>
<tr>
<td>Canalicular Number</td>
<td>93.4 ± 23.1</td>
<td>85.53 ± 16.3</td>
<td>-8.5% p=0.13</td>
</tr>
<tr>
<td>Canalicular Number Density (/μm²)</td>
<td>0.210 ± 0.013</td>
<td>0.205 ± 0.022</td>
<td>-2.4% p=0.29</td>
</tr>
</tbody>
</table>

Note: Measurements were made on 20-wks-old C57BL/6J (WT) and C57BL/6-Ins2Akita/J (Akita) male mice (three animals per group).
### Table 3.5. TEM-Based LCS Measurements—Compartmental Comparisons

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>C57BL/6J Adult Wildtype (15wks)</th>
<th>C57BL/6J Aged Wildtype (32wks)</th>
<th>C1532Yneo Aged Perlecan Hypomorph (32wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Compartment</td>
<td>Cortical</td>
<td>Cancellous</td>
<td>Difference Between Compartments</td>
</tr>
<tr>
<td></td>
<td># of Lacuna</td>
<td></td>
<td># of Lacuna</td>
</tr>
<tr>
<td></td>
<td>Cortical</td>
<td>Cancellous</td>
<td>Difference Between Compartments</td>
</tr>
<tr>
<td></td>
<td>Lac. Pericellular Area (µm²)</td>
<td></td>
<td>Lac. Pericellular Area (µm²)</td>
</tr>
<tr>
<td></td>
<td>9.28 ± 1.17</td>
<td>7.15 ± 1.87</td>
<td>7.72 ± 1.88</td>
</tr>
<tr>
<td></td>
<td>7.15 ± 1.87</td>
<td>5.27 ± 1.77</td>
<td>6.85 ± 1.85</td>
</tr>
<tr>
<td></td>
<td>-22.9% p&lt;0.005</td>
<td>-21.7% p&lt;0.005</td>
<td>2.93 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>6.85 ± 1.85</td>
<td>2.93 ± 0.87</td>
<td>-57.2% p&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>0.49 ± 0.15</td>
<td>0.25 ± 0.04</td>
<td>0.47 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>0.25 ± 0.04</td>
<td>0.33 ± 0.12</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>-48.9% p&lt;0.005</td>
<td>-31.7% p&lt;0.005</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
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<td>-29.8% p&lt;0.005</td>
<td>-29.8% p&lt;0.005</td>
<td>-51.2% p&lt;0.005</td>
</tr>
<tr>
<td># of Canaliculi</td>
<td>721</td>
<td>718</td>
<td>506</td>
</tr>
<tr>
<td></td>
<td>718</td>
<td>334</td>
<td>475</td>
</tr>
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<td>721</td>
<td>506</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>718</td>
<td>334</td>
<td>475</td>
</tr>
<tr>
<td>Canalicular Area</td>
<td>0.071 ± 0.029</td>
<td>0.072 ± 0.033</td>
<td>0.081 ± 0.040</td>
</tr>
<tr>
<td>(µm²)</td>
<td>0.072 ± 0.033</td>
<td>0.072 ± 0.040</td>
<td>0.081 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>1.4% p=0.54</td>
<td>0.081 ± 0.040</td>
<td>-11.1% p&lt;0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.081 ± 0.032</td>
<td>0.066 ± 0.031</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.081 ± 0.040</td>
<td>0.062 ± 0.035</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4% p=0.54</td>
<td>-6.1% p=0.10</td>
</tr>
<tr>
<td>Cell Process Area</td>
<td>0.015 ± 0.008</td>
<td>0.021 ± 0.012</td>
<td>0.017 ± 0.013</td>
</tr>
<tr>
<td>(µm²)</td>
<td>0.021 ± 0.012</td>
<td>0.021 ± 0.013</td>
<td>0.021 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>40% p&lt;0.005</td>
<td>0.021 ± 0.013</td>
<td>23.5% p&lt;0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.021 ± 0.013</td>
<td>0.015 ± 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40% p&lt;0.005</td>
<td>0.021 ± 0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20% p&lt;0.005</td>
<td>0.021 ± 0.013</td>
</tr>
<tr>
<td>Can. Pericellular Area (µm²)</td>
<td>0.056 ± 0.025</td>
<td>0.052 ± 0.025</td>
<td>0.065 ± 0.034</td>
</tr>
<tr>
<td></td>
<td>0.052 ± 0.025</td>
<td>0.052 ± 0.024</td>
<td>0.053 ± 0.026</td>
</tr>
<tr>
<td></td>
<td>-7.1% p&lt;0.005</td>
<td>-20% p&lt;0.005</td>
<td>-22.6% p&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>-7.1% p&lt;0.005</td>
<td>-20% p&lt;0.005</td>
<td>-22.6% p&lt;0.005</td>
</tr>
<tr>
<td>Can. Pericellular Thickness (µm)</td>
<td>0.095 ± 0.028</td>
<td>0.084 ± 0.027</td>
<td>0.101 ± 0.032</td>
</tr>
<tr>
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<td>0.084 ± 0.027</td>
<td>0.084 ± 0.028</td>
<td>0.089 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>-11.6% p&lt;0.005</td>
<td>-16.8% p&lt;0.005</td>
<td>-25.8% p&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>-11.6% p&lt;0.005</td>
<td>-16.8% p&lt;0.005</td>
<td>-25.8% p&lt;0.005</td>
</tr>
</tbody>
</table>

Note: Difference between compartments was calculated as (Cancellous-Cortical)/Cortical
### Table 3.6. TEM-Based LCS Measurements—Effects of Age

<table>
<thead>
<tr>
<th>Bone Compartment</th>
<th>Cortical</th>
<th>Cancellous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac. Pericellular Area (μm²)</td>
<td>-16.8% p&lt;0.005</td>
<td>-26.3% p&lt;0.005</td>
</tr>
<tr>
<td>Lac. Pericellular Thickness (μm)</td>
<td>-4.1% p=0.48</td>
<td>32% p&lt;0.005</td>
</tr>
<tr>
<td>Canalicul Area (μm²)</td>
<td>14.1% p&lt;0.005</td>
<td>0 p&gt;0.99</td>
</tr>
<tr>
<td>Cell Process Area (μm²)</td>
<td>13.3% p&lt;0.005</td>
<td>0 p&gt;0.99</td>
</tr>
<tr>
<td>Can. Pericellular Area (μm²)</td>
<td>16.1% p&lt;0.005</td>
<td>0 p&gt;0.99</td>
</tr>
<tr>
<td>Can. Pericellular Thickness (μm)</td>
<td>6.3% p&lt;0.005</td>
<td>0 p&gt;0.99</td>
</tr>
</tbody>
</table>

Note: Comparisons were made between 15 wks and 32 wks WT male mice.

### Table 3.7. TEM-Based LCS Measurements—Effects of Perlecan Deficiency

<table>
<thead>
<tr>
<th>Bone Compartment</th>
<th>Cortical</th>
<th>Cancellous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac. Pericellular Area (μm²)</td>
<td>-11.3% p=0.04</td>
<td>-44.4% p&lt;0.005</td>
</tr>
<tr>
<td>Lac. Pericellular Thickness (μm)</td>
<td>-12.8% p&lt;0.005</td>
<td>-39.4% p&lt;0.005</td>
</tr>
<tr>
<td>Canalicul Area (μm²)</td>
<td>-18.5% p&lt;0.005</td>
<td>-13.9% p&lt;0.005</td>
</tr>
<tr>
<td>Cell Process Area (μm²)</td>
<td>-11.8% p=0.006</td>
<td>4.7% p=0.39</td>
</tr>
<tr>
<td>Can. Pericellular Area (μm²)</td>
<td>-18.5% p&lt;0.005</td>
<td>-21.2% p&lt;0.005</td>
</tr>
<tr>
<td>Can. Pericellular Thickness (μm)</td>
<td>-11.9% p&lt;0.005</td>
<td>-21.4% p&lt;0.005</td>
</tr>
</tbody>
</table>

Note: Comparisons were made between 32 wks-old WT and perlecan deficient (Hypo) male mice.
Table 3.8. TEM-Based LCS Measurements—Effects of Diabetes

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>C57BL/6J Adult Wildtype (20wks)</th>
<th>C57BL/6-Ins2Akita/J Adult Akita (20wks)</th>
<th>Difference (%) (Akita-WT)/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Compartment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of Lacuna</td>
<td>68</td>
<td>69</td>
<td></td>
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<tr>
<td>Lac. Pericellular Area (μm²)</td>
<td>2.97 ± 1.74</td>
<td>3.78 ± 3.03</td>
<td>27.3%  p=0.06</td>
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<tr>
<td>Lac. Pericellular Thickness (μm)</td>
<td>0.23 ± 0.14</td>
<td>0.28 ± 0.21</td>
<td>21.7%  p=0.10</td>
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<tr>
<td># of Canaliculi</td>
<td>206</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>Canalicular Area (μm²)</td>
<td>0.059 ± 0.023</td>
<td>0.054 ± 0.019</td>
<td>-8.5%  p=0.02</td>
</tr>
<tr>
<td>Cell Process Area (μm²)</td>
<td>0.015 ± 0.007</td>
<td>0.013 ± 0.006</td>
<td>-13.3%  p&lt;0.005</td>
</tr>
<tr>
<td>Can. Pericellular Area (μm²)</td>
<td>0.045 ± 0.018</td>
<td>0.042 ± 0.015</td>
<td>-6.7%  p=0.06</td>
</tr>
<tr>
<td>Can. Pericellular Thickness (μm)</td>
<td>0.079 ± 0.020</td>
<td>0.078 ± 0.018</td>
<td>-1.3%  p=0.59</td>
</tr>
</tbody>
</table>

Note: Measurements were made on 20-wks-old C57BL/6J (WT) and C57BL/6-Ins2Akita/J (Akita) male mice (three animals per group).
3.5 Discussion

Measurements of the morphology of lacunae and canaliculi in five experimental animal groups presented comprehensive snap-shots of the dimensions of the osteocyte network, from which the invariant and variant features of the network were derived by comparisons.

The most revealing finding from this study is that the canalicular number density emanated from individual lacunae was highly consistent, regardless of the size and location of the lacunae, animal age, and the status of diabetes or perlecan deficiency (Tables 3.1-3.4). This suggests that, as an osteoblast differentiates from a surface lining cell to an osteocyte embedded in bone matrix, it is likely programmed to incorporate into the pre-existing LCS network via a specified number of canaliculi, and the number is likely determined mainly by the surface area of the lacuna containing the cell body. The number of canalicular connections appears to be quite conserved (~1 canaliculus per 5 μm² lacunar surface) within the age range examined here. In contrast, the synapse connections found in neural networks are susceptible to age-related loss [164-166]. Furthermore, we did not find any difference in canalicular number density between cancellous and cortical compartments, although the average age of bone tissue (and hence the age of osteocytes) was likely younger within the cancellous compartment due to higher bone turnover [11]. We did detect a change in lacunar shape (as shown in the longer major axis and thus more elongated osteocyte shape in cortical bone) among compartments, which was consistent with previous
studies where the shape of osteocyte was found to depend on local stress distribution [167]. As for the effects of diabetes on the LCS, we did not expect to see significant changes in the morphology of cell processes because the integrity of an osteocyte process is mainly dependent on actin and microtubules [168, 169], which may not be altered in the presence of diabetes. However, the number density of lacunae in diabetic cortical bone was 10% lower than that in the normal age-matched mice, possibly due to impaired osteoblast differentiation commonly associated with diabetes [170, 171]. In the other disease condition examined in this study, a reduction in osteocyte canalicular density was found in bones from mice deficient in perlecan, an extracellular matrix heparan sulfate extracellular matrix proteoglycan. While it remains unclear how perlecan regulates canalicular density, these results are consistent with our previous findings [94]. The consistent canalicular number density demonstrates that the topology of the overall canalicular connections is an invariant feature of the osteocyte network. This agrees with the notion that one of the major purposes of the extensive LCS network is to ensure the survival of osteocytes encased in mineralized matrix by facilitating the transport of nutrients and cell-cell signaling molecules through the network.

Although the architecture of the canalici branches, i.e., topology, of the LCS network appears to be very robust, the dimension of the pericellular space surrounding the osteocyte cell body and cell process, a critical parameter determining the overall permeability of fluid/solute transport in the LCS and analogous to the bandwidth of an individual network link, is quite sensitive to changes in age and the composition
of the pericellular matrix (Tables 3.5-3.8). With increasing age, the gap of the pericellular space increases in cortical canaliculi, mainly due to an expansion of the canicular wall that exceeds the enlargement of the cell process (Table 3.6). In young wild-type animals (15 wks), larger pericellular gaps were found in the cortical compartment than the cancellous compartment. However, the compartmental difference disappeared in older animals (Table 3.5). Among all factors considered, perlecan deficiency is associated with the most profound changes in the pericellular space observed in this study—the pericellular fluid annulus around bone lacunae and canaliculi of the perlecan deficient bone decreased both compartments (Table 3.7). This result agrees with our previous findings that perlecan is an essential component of the pericellular matrix, which maintains adequate fluid/solute transport pathway by possibly inhibiting mineralization of the pericellular space [94]. In contrast, diabetes shows no effect on the pericellular space, while it does reduce the areas of the canicular wall and cell process simultaneously (Table 3.8).

These data suggest that the osteocyte LCS is a network with relatively stable nodes (lacunae) connected by canaliculi with a consistent number density (Figure 3.5). While metabolic factors such as diabetes can influence the spacing of the lacunae, the overall structure of the network is robustly determined within a very narrow range of the number density of canaliculi branching out of individual lacunae (~ 1 canaliculi per 5μm² lacunar surface in adult mice). Such conservation of canalicular number is likely achieved through genetic pre-programing to meet the metabolic requirements for osteocyte survival so that larger lacunae are wired with
more canaliculi. Meanwhile, the LCS network is very dynamic, demonstrating significant variations and modifications on the pericellular fluid annulus around each canalicular connection or lacunar node (Figure 3.5). It becomes increasingly clear that osteocytes are capable of releasing osteolytic agents such as MMPs to modify their surroundings [14], especially under lactation, hibernation or pregnancy due to the need of releasing mineral from the skeleton [137]. Furthermore, our results show that proper composition of the secreted osteocyte pericellular matrix plays an important role in maintaining the pericellular fluid annulus, as shown by the profound changes in the perlecan deficient LCS. Since aging induces catabolic and anabolic cellular changes, the age-related differences in the LCS morphology may be attributed to increased osteolytic and decreased anabolic activities in aged osteocytes [154, 172, 173].
Figure 3.5. Osteoblasts are differentiated into osteocytes, which form an orderly network through the connecting canaliculi. The number of canaliculi is determined mainly by the surface area of lacuna \((N=S [\mu m^2]/5)\) regardless of bone compartment, age, and disease conditions. In cortical bone, aging is associated with larger canalicular annulus area and lower fiber density, while perlecan (Pln) deficiency reduces both annulus area and fiber density compared with normal sample (Tables 4.6 & 4.7).

There were several limitations in the study. Artifacts such as tissue shrinking during TEM sample processing are always a concern in such quantitative studies. To minimize the potential artifacts, we sectioned the mouse femora into smaller segments for faster and better penetration of fixatives. The addition of RHT in the fixatives helped stabilize the pericellular matrix as well [162]. We also excluded sections when the pericellular spaces around lacunae were devoid of pericellular matrix, indicating poor fixation and possible tissue shrinkage. Since the TEM measurements were performed on 2D sections, the orientation of the samples could
impact the data, especially the overall shape and dimension of the lacunae and canaliculi. To minimize this effect, we sampled a large number canaliculi (211-721) and excluded those with shape factor larger than 2.5. However, we should treat the data of lacunar pericellular area and thickness with caution, since they might be more susceptible to orientation artifacts. It is also noted that the confocal measurements using basic fuchsin-stained samples reflected more on the structures of LCS pore system rather than those of osteocytes, because the dye could stain both the cellular components but also the pericellular space encased in the mineralized bone matrix. However, due to the submicron thickness of the pericellular space in lacunae and canaliculi, the measurements of lacunae should be very close to those of osteocytes. The other limitation was that the data were derived from a small sample size of animals (three mice per group). However, the numbers of osteocytes studied were much higher (N=30) and we did not find any difference among individual mice within the same group. Additional limitation was the small span of age (15 to 32 weeks) investigated herein. It would be better to know how LCS evolves during the entire developmental and aging process. Due to the technical limitation, all data have to be derived from individual time points and from different osteocyte populations. It would be ideal if the LCS network can be tracked longitudinally and quantified in the same cells. Advances in bioimaging in combination of transgenic mouse models where osteocytes are labeled with green fluorescent proteins could potentially make this feasible in the future. In summary, key features such as the canalicular number density and pericellular fluid annular gap of the osteocyte lacunar-canalicular network were
analyzed in trabecular and cortical compartments, in young and old bones, as well as from two diseased conditions (perlecan deficiency and diabetes). The LCS network showed both topological stability in terms of conserved numbers of connections, and considerable variability of “bandwidth” (communication capacity) of individual connections in terms of the pericellular annular fluid gap around lacunae and canaliculi. Our examination of diabetic cortex and perlecan deficient bones revealed that the former condition mainly impact the spacing of the lacunae (the nodes in the LCS network), while the latter had profound influence in the pericellular fluid annulus (the connection’s bandwidth). Age, in the range of our study (15-32 weeks) affected mainly the pericellular fluid annulus, possibly through altered osteolytic and anabolic activities in osteocytes. Quantifying the morphology and structure of the osteocyte lacunar-canalicular network will help enhance our understanding of osteocyte physiology and alterations associated with aging and skeletal disease conditions. The osteocyte LCS networking characteristics obtained in this study shed new insights onto not only the intercellular communication among bone cells but also the role of bone fluid flow in bone’s response to mechanical forces, a potent anabolic factor regulating bone growth and adaptation.
Chapter 4

REDUCED HEPARAN SULFATE PROTEOGLYCAN (HSPG) IN CALVARIA----A POTENTIAL MECHANISM FOR CALVARIA’S RESISTANCE TO DISUSE?

4.1 Abstract

In order to make higher efficiency of mechanical performance, bone is known to be able to adapt its mass and structure to mechanical demands. Removal of such loading stimulus as experienced under microgravity and bed-rest conditions is associated with the loss of bone and increased susceptibility to fracture. Interestingly, bone adaptation to disuse is site-specific with significant bone loss in long bones, while bone mass maintains in the skull bones. Recently, we identified perlecan, a large secreted HSPG, as an essential structural component of the osteocytic pericellular matrix in the lacunar-canalicular system. In our in vivo tibial loading study, we found that perlecan deficient mice did not respond to anabolic loading stimulation as compared with normal wild-type controls. Meanwhile these mice demonstrated reduced bone loss under hind-limb suspension. These intriguing results let us hypothesize that perlecan molecule acts as a mechanosensor for osteocytes to detect the mechanical environment. To test this hypothesis, Alcian Blue staining and FRAP diffusion experiments were performed on both murine calvaria and tibia. We found calvaria showed significantly less Alcian Blue staining when compared to the femur in
both B6 and perlecan deficient mice, which indicates murine long bones express higher content of HSPG than calvaria. These results support our hypothesis and are helpful to uncover the mechanisms regulating calvaria’s resistance to bone loss.

4.2 Introduction

Bone tissue is known to be able to alter its mass and structure in response to mechanical demands during life and this adaptation could make more efficient mechanical performance. Bone’s response depends on loading magnitude, loading pattern, genetics background, age, sex and bone sites [174, 175]. Removal of such loading stimulus as experienced under microgravity and bed-rest conditions is associated with the loss of bone and increased susceptibility to fracture [176-179]. Loss of bone mass is not uniform throughout the skeleton, but varies at different bone sites [178, 179]. From a previous study of bone mineral change after 17 weeks of bed rest, bone loss was significant in the calcaneus, proximal femur and lumbar spine, while there was significant gain in bone mass within the head [178]. This site-specific bone loss to disuse raises an interesting question of how the bone tissue senses mechanical loads. Mechanical adaptation of bone is a cellular process and the cellular mechanisms remained poorly understood.

Heparan sulfate proteoglycans (HSPG), a class of negatively charged proteins found in the extracellular matrix, play important roles in morphogenesis and growth-factor signaling during bone development, tissue repair, and remodeling/adaptation. Recently, we identified perlecan, a large secreted HSPG, as an essential structural
component of the osteocytic pericellular matrix in the lacunar-canalicular system [94]. Decreased expression of perlecan, as occurs in Schwartz-Jampel Syndrome, was found to alter mechanical loading-induced solute transport, fluid flow, and mechanosensing of osteocytes as detailed in Chapter 2. In our in vivo tibial loading study, we found that perlecan deficient C1532Yneo mice did not respond to anabolic loading stimulation as compared with normal wild-type controls. Meanwhile these mice demonstrated reduced bone loss under hind-limb suspension [180]. These intriguing results suggested that the large modular perlecan molecule may serve as a mechanosensor for osteocytes to detect the mechanical environment. Thus the local expression/density of the osteocytic pericellular perlecan mechanosensors may determine an individual bone’s sensitivity to mechanical loading as well as, inversely its lack of response to removal of such loading stimulus as experienced under microgravity and bed-rest conditions. We hypothesize that long bones have higher HSPG content (higher mechanosensitivity), while calvariae bones have lower content of HSPG (lack of response to mechanical factors), which contributes to its surprisingly high resistance to disuse bone loss.

To test our hypothesis, two lines of experiments were conducted in the current study: Alcian Blue staining and FRAP diffusion experiments. Alcian Blue binds to the highly charged glycosaminoglycans of the HSPGs and results in blue staining of the bone matrix and cells. The diffusivity of tracers could be an indicator of the expression/density of the osteocytic pericellular perlecan mechanosensors. The objective of the present study was to quantitatively examine and compare HSPG
expression and distribution in murine long bone and calvaria. The long-term goal of our study is to elucidate the role of osteocyte PCM in bone mechanical sensing and signal transduction in vivo and in situ.

4.3 Methods

4.3.1 Alcian Blue staining

4.3.1.1 Animals

Murine calvariae and femurs were harvested from adult (age 7–13 months) C57BL/6J mice (B6, n = 2) and perlecan deficient mice (PLN, Hypo, n = 4).

4.3.1.2 Sample preparation

The harvested samples were fixed in buffered formalin for 2 days, decalcified in a mixture of formic acid, HCl and EDTA for 1 week, embedded in OCT media in plastic molds, and frozen at -50°C. The samples were then cut into 10µm thick sections using the cryostat Cryojane system between -35°C to -25°C. Sagittal cross-sections of calvariae and transverse cross-sections of femoral mid-shafts (Figure 4.1) were obtained. Thirty slides containing three cross-sections each were obtained per bone sample. Half of the slides were stored at room temperature for Alcian Blue staining (detailed below) while the other half were kept frozen for future studies.
4.3.1.3 Staining

The slides were stained for 30 minutes in freshly made 0.05% Alcian Blue GX in 0.025M acetate buffer solution containing 0.5M MgCl2 at a final pH of ~5.6, modified from protocols published previously [181, 182]. Alcian Blue binds to the highly charged glycosaminoglycans of the HSPGs and results in blue staining of the bone matrix and cells. The slides were then rinsed in 70%, 90%, 100%, and 100% ethanol, Safe Clear/xylenes, and mounted with EUKITT.

![Image](image.png)

**Figure 4.1.** Representative images of Alcian Blue staining in B6 calvaria (A) and PLN Hypo femur (C). Blowup images of the boxed areas in (A) and (C) are shown in (B) and (D), respectively. Scale bars= 500µm in (A, C); Scale bars= 50µm in (B, D).

4.3.1.4 Data collection and analysis

Images were collected with a CCD color camera at 20x magnification. A randomly chosen cross-section was used to obtain 5-10 images per sample. Camera
and microscope settings were held constant throughout the imaging. The colored images were converted to an inverted gray scale, where darker blue staining corresponded to pixels with lower intensity. Image J was then used to collect the average intensity of the bone matrix and background of the slide. Approximately 30-50 boxes with a size of 240 x 240 pixels were used to collect the data for the matrix per sample and around 20 boxes of variable size were used to collect data from the background. The semi-quantitative data of HSPG value for each bone sample was reported by subtracting the average intensity of the bone matrix from that of background.

4.3.2 FRAP diffusion study

4.3.2.1 Specimen preparation

Adult (age 4-month) C57BL/6J mice (n = 5) were used in this study. The mice were injected via the tail vein with 0.5 mL of phosphate buffered saline (PBS) containing 5mg sodium fluorescein (Sigma-Aldrich, St. Louis, MO, USA), allowed to circulate for 0.5h prior to sacrifice. After the pre-defined circulation time, the animals were sacrificed with carbon dioxide. The tibiae and calvariae were then harvested, cleansed of soft tissue, and tested within 0.5-3h post-mortem. All the animal procedures were approved by the Institutional Animal Use and Care Committee.
4.3.2.2 FRAP diffusion experiments

Tibiae were fixed and tested in a custom-made round chamber filled with PBS solution. Calvariae were fixed using grease and tested in a 35-mm petri-dish filled with PBS solution. Among the five mice, three mice were tested with tibiae first while two mice were tested with calvariae first to avoid the potential effect of the experimental sequence. The FRAP experiments were performed as detailed in Chapter 2. A total of ~10 FRAP tests were performed in each bone site.

4.3.2.3 Histological measurements of lacunar-canalicular microanatomy of calvaria

Certain anatomical measures were obtained from histological specimens of murine tibia in previous study [107], and these measurements need to be quantified in calvaria as well. Calvaria samples from three age-matched C57BL/6J mice were processed for basic fuchsin staining, plastic embedding, sectioning and confocal imaging as detailed in Chapter 3. The shape index major axis $a$, minor axis $b$ and minor axis $c$ were analyzed and the ratio was found to be 3:1.5:1. Thus the three axis radii were calculated from the lacunar perimeter $P$ and area $A$ of the two-dimensional FRAP images, which were used to determine the 3D volume ($\frac{4}{3}\pi abc$) and the surface area ($\frac{4}{3}\pi (ab+bc+ca)$). From the three-dimensional reconstruction images of lacuna and canaliculi, the canalicular number density was calculated to be $0.20 \pm 0.04/\mu m^2$ (mean ± SD), and the fraction of canaliculi contributing to solute transport in calvaria.
was found to be 24 ± 3% (mean ± SD) using the method described in our previous paper [107].

4.3.2.4 Data analysis

Tracer diffusion coefficients were extracted from the FRAP image series using the methods previously described [107]. The time course of the fluorescence intensity in the photobleached lacunae \((I(t'))\), including the intensities before photobleaching \((I_0)\), immediately after photobleaching \((I_b)\), the new equilibrium after recovery \((I_\infty)\), were calculated from the image sequence. Autofading during the recovery imaging was corrected using a reference lacuna that was far away from the photobleached one and assumed to have a constant intensity. The two-compartment model [107] predicted that the experimental data followed this equation:

\[
\ln \left( \frac{I_\infty - I(t')}{I_\infty - I_b} \right) = -V_r t' D_{LCS}/d^2
\]

\((4.1)\)

\(V_r\) is the fluid volume ratio between the canaliculi and the sink lacuna. All parameters except for the diffusion coefficients were obtained by analyzing FRAP images or histological sections. The experimental data were analyzed using linear regression with time and the slope of the fitting line \(k = -V_r D_{LCS}/d^2\) was used to determine the diffusion coefficient \(D_{c,s}\).

Since the recovered fluorescence intensities at new equilibrium \((I_\infty)\) were reduced compared with pre-bleach intensities \((I_0)\), unbound fraction \((\gamma)\) and bound
fraction ($\partial$) was used in the study. These two fractions describe percentage of tracers that contribute to the recovery and those not contribute to the recovery, respectively. They are determined as below:

\[
\gamma = \frac{I_\infty - I_b}{I_0 - I_b}
\]  
(4.2)

\[
\partial = 1 - \gamma = \frac{I_0 - I_\infty}{I_0 - I_b}
\]  
(4.3)

4.4 Results

4.4.1 Alcian Blue staining

Calvaria showed significantly less Alcian Blue staining when compared to the femur in both B6 and perlecan deficient mice ($p<0.01$, Figure 4.2), which was also obvious in representative images shown in Figure 4.1. There was no significant difference in Alcian Blue staining intensity between the B6 and PLN hypo bones.
Figure 4.2. HSPG content in calvaria and femurs in B6 (blue color) and PLN Hypo mice (red color). Significant difference was found between femur and calvaria regardless of genotype. No significant difference between PLN Hypo and B6 bones.

4.4.2 FRAP diffusion study

Detailed results of anatomical parameters and the measured tracer diffusion coefficient were obtained in both calvaria and tibia (Table 4.1&4.2). Significant difference was detected in lacunar area $A$, lacunar volume $V_s$, lacunar surface area $S$, lacunar spacing $d$ and ratio of fluid volumes of the canaliculi and the lacuna $V_r$ between calvaria and tibia using student’s $t$ tests. Measurements of $D$ obtained by FRAP were reproducible with reasonable intra- and inter-animal variations. The coefficient of variation for replicate measurements from individual animals was typically 13–43%, 20–34% in calvaria and tibia, separately, whereas inter-animal variation was found to be 36% and 34% in calvaria and tibia, separately. The diffusion coefficients of sodium fluorescein was measured to be $417.4 \pm 151.1 \, \mu m^2/sec$. 
based on 40 FRAP measurements in calvaria (Table 4.1) and 365.2 ± 122.6 µm²/sec based on 33 FRAP measurements in tibia (Table 4.2). No significant difference was detected between the two bone sites using student’s \( t \) test.
<table>
<thead>
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<th>$b$, $\mu$m</th>
<th>$c$, $\mu$m</th>
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Table 4.1. Anatomical parameters and tracer diffusion coefficients measured in the FRAP experiments in murine calvaria.
Table 4.1 (Continued)

| Animal no. | FRAP no. | \( A \), \( \mu m^2 \) | \( P \), \( \mu m \) | \( a \), \( \mu m \) | \( b \), \( \mu m \) | \( c \), \( \mu m \) | \( V_s \), \( \mu m^3 \) | \( S \), \( \mu m^2 \) | \( N \) | \( d \), \( \mu m \) | \( V_r \) | \( I_0 \) | \( I_\infty \) | \( I_b \) | \( \gamma \) | \( \partial \) | \( k \) | \( D \mu m^2 s^{-1} \) |
|------------|---------|--------------------|----------------|----------------|----------------|----------------|----------------|----------------|-----------|----------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 2          | 96      | 36                 | 7.0            | 3.5            | 2.3            | 235            | 203            | 10              | 31        | 0.06           | 203    | 149    | 120    | 0.35   | 0.65   | 0.03   | 512.0  |
| 3          | 105     | 39                 | 7.6            | 3.8            | 2.5            | 305            | 241            | 12              | 33        | 0.06           | 216    | 190    | 142    | 0.65   | 0.35   | 0.04   | 675.3  |
| 4          | 133     | 42                 | 8.2            | 4.1            | 2.7            | 386            | 282            | 14              | 37        | 0.06           | 178    | 140    | 93     | 0.56   | 0.44   | 0.03   | 697.2  |
| 1          | 99      | 40                 | 7.7            | 3.9            | 2.6            | 320            | 249            | 12              | 28        | 0.05           | 189    | 160    | 70     | 0.76   | 0.24   | 0.03   | 591.1  |
| 2          | 76      | 33                 | 6.4            | 3.2            | 2.1            | 181            | 170            | 8               | 29        | 0.06           | 111    | 106    | 73     | 0.86   | 0.14   | 0.02   | 257.3  |
| 3          | 87      | 36                 | 7.0            | 3.5            | 2.3            | 237            | 204            | 10              | 30        | 0.06           | 192    | 167    | 111    | 0.69   | 0.31   | 0.01   | 219.1  |
| 4          | 88      | 36                 | 7.0            | 3.5            | 2.3            | 235            | 203            | 10              | 17        | 0.03           | 148    | 135    | 98     | 0.72   | 0.28   | 0.02   | 166.3  |
| 5          | 83      | 34                 | 6.7            | 3.4            | 2.2            | 210            | 188            | 9               | 21        | 0.04           | 180    | 139    | 115    | 0.36   | 0.64   | 0.05   | 515.7  |
| 6          | 79      | 33                 | 6.4            | 3.2            | 2.1            | 184            | 172            | 8               | 23        | 0.05           | 218    | 161    | 130    | 0.35   | 0.65   | 0.05   | 588.6  |
| 7          | 106     | 39                 | 7.7            | 3.8            | 2.6            | 315            | 246            | 12              | 35        | 0.06           | 199    | 158    | 82     | 0.65   | 0.35   | 0.03   | 578.8  |
| 8          | 92      | 37                 | 7.2            | 3.6            | 2.4            | 259            | 216            | 10              | 34        | 0.06           | 224    | 181    | 112    | 0.62   | 0.38   | 0.03   | 578.7  |
| 9          | 72      | 32                 | 6.3            | 3.2            | 2.1            | 175            | 167            | 8               | 30        | 0.06           | 214    | 190    | 129    | 0.72   | 0.28   | 0.02   | 307.6  |
| 5          | 1        | 141                | 8.4            | 4.2            | 2.8            | 418            | 298            | 14              | 23        | 0.03           | 183    | 156    | 81     | 0.73   | 0.27   | 0.02   | 372.6  |
| 2          | 91      | 35                 | 6.9            | 3.5            | 2.3            | 230            | 200            | 10              | 23        | 0.04           | 122    | 102    | 64     | 0.66   | 0.34   | 0.03   | 379.8  |
| 3          | 123     | 39                 | 7.6            | 3.8            | 2.5            | 311            | 244            | 12              | 23        | 0.04           | 208    | 182    | 129    | 0.67   | 0.33   | 0.03   | 432.7  |
| 4          | 90      | 37                 | 7.3            | 3.6            | 2.4            | 267            | 221            | 11              | 24        | 0.04           | 211    | 190    | 102    | 0.81   | 0.19   | 0.02   | 274.6  |
| 5          | 142     | 46                 | 9.0            | 4.5            | 3.0            | 506            | 338            | 16              | 31        | 0.04           | 141    | 118    | 67     | 0.69   | 0.31   | 0.02   | 507.3  |
| 6          | 126     | 40                 | 7.9            | 3.9            | 2.6            | 339            | 259            | 12              | 22        | 0.03           | 184    | 171    | 100    | 0.85   | 0.15   | 0.02   | 232.4  |
| 7          | 102     | 38                 | 7.3            | 3.7            | 2.4            | 274            | 224            | 11              | 22        | 0.04           | 172    | 113    | 81     | 0.36   | 0.64   | 0.03   | 381.9  |
| 8          | 101     | 37                 | 7.1            | 3.6            | 2.4            | 252            | 212            | 10              | 23        | 0.04           | 163    | 151    | 87     | 0.84   | 0.16   | 0.01   | 175.3  |
| Mean       |         | 95                 | 7.2            | 3.6            | 2.4            | 263            | 216            | 10              | 29        | 0.05           | 182    | 157    | 97     | 0.69   | 0.31   | 0.03   | 417.4  |
| St dev     | 19       | 4                  | 0.7            | 0.3            | 0.2            | 77             | 42             | 2               | 5         | 0.01           | 26     | 26     | 21     | 0.16   | 0.16   | 0.01   | 151.1  |

\( A \), lacunar area; \( P \), lacunar perimeter; \( a, b, c \), lacunar radii in the three axes; \( V_s \), lacunar volume; \( S \), lacunar surface area; \( N \), number of contributing canaliculi; \( d \), mean canalicular length; \( V_r \), ratio of fluid volumes of the canaliculi and the lacuna; \( I_0 \), pre-bleach intensity; \( I_\infty \), recovered intensity at new equilibrium; \( I_b \), intensity after photobleaching; \( \gamma \), unbound fraction; \( \partial \), bound fraction; \( k \), recovery rate; \( D \), diffusion coefficient.
Table 4.2. Anatomical parameters and tracer diffusion coefficients measured in the FRAP experiments in murine tibia

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$A$, lacunar area; $P$, lacunar perimeter; $a$, lacunar radius in the long axis; $b$, lacunar radius in the short axes; $V_r$, lacunar volume; $S$, lacunar surface area; $N$, number of contributing canaliculi; $d$, mean canalicular length; $V_r$, ratio of fluid volumes of the canaliculi and the lacuna; $I_0$, pre-bleach intensity; $I_\infty$, recovered intensity at new equilibrium; $I_b$, intensity after photobleaching; $\gamma$, unbound fraction; $\partial$, bound fraction; $k$, recovery rate; $D$, diffusion coefficient.
4.5 Discussion

The Alcian Blue staining data confirmed that murine long bones express higher content of HSPG than calvaria, which agreed with a previous study [181]. Although we were unable to define pericellular boundaries and to quantify pericellular HSPG contents in the current setup, differences in pericellular HSPG content are expected between the femur and the calvaria, because both extracellular and pericellular HSPG are secreted by osteoblasts and osteocytes (retired osteoblasts). We also used the FRAP diffusion approach to quantify the tracer diffusion coefficients in long bone vs. skull bone, which is an indicator of the expression/density of the osteocytic pericellular perlecan mechanosensors. These studies are required to thoroughly test our hypothesis that pericellular HSPG matrix serves as mechanosensor that allows osteocytes to detect the presence and absence of mechanical stimulation. This novel hypothesis may provide a potential explanation of calvaria’s surprising resistance to disuse bone loss. From an evolutionary point of view, since cavarial bone appeared first in fish living in ocean, where gravity and body weight are countered by buoyance force, calvaria’s resistance to disuse bone loss may be an intrinsic property required to fulfill its main function: protecting the most vital organ (brain) in the body. This study aimed to test whether HSPG, a potential mechanosensor, is reduced in calvaria, which was supported by our results.

The diffusion coefficient measured in my study was comparable to previous data in the murine tibia [88, 107]. Although my results demonstrated that the diffusion
coefficients were not significantly different between murine long bones and calvaria, I did observe significant different immobilized fractions for the tested tracer as shown in the reduced recovered fluorescence intensities ($I_\infty$) compared with pre-bleach intensities ($I_0$) (data shown in Table 4.1 & 4.2, Figure 4.3). The hindered recovery experienced by sodium fluorescein in the bone LCS can be attributed to multiple molecular interactions between the tracers and the extracellular solid and fluid phases, including (but not limited to) electrostatic interactions, hydrophobic/hydrophilic compartmental partitioning, and physical tangling [88]. This immobilized fraction was found to be 19 ± 13%, 31 ± 16% for tibia and calvaria, respectively. The significant higher immobilized fraction in calvaria compared to tibia could be due to a complicated binding mechanism associated with the fiber matrix in the annular region between the osteocytic cell membrane and the surface of the canalicular wall. The canalicular length (lacunar spacing) was significantly longer in calvaria than in tibia (Table 4.1 & 4.2), which may also lead to a more severe binding in calvaria canalicular annular region since the tracer needs to pass through a longer path.
Figure 4.3. Representative curves for calvaria and tibia FRAP. $I_0$, pre-bleach intensity; $I_\infty$, recovered intensity at new equilibrium; $I_b$, intensity immediately after photobleaching. Bound fraction ($\vartheta$) is shown in the figure, and it is significantly higher in calvaria than in tibia.

The current study has certain limitations. We did not detect any Alcian Blue staining difference between PLN Hypo and B6 bones, which may due to the lack
of specificity of the staining method. We are planning to use HSPG-specific antibodies and immunohistochemistry (IHC) to address this limitation. In addition, a more severe binding effect was detected in the skull bones compared with long bones. We plan to use in future studies our newly developed tracer velocimetry approach detailed in Chapter 2 to quantify the differential distribution of pericellular HSPG matrix content in long bone vs. skull bone. Mechanical loading might alleviate the binding effect and the obtained transport enhancement results would better describe the tracer movement in the bone LCS.

In summary, our experimental data suggest differential expressions of HSPG between weight-bearing long bone and calvaria bone. Although we did not detect the changes of diffusion of fluorescein, a small molecule, through the LCS of the two types of bone, we did observe a significant increase of immobile fraction of the tracers onto the pericellular matrix, suggesting the reduction of the overall mobility of the tracers in calvaria than tibia. Transport of larger molecules and transport in the presence of mechanical loading are yet to be determined. Additional studies are needed to uncover the mechanisms regulating calvaria’s resistance to bone loss, which may be applied to other bone sites. In addition, reliable and accurate methods to quantify pericellular matrix HSPG will be valuable research and clinical diagnostic tools.
Chapter 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Significance

The studies presented in this dissertation made several contributions to the field of bone biology, including a novel hypothesis that the osteocyte pericellular matrix (PCM) fibers act as osteocyte’s sensing antennae. I obtained quantitative data on osteocytic PCM fiber density, cellular-level mechanical stimulations and key features of the LCS network including the topological parameter and the detailed structure of individual connections of both normal and pathologic bones. In addition, I found that murine long bones express higher content of HSPG than calvaria, which provides a potential explanation of calvaria’s surprising resistance to disuse bone loss.

My first project quantified osteocytic PCM fiber spacing using the recently developed tracer velocimetry approach. From the obtained PCM fiber spacing, my study elucidated how the density of this matrix regulates cellular stimulations, the shear stress and the fluid drag. I found the sieving properties and the ultrastructure of the osteocytic PCM were altered among young adult, aged, and perlecan-deficient bones, as well as the levels of mechanical stimulation forces experienced by osteocytes in situ. Since the LCS structure is also very important for bone fluid flow, my second study elucidated the features of LCS at different bone compartments, age,
and diseased conditions using confocal imaging and TEM imaging approach. The LCS anatomy such as canalicular number density and lacunar major axis radius are key parameters in the mathematical simulation to obtain the solute velocities, which determine the magnitude of the cellular stimulation forces. For the PCM parameters obtained from TEM imaging, when the cell process radius increases, the fluid shear stress would increase while the drag force would decrease. Alternatively, when the canalicular wall radius increases, the fluid shear stress would decrease while the drag force would increase. Besides the response to mechanical loading, PCM density was also found to determine bone adaptation to disuse. The Alcian Blue staining data confirmed that murine long bones express higher content of HSPG than calvaria. The FRAP diffusion study indicated different binding effect between the two bone sites. These results will help uncover mechanisms regulating calvaria’s resistance to bone loss, which may be applied to other bone sites.

Taken together, my three projects support the idea that the PCM fibers act as osteocyte’s sensing antennae by regulating the hydrodynamic forces experienced by the cell and is therefore an essential player in bone’s sensitivity and in vivo adaptation to its mechanical environments. Altogether, my work provide a solid foundation and powerful tools for better characterizing the osteocytic PCM and LCS and their physiological roles in bone mechanosensing and contributions to the maintenance of bone health and quality.
5.2 Future directions

The studies that have been done in this dissertation indicated that the pericellular matrix (PCM) fibers, acting as flow-sensing antenna on osteocytes, regulate bone’s sensitivity to mechanical loading. We have begun to elucidate the critical role of the PCM using a novel tracer velocimetry approach, which combined FRAP-based confocal imaging and a hydrodynamic sieving model, where the velocities of the fluid and a large protein through the PCM were measured separately using two tracers in adult murine tibial cortex. We also probed into the expression of HSPG content in different bone sites, which may help to uncover mechanisms regulating calvaria’s resistance to bone loss. The hypothesis is that the pericellular HSPG matrix serves as mechanosensor that allows osteocytes to detect the presence and absence of mechanical stimulation. A series of studies need to be performed to further test this novel hypothesis, with the ultimate goal of revealing the cellular mechanisms responsible for the osteocyte mechanotransduction and bone adaptation.

In the one-tracer velocimetry approach, velocities of fluid and solute were measured separately in different bone samples, which introduced variability from both anatomical and loading inconsistency. To address this limitation, a novel dual-colored FRAP approach, which allows simultaneous measurements of both fluid and solute velocities, has been testing. Two fluorescent tracers are injected into the same mouse, with the small green tracer used to track fluid velocity and the large red tracer to track solute velocity. PCM ultrastructure and the corresponding cellular stimulation forces will be obtained. The dual-colored FRAP approach will be applied to normal and aged
mice as well as mice with decreased PCM components and validated by comparing its results with previous one-colored FRAP approach. After validation, we can apply the dual-colored FRAP approach to other bone sites and pathological bones. The potential challenge is that there might be interaction between the small tracer and the large tracer, which could make the small tracer move slower.

Furthermore, currently only diffusive studies were performed on the murine long bone and skull bone with significant binding issues. Mechanical loading might alleviate the binding effect and the obtained transport enhancement results would possibly better describe the tracer movement in the bone LCS. Using the novel dual-colored FRAP approach, the ultrastructure of PCM, membrane shearing force and total drag force on the PCM fibers will be examined and compared between long bone vs. skull bone. Because of the unique shape of the calvaria, difficulty is anticipated on how to apply the mechanical loading on the calvaria. Also comparable surface strains are required apply on the two bone sites.

Upon the successful completion of these studies, I anticipate that the cellular mechanism for osteocyte’s mechanosensing and bone adaptation will be understood more deeply. Quantification of the PCM fiber density or related characteristics could be a powerful tool to identify an individual’s sensitivity to loading and provide new targets to promote bone formation in osteoporotic patients.
REFERENCES


Appendix A

IMAGING AND QUANTIFYING SOLUTE TRANSPORT ACROSS PERIOSTEUM: IMPLICATIONS FOR MUSCLE–BONE CROSSTALK
Original Full Length Article

Imaging and quantifying solute transport across periosteum: Implications for muscle–bone crosstalk

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A B S T R A C T

Muscle and bone are known to act as a functional unit and communicate biochemically during tissue development and maintenance. Muscle-derived factors (myokines) have been found to affect bone functions in vitro. However, the transport times of myokines to penetrate into bone, a critical step required for local muscle–bone crosstalk, have not been quantified in situ or in vivo. In this study, we investigated the permeability of the periosteum, a major barrier to muscle–bone crosstalk by tracking and modeling fluorescent tracers that mimic myokines under confocal microscopy. Periosteal surface boundaries and tracer penetration within the boundaries were imaged in intact murine tibia using reflected light and time-series xz confocal imaging, respectively. Four fluorescent tracers including sodium fluorescein (376 Da) and dextrans (3 kDa, 10 kDa and 40 kDa) were chosen because they represented a wide range of molecular weights (MW) of myokines. We found that (i) murine periosteum was permeable to the three smaller tracers while the 40 kDa could not penetrate beyond 40% of the outer periosteum within 8 h, suggesting that periosteum is semi-permeable with a cut-off MW of approximately 40 kDa, and (ii) the characteristic penetration time through the periosteum (~60 μm thick) increased with tracer MW and fit well with a relationship $t_{\%} = \left(\frac{4.43 \times 10^{-3}}{\text{MW}} - 0.57 \right) \times 10^9 + 1.19 \times 10^{-4} - 0.47$, from which, the characteristic penetration times of various myokines were extrapolated. To achieve effective muscle–bone crosstalk, likely signaling candidates should have shorter penetration time than their bioactive time, which we assumed to be 5 times of the molecule's half-lifetime in the body. Myokines such as PGE2, IGF-1, IL-15 and FGF-2 were predicted to satisfy this requirement. In summary, a novel imaging approach was developed and used to investigate the transport of myokine mimicking-tracers through the periosteum, enabling further quantitative studies of muscle–bone communication in physiologically normal and pathological conditions.

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Introduction

Emerging evidence suggests that muscle and bone, being a functional unit, communicate with each other during tissue development and maintenance [1–3]. Soluble factors secreted by skeletal muscles (termed myokines) can affect the bone metabolism [4–7]. These myokines include growth factors (e.g., insulin like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), and transforming growth factor-β (TGFB), cytokines (e.g., interleukins (IL-9 and IL-15)), and other small signaling molecules (e.g., prostaglandin E2 (PGE2)). A recent study demonstrated that myokines secreted by skeletal muscles protected osteocytes from glucocorticoid-induced apoptosis through the activation of Wnt/β-catenin pathway, and such protective effect was greatly enhanced when the muscle was electrically stimulated [8]. Muscle also serves as an important source of cells and growth factors that promote bone healing [9], and muscle progenitor cells even retain the capacity to differentiate into osteogenic cell lineages [10,11]. The communication between bone and muscle can operate in both directions. Bone marrow-derived mesenchymal stromal cells (MSCs) were found to stimulate skeletal myoblast proliferation by releasing growth factors [12]. In addition, mechanically stimulated MLO-Y4 osteocytes were found to express anabolic factors, such as IGF-1, vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF), which are known to affect muscle mass and its adaptation to mechanical loading [13]. Therefore, understanding the functional interactions between muscle and bone will be crucial to the development of new and efficient bone/muscle repair strategies.

The periosteum, a fibrous membrane that physically separates bone and muscle tissues, acts both as a functional target for muscle/bone...
derived growth factors as well as an important gatekeeper for fluid and solute exchange between muscle and bone [6,14]. The periosteum is composed of two distinct layers with the outer fibrous layer containing fibroblasts, collagen, Sharpey’s fibers and an extensive nerve and microvascular network and the inner cambium layer containing nerves, capillaries, and adult mesenchymal progenitor cells [15-19]. As a reservoir of pluripotent mesenchymal stem cells, which can differentiate into bone cells, chondrocytes, adipocytes, and skeletal myocytes [20, 21], periosteum could be a target of muscle-bone-derived factors. A recent study found that periosteum expressed abundant receptors for myokine IGF-1 and IGF-2, which are known to be potent regulators of bone formation [6]. Furthermore, as an interface, the periosteum can act as a molecular sieve, controlling the penetration and transport rates for signaling molecules involved in bone–muscle crosstalk. To date, the periosteum has been described as ranging from a completely sealed impermeable barrier for fluid perfusion [22-25] to a semi-permeable membrane [26]. A recent study [27] characterized the hydraulic conductance of ovine femoral and tibial periosteum using isolated samples and Darcy’s law. However, the sieving property of the periosteum to signaling molecules has not been determined in situ.

To fill the knowledge gap regarding periosteum permeability and to further elucidate the mechanisms by which muscle and bone communicate with each other, a novel imaging approach was developed to quantify the molecular transport through intact periosteum in situ using confocal microscopy. The murine tibial periosteum was first identified through reflected-light imaging, followed by temporally tracking the penetration of fluorescent tracers into the periosteum, from which the sieving property of the periosteum was obtained. An empirical relationship was established between molecular weight and the characteristic penetration time across the periosteum. These data helped us to analyze the rate-limiting steps controlling bone–muscle crosstalk and the likely molecular candidates responsible for such communication. The new approach and the quantitative data laid the foundation for further investigation of muscle–bone interactions in physiological and pathological conditions.

Materials and methods

Fluorescent tracers

Four fluorescent tracers including sodium fluorescein (376 Da, Sigma-Aldrich, St. Louis, MO), and fluorescein-conjugated dextran with nominal molecular weights (MW) of 3 kDa, 10 kDa, and 40 kDa (Molecular Probes/Invitrogen Corp., Eugene, OR) were selected as representatives of a spectrum of myokines. The tracer concentration of the tracer solution (0.01 mg/mL for sodium fluorescein and 0.02 mg/mL for the dextrans, respectively) was chosen based on our preliminary tests to ensure (i) adequate fluorescence signal for tracer detection in the periosteum and (ii) fluorescence intensity being proportional to the concentration of the tracer (no saturation or loss of fluorescence due to overcrowding [28]).

Specimen preparation

Twelve skeletally mature mice (C57BL/6J or Balb/c, five- to eight-month old) were sacrificed with carbon dioxide. Left tibiae were immediately harvested and cleansed of soft-adherent tissues using surgical scissors with care to keep the periosteum structurally intact (Fig. 1). Occasionally, the periosteum might become perforated during preparation. This could be identified easily by the presence of abnormally high-intensity spots in an otherwise uniform background during the tracer-tracking xz imaging (described below). Damaged samples were discarded. The samples were tested within 3 h post-mortem to minimize permeability and other postmortem changes. For each tracer, three mice were tested. All animal procedures were approved by the Institutional Animal Use and Care Committee of the University of Delaware.

Experimental set-up

The two ends of left tibiae were rigidly fixed in a custom-made holder placed inside in an imaging chamber containing phosphate buffered saline (PBS, ~120 mL) at room temperature (25°C) (Fig. 2A). The anterior–medial periosteal surface of the tibial shaft, located 20–30% distal from the proximal end, was selected for the experiment (Fig. 2B). This region was chosen because (i) it is relatively flat and in focus within a microscope-imaging plane (xy) and (ii) the local tracer penetration could be simplified as a one-dimensional (z) mathematical problem by neglecting bone curvature. After the region of interest on the periosteal surface was identified, the bone was imaged with an inverted confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss Inc., Thornwood, NY) equipped with a lens inverter (LSM Technology, Eitters, PA) and a 20 × 1.0 numerical aperture water dipping lens (W Plan-Apochromat, Zeiss) (Fig. 2A). The "L" shaped lens inverter alters

Fig. 1. Left murine tibia was shown with associated muscles intact (A) and muscles removed (B) to expose the imaging area on periosteum (anterior–medial surface 20%–30% distal from the tibial proximal end).
the laser path so that the tibia with its periosteal surface facing up could be imaged from the top.

Validation and identification of the periosteum

To identify the boundaries of the tibial periosteum, a z-axis scan under reflected light was captured using a series of line scans (512-pixels) at a rate of 3.93 s/frame as the focus plane was moved incrementally in the z direction, which was perpendicular to the periosteal surface, from the bathing solution towards the interior bone (Fig. 2C). The z-step was chosen to be 1.19 μm. Due to the presence of dense connective tissue in the periosteum [16–19], the exciting laser is easily reflected and a strong signal could be collected during the z-axis scanning using the reflected mode. To further confirm that this high-intensity layer in the z-axis reflected image was the periosteum, amine-targeted tissue staining was performed on samples in a separate experiment. Three freshly isolated tibiae with intact periosteum were first imaged under reflected light mode using 488-nm excitation at 7.53 laser power as described above (Fig. 3A), followed by immersion into Texas red C2-dichlorotriazine dissolved in PBS (2 mg/mL). This reactive dye, with absorption/emission maxima of ~588/601 nm, readily reacts and binds to amine groups that are abundant in proteins found in periosteum. After 24 h of dye incubation, the tibiae were washed multiple times with PBS for 0.5 h to remove any residual dye solution and imaged under the confocal microscope using a 561-nm excitation wavelength (3% transmission). The periosteum, with its high content of proteins/
amines, was stained red with the Texas red C2-dichlorotriazine dye (Fig. 3B). The position of the tip and the region of focus remained unchanged in the imaging chamber during the entire process, so that the red fluorescent and reflected signal channels could be overlaid for comparison (Fig. 3C). Normalized spatial intensity profiles were obtained for the two channels (detailed in the subsequent section of Characteristic Tracer Penetration Time). Our results clearly indicated the fidelity of using reflected light imaging to identify the periosteum (Figs. 3C and D). From the spatial intensity profiles (> 100 z-steps), the two peristomial boundaries (vertical red lines) were identified at locations with the half maximal intensity value (red horizontal dotted lines, Fig. 3D), from which the periosteum thickness was calculated between the boundaries. These boundaries were also overlaid onto the time-series images of tracer penetration to define the transport characteristics (detailed below).

**Time-series imaging of tracer penetration**

Prior to imaging the tracer penetration into the periosteum, tibiae were isolated as described earlier in Specimen Preparation, followed by reflected light imaging of the periosteum. The bathing PBS was then removed from the imaging chamber and a pre-mixed fluorescent tracer solution (120 mL) was added to the imaging chamber using two 60 mL syringes. The fluid change process was performed with caution to avoid vibration and/or disturbance in the imaging system, and with a high speed (>seconds) to minimize any drying that may alter the permeability of the periosteum. Continuous xz scanning along the depth of the periosteum was initiated immediately following the introduction of the tracer solution into the imaging chamber. The 488 nm laser power was set to be 5.5% and 7.5% for sodium fluorescein and dextran, respectively. The length of scan lines was 256 pixels and the z-step was set to 6.15 μm. It usually took ~7 s to acquire a single xz scan consisting of 30 z-steps. The xz scan was repeated for up to 400 times, during which the tracer penetration into the periosteum was tracked for up to 8 h. Representative snapshots of the tracer penetration across the periosteum were grouped at various time points using ImageJ software package (NIH) (Fig. 4).

**Characteristic tracer penetration time**

To quantify and compare penetration processes among various tracers, a characteristic transport time was obtained from each time-series scanning test. First, the two-dimensional xz scans at various time points (Fig. 4) were collapsed into one-dimensional intensity profiles along the periosteal depth (z) by averaging the intensity values for the 256 pixels in each x line. The intensity profiles were then normalized with the fluorescence intensity of the bath solution. For each test, the bath-periosteum and periosteum-bone interfaces were identified from the reflected-light imaging as described in the preceding section (Fig. 3D), and overlaid on the tracer penetration profiles, where the interface with the bathing liquid was denoted as z = 0 and the interface with bone as z = 1 (Fig. 5A). Second, the penetration depth at each time point (vertical red lines d1 and d2) was defined as the depth where tracer intensity reached 50% of the normalized tracer intensity (the red dotted line, Fig. 5A). The penetration depths were plotted as the function of time (Fig. 5B). The characteristic penetration time (τ), defined as the time lag when the penetration depth reached the midplane of the periosteum (horizontal line at z = 0.5, Fig. 5B), were obtained and compared among groups.

**Effective transport rate for tracer penetration into periosteum**

To further quantify transport dynamics, transport characteristics at the periosteum–solution interface (z = 0) were calculated from the spatiotemporal profiles (Fig. 5A). The concentration gradient (dC/dz) at the interface was calculated at each time point. As a first approximation, we assumed that solute influx through this interface followed Fick’s law and was proportional to the concentration gradient and a transport rate constant k. As widely used in membrane transport problems [29-31], the first principle (mass conservation) can be applied to the half space consisting of the periosteum and underlying bone (Eq. (1)), i.e., the influx of tracer from the bath through the interface z = 0 (the left side) equaled the tracer accumulation within the region of interest during each time step (the right side):

\[
k(t) \int_{z=0}^{z_m} \frac{dC}{dz}dz = \int_{z=0}^{z_m} C(t,z)dz - \int_{z=0}^{z_m} C(t,z_{m-1})dz.
\]  

From Eq. (1), the effective transport rate k could be obtained as a function of time. Please note that although it has the same unit as diffusivity, k is an effective constant describing the total transport resulting from diffusion and other transport modes such as binding. The initial effective transport rates (k0) at time zero were obtained first. To compare the time-dependent changes in the transport process, normalized transport rate (k/k0) was plotted as a function of dimensionless time (τ), where τ is defined as t = h²k0 and h is the periosteal thickness.

**Statistical analysis**

Data were reported as means and standard deviations. One-way ANOVA followed by Tukey's post hoc tests was performed to test the differences among four tracer groups, with p < 0.05 indicating a significant difference. GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA) was used.

**Results**

The spatiotemporal profiles demonstrated that tracer concentration increased inside the periosteum with time and the penetration depth also increased with time but with a decreasing rate (Figs. 5A and B). The characteristic penetration time for the particular dextran-3 k experiment shown in Fig. 5B was found to be 66 s.

The periosteal thickness was comparable with no significant difference among the four tracer groups (sodium fluorescein: 59.5 ± 6.0 μm, dextran-3k: 59.2 ± 8.4 μm, dextran-10k: 62.8 ± 7.6 μm, and dextran-40k: 67.4 ± 9.5 μm) (Fig. 6).

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![Image](https://example.com/fig4.png)

**Fig. 4** A representative time-series of xz scans showing the gradual penetration of dextran-3k (green color) into the periosteum at various time points. The periosteal boundaries were identified in reflected light imaging (not shown). Scale bar = 50 μm.
Periosteum exhibited the properties as a semi-permeable membrane, allowing the penetration of smaller tracers (Fig. 5) but effectively blocking the passage of 40 kDa-dextran, the largest tracer tested in the study (Fig. 7). The penetration depth of 40 kDa was limited to 40% of the outer periosteum, suggesting that the cut-off MW for periosteum was approximately 40 kDa.

For the three smaller tracers, a significant difference was found in their characteristic penetration times, with sodium fluorescein penetrating the fastest (9.9 ± 1.6 s), followed by dextran-3k (57.7 ± 49.5 s), and dextran-10k (547.3 ± 5933 s) (Fig. 8).

The initial effective transport rate $k_b$ at the periosteal surface was found to decrease with tracer molecular weight (Fig. 9). Sodium fluorescein showed the highest initial transport rate (361 ± 104 μm²/s) while dextrans showed progressively reduced transport rates with increasing molecular weights (175 ± 55 μm²/s for dextran-3k, 101 ± 93 μm²/s for dextran-10k, and 17 ± 15 μm²/s for dextran-40k). As time went on, the effective transport rate declined, with the fastest drop for dextran-40k (Fig. 10).

**Discussion**

The present study aimed to quantify the sieving properties of the periosteum, a dense connective tissue that acts as a transport barrier as well as a potential target for the signaling molecules. Bone and adjacent muscles are mechanically and biochemically coupled. In this study we focused on quantifying transport of signaling molecules from muscle towards bone. Although it is known that muscles release various functional molecules that are capable of regulating bone metabolism in vitro (Table 1), candidates responsible for the in vivo muscle–bone crosstalk have not been identified. Nor are the mechanisms elucidated, by which these myokines move between the tissues. Presumably, these signaling molecules can be carried away from muscle through extracellular space and/or through systemic vasculature [4,6]. The present study addressed the first transport pathway, especially signaling transport across periosteum covering the cortical bone.

Because periosteal permeability determines how quick molecules move within the tissue and periosteal thickness is an important parameter for permeability, we first determined the periosteal thickness in murine tibiae. Using the x2 reflected light imaging, we found that the periosteal thickness was ~69 μm in adult mice (five to eight-month old, Fig. 6). This result was consistent with previous findings of periosteal thickness (~60 μm) from murine femurs using hematoxylin and eosin (H&E) staining and DIC light microscopy [17]. A thinner cambium layer (~40 μm) was obtained using confocal z-stack imaging in intact murine tibiae treated with calcine labeling (to stain the bone surface) and nuclei staining (to stain cells) [32]. The consistency of the periosteal thickness measurements obtained using histological sections [17] and confocal microscopy (this study and ref. [34]) alleviated the concerns of "stretching" artifacts in the axial imaging [33] and confirmed the fidelity of the x2 confocal scanning imaging approach. This may be attributed to the fact that periosteum is a non-mineralized tissue and thus the optical index mismatch between the tissue and the aqueous solution is not as severe as that presented in bone [33].

The most important result from this study was that periosteum serves as a semi-permeable sieve with a cut-off MW of ~40 kDa. Our

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$\text{Fig. 6. The periosteal thickness was comparable with no significant difference among the four tracer groups (N = 3/group).}$

---

$\text{Fig. 7. Semi-permeable periosteum limited the penetration of dextran-40k within 40% of outer layer even after 6 h of testing, suggesting the cut-off MW of periosteum to be ~40 kDa.}$
tests demonstrated that the penetration of molecules into the periosteum took a longer time with increasing molecular weight and the transport was effectively blocked for dextrans with MW of 40 kDa (Figs. 7 and 8). Although we only performed three experiments for each tracer, the achieved power in statistical analysis was close to 1 because of the high effective sizes in the characteristic transport time among the four tracer groups (Fig. 8). The following empirical relationship was found to best fit the experimental data with the cut-off behavior:

\[
\tau_c(t) = \left(4.43 \times 10^5\right) \cdot 0.57 \cdot \left(\frac{MW}{Da} - 4 \times 10^3\right) \cdot \left(\frac{MW}{Da} - 4 \times 10^3\right) - 8.65 \times 10^5 \cdot \left(\frac{MW}{Da} - 4 \times 10^3\right)
\]

The equation stipulates that the characteristic transport time through periosteum approaches infinite as MW increases towards 40 kDa. Applying Eq. (2) to myokines identified in vitro and in vivo [4–6], their characteristic penetration times were found to vary between 10 s (FGF-2) and 6 h (FGF-2) (Fig. 11, Table 1).

During bone–muscle crosstalk, there are two competing time constraints for myokines, i.e., they should be able to penetrate and reach their target bone (described by penetration time) before the ending of their bioactive lifetime. Assuming that these molecules are degraded and cleared from the body in an exponential fashion, their bioactive concentration will be decreased to a negligible value (3.1%) after five half-lifetime (5 \( \tau_c \)). It is thus reasonable to assume for an effective messenger that its penetration time \( \tau_c \) should be less than 5 \( \tau_c \). We find that some myokines including PGE_2, IGF-1, IL-15 and FGF-2 are predicted to satisfy this criterion and may be likely candidates for muscle–bone crosstalk, while IL-6 and FGF-2 do not meet the criterion (Table 1). Interestingly, peristeme was shown to express abundant receptors for IGF-1 and FGF-2 [6]. Although this result provides preliminary guidelines for designing future in vivo experiments to identify candidate myokines for muscle–bone crosstalk, we should be aware of the assumptions and limitations for the current result. The predicted transport times for the myokines are extrapolated from the tracer data based on molecular weight only (Eq. (2)). The structure, chemistry, polarity, and charge of the myokines, which may be quite different from those of the tracers used herein, could affect their interactions with extracellular matrix and cell surface receptors and impact their transport, storage, and bioavailability during tissue crosstalk. The efficacy of muscle–bone crosstalk is also dependent on the relative abundance and potency of the signaling molecules. In addition, constrictions induced by muscle contraction could possibly make the larger molecules move faster. These confounding factors, although beyond the scope of this study, need to be studied further in vivo.

Despite being a molecular sieve, the periostuem is unlikely to be the rate-limiting step for smaller signaling molecules during muscle–bone crosstalk through the extracellular transport pathway consisting of muscle tissue, periosteum, and cortical bone tissue. Without any available solute permeability measurements in muscle tissue, it is impossible to identify which tissue is the rate-limiting step in this pathway. However, comparing the initial transport rates in periosteum (\( \tau_c = 0 \)) and those in bone (Table 2), the permeability of the two tested smaller tracers (sodium fluorescein and dextran-3k) is more than 10-fold higher in periosteum than in bone. For the larger linear dextran tracers (10 kDa and 40 kDa), their transport rates were not measured in bone. As demonstrated in previous study [41], they are expected to have smaller permeability than those of globular proteins with similar MWs (parvalbumin 12.3 kDa, 15.7 \( \mu \)m2/s) and ovalbumin 43 kDa, 6.5 \( \mu \)m2/s). Therefore, the initial transport rates of the dextran-10 k and dextran-40 k in periosteum are at least 2.5- and 6-fold higher than those in bone, respectively (Table 2). However, after the lapse of one time constant \( \tau = \bar{h}/\bar{a}_0 \) the peristeme transport rates for smaller tracers (376k Da, 3 kDa) remain higher than those in bone, but those for larger tracers decrease to the levels that are comparable to those in bone (Table 2). Overall, peristeme may be a major barrier to larger molecules (\( \geq 10 \) kDa) much like bone, while it is much more permeable to small molecules (\( \leq 3 \) kDa) than bone is.

It is not clear why the effective transport rates in peristeme declined with time, especially for larger dextran molecules (Fig. 10). As mentioned earlier, the effective transport rate accounts for diffusion as well as other transport modes involving, for example, binding of the tracer molecules to the fibrous extracellular matrix and to the surface receptors of the resident cells in periosteum. In fact, the observation of temporal decrease of the effective transport rate suggests the presence

![Figure 8](image1.png)

**Fig. 8.** The characteristic penetration time increased with tracer molecular weight. Sodium fluorescein: 376 Da; dextran-3k: 3000 Da; dextran-10k: 10,000 Da; dextran-40k: 40,000 Da; pairs denoted with different letters (a/b/c) were significantly different (p < 0.05).

![Figure 9](image2.png)

**Fig. 9.** Initial transport rate at the periosteum surface decreased with tracer molecular weight. Sodium fluorescein: 376 Da; dextran-3k: 3000 Da; dextran-10k: 10,000 Da; dextran-40k: 40,000 Da; pairs denoted with different letters (a/b) were significantly different (p < 0.05).

![Figure 10](image3.png)

**Fig. 10.** Transport rate at the periosteum surface decreased with time. Time is normalized with \( \tau = h/\bar{a}_0 \), where \( h \) is the periosteum thickness and \( \bar{a}_0 \) is the initial transport rate.
of the non-diffusion transport modes. Previous work demonstrated that dextran could specifically bind to the mannose and DC-SIGN family receptors [43,44]. As more and more mobile molecules are bound to ECM or taken inside cells through endocytosis, the available molecules decrease for diffusive transport, leading to the decline of the effective transport rate (Fig. 10). Note that the experiments were undertaken in room temperature (25 °C), at which solute diffusivity was anticipated to be 4% smaller and the cell-mediated transport might also be lower compared with measurements in body temperature (37 °C). Therefore, there is a need to test the transport of myokines under in vivo conditions in the future.

There are several additional limitations in the present study. i) We focused only on the local pathways for the muscle-derived factors entering the bone tissue through the periosteum. The other complementary pathway is that myokines, after being released to extracellular space, are resorbed into the vascular space, through which they are delivered into bone tissue. Although the semi-permeable capillary wall (~1–2 μm thick) is one-order of magnitude thinner than the periosteum (~60 μm), the plasma concentrations of the myokines are expected to be much lower than those in local muscle tissues due to molecular diffusion and binding to plasma proteins. Therefore, we believe that the myokines are more likely to exert their effects on adjacent bone tissues through the local (i.e., direct penetration into/bone tissue) rather than via a systemic vascular effect. ii) The transport of myokines within muscles was not considered in this study. We only investigated their penetration into the periosteum after released from the muscles. Solute transport in muscles at either resting or activated situation is warranted for future study. iii) The dependency of the periosteum permeability on animal age, bone type/site and anatomical location, although beyond the scope of the current study, needs further investigation. iv) The current study utilized fluorescent tracers as surrogates for myokines and mathematical extrapolation (Eq. (2)) to predict the transport times of various myokines. In vivo experiments using fluorescently-tagged myokine molecules are needed to confirm the results obtained herein. These experiments will allow more accurate modeling of the sieving properties of periosteum to bioactive globular signals.

Despite these limitations, the present study provides quantitative data on the transport of myokine-mimicking tracers through the periosteum, which helped to identify likely signaling candidates for muscle–bone cross-talk. The current approach could be expanded to further study muscle–bone cross-talk under more physiological conditions in healthy and diseased states. In particular, the study provides a foundation for further studies of cellular and molecular interactions between muscle and bone, which may lead to new therapeutic strategies for musculoskeletal repair.

Acknowledgments

This study was supported by NIH grants (RO1AR054385 and P30GM103333).

References


Table 1

<table>
<thead>
<tr>
<th>Myokine</th>
<th>MW (Da)</th>
<th>Serum concentration (nmol/L)</th>
<th>Permeation across Laplace membrane (μm/s)</th>
<th>Penetration time (h)</th>
<th>Likely candidate?</th>
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<tr>
<td>FGF-1</td>
<td>50000</td>
<td>1000</td>
<td>100 μm/h</td>
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<tr>
<td>FGF-2</td>
<td>18,000</td>
<td>1000</td>
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<td>10 h</td>
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<tr>
<td>IL-1β</td>
<td>21,000</td>
<td>1000</td>
<td>100 μm/h</td>
<td>10 h</td>
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<td>TGF-β1</td>
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<tr>
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<td>23,000</td>
<td>1000</td>
<td>100 μm/h</td>
<td>10 h</td>
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Fig. 11. Penetration times for several myokines in periosteum were predicted based on the empirical relationship (Eq. (2)) in the text, which accounted for the experimental data using four tracers of various molecular weight (MW) as well as the observed cut-off MW of 40 kDa for periosteum.

Table 2

<table>
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<tr>
<th>MW (Da)</th>
<th>Tracers</th>
<th>Permeation across Laplace membrane (μm/s)</th>
<th>Permeation across Laplace membrane (μm/s)</th>
<th>Cortical bone (μm/s)</th>
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</thead>
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<tr>
<td>375</td>
<td>Sodium fluoride</td>
<td>300 ± 103.7</td>
<td>2701 ± 93.3</td>
<td>31.0 ± 6.0 [35]</td>
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<tr>
<td>300</td>
<td>Dextran-3k</td>
<td>156 ± 10.4</td>
<td>472 ± 19.2</td>
<td>12.8 ± 3.2 [41]</td>
</tr>
<tr>
<td>10,000</td>
<td>Dextran-7k</td>
<td>105 ± 9.3</td>
<td>69 ± 11.0</td>
<td>15.7 ± 4.5 [43]</td>
</tr>
<tr>
<td>40,000</td>
<td>Dextran-40k</td>
<td>165 ± 13.3</td>
<td>16 ± 2.1</td>
<td>6.5 ± 4.3 [43]</td>
</tr>
</tbody>
</table>

* For periosteum, the effective transport rate at time t = 0 (k0) and t = r (kr) are given. The time constant r is defined as b2/k0, and h is the periosteal thickness.
* Permeability over the cortical tissue was estimated based on the permeability measured in the lacunar–canalicular system (LCS), as indicated in the references and the porosity of the LCS in cortical bone, assumed to be 10% [42].

158
Appendix B

PERLECAN/HSPG2 DEFICIENCY ENHANCES THE DIFFERENTIATION AND MINERALIZATION OF MESENCHYMAL STROMAL CELLS BUT REDUCES THEIR INTRACELLULAR CALCIUM SIGNALING RESPONSES TO FLUID FLOW STIMULATION
Perlecan/HSPG2 Deficiency Enhances the Differentiation and Mineralization of Mesenchymal Stromal Cells but Reduces Their Intracellular Calcium Signaling Responses to Fluid Flow Stimulation

Lai, Xiaohua; Lv, Mengxi; Kim-Safran, Catherine; Lu, X. Lucas; Wang, Liyuan
University of Delaware, Newark, DE, United States

INTRODUCTION: Osteocytes are believed to detect external mechanical loads by sensing the interstitial fluid flow through their pericellular matrix (PCM) [1, 2]. In addition, the PCM can modulate the transport of signaling molecules and nutrients in bone [3, 4]. In our recent studies, perlecan/HSPG2 (ph), a large heparan sulfate (HS) proteoglycan, was discovered residing in the osteocyte PCM [5]. Ph’s critical role in maintaining the osteocyte PCM structure was further confirmed by TEM imaging of osteocyte cytoarchitecture from C1532Yeo mice [5], a transgenic model developed to recapitulate the reduced phn expression associated with Schwartz-Jampel Syndrome (SJS) [6]. Significant decrease in phn secretion and in the number of tethering elements in PCM (~35%) were reported in these phn-deficient (hypomorphic, Hypo) mice. Through a newly developed tracer velocimetry approach [7], our lab found the Hypo PCM showed a ~30% increase in fiber spacing and a greatly attenuated response to in-vivo tilting load compared with wildtype control (CTL) mice (C57BL/6J) [7]. The objective of this study was to test if Hypo bone’s lack of responses to loading is due to impaired osteocyte mechanosensing or defective osteoblastic function.

METHODS: Two studies were performed in vitro. (i) Differentiation and Mineralization Assays: Bone marrow mesenchymal stromal cells (MSCs), osteoblastic progenitor cells, were isolated from 4-6 month-old Hypo and CTL male mice (n=2 mice/group). Immediately after the sacrifice, the femurs and tibiae were dissected and cleaned of muscle and soft tissue. A small opening was cut at one end of the long bone, from which MSCs were flushed out using a syringe and culture medium [8]. The cell-containing medium was then centrifuged, and cells were re-suspended and plated onto gelatinized 24-well plates. For each group, 5×10^5 cells were seeded and cultured in an incubator with osteogenic culture medium changed every 3 days as described previously [9]. After 7 days of cell culture, a potent osteogenic inducer BMP-2 (100ng/mL, R&D Systems) was added to stimulate mineralization. After 12 more days of culture with and without BMP-2 stimulation, cells were fixed in 95% ethanol, rinsed gently in DI water, stained with 2% alizarin red solution for 30min, rinsed in DI water, and allowed air dry before imaging. (ii) Calcium signaling under fluid shear stress: Using the same methods above, MSCs were harvested from long bone marrow of adult male Hypo and WT mice, with five mice in each group. 1.3×10^6 cells were seeded on a glass slide, stained with Fluo-8AM, exposed to 1.2 Pa fluid shear stress in a laminar flow chamber, and the number of responding cells showing calcium responses and the non-responding cells were recorded and counted as described previously [10]. The difference of the responding fractions of Hypo and CTL MSCs was examined using chi-squared tests (Excel, Microsoft). Approximately 100 cells were examined in duplicated tests for each group.

RESULTS: In contrast to the CTL group that showed minimal calcium staining in the absence of BMP-2 treatment, Hypo MSCs showed enhanced mineralization with strong red staining, which was further elevated under BMP-2 stimulation (Fig. 1). Both Hypo and CTL MSCs showed a typical spindle-like shape (Fig. 2A). Fewer Hypo MSCs exhibited calcium responses (Fig. 2B) and their responsive rate (0.08%) was significantly reduced vs. that of CTL MSCs (0.28%, p<0.02, Fig. 2C).

DISCUSSION: Our results demonstrated that the perlecan-deficient osteoblastic progenitor cells have higher differentiation/mineralization potentials than CTL cells (Fig. 1), which is consistent with our recent findings of enhanced differentiation and mineralization of the Hypo embryonic osteogenitor cells [9]. Both studies suggest that the osteoblastic bone formation function is not impaired in Hypo mice and is unlikely to be the main reason for Hypo bone’s attenuated bone formation in response to tilting load. Instead, impaired mechanosensing in osteocytes is strongly supported by our preliminary intracellular calcium imaging data, where the responsive rate of Hypo MSCs to fluid shear stress was significantly decreased (Fig. 2). Together, our data suggest that impaired osteocyte mechanosensing could lead to the lack of responses to loading in phn deficient bone. Because the current studies utilized in vitro models where intact 3D PCM was not present in osteocytes, the results from the present study need to be confirmed in situ and in vivo. We are currently applying in situ dynamic loading [10] on intact Hypo long bones to test whether the in situ intracellular calcium responses to mechanical loading are indeed compromised in perlecan deficient osteocytes. Our long-term goals are i) to elucidate the molecular mechanisms responsible for the osteocyte mechanotransduction and ii) to test the novel hypothesis that PCM fibers serve as osteocyte mechanosensors and thus regulate bone adaptation and remodeling.

SIGNIFICANCE: Elucidating the role of perlecan in osteocyte mechanosensitivity will help to establish PCM as a potential new target that allows us to manipulate osteocytes’ mechanosensitivity to mechanical signals for treating osteoporosis.

ACKNOWLEDGEMENT: NIH P30GM103333; RO1AR854385.

Appendix C

UNIVERSITY OF DELAWARE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPLICATION TO USE ANIMALS IN RESEARCH AND TEACHING
Title of Protocol: Alterations of osteocyte pericellular space and cellular responses in fragile bones

AUP Number: 1149-2015-0 (4 digits only — if new, leave blank)

Principal Investigator: Liyun Wang, Ph.D

Common Name: Mice

Genus Species: Mus Musculus

Pain Category: (please mark one)

<table>
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<th>Category</th>
<th>Description</th>
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<tr>
<td>□ B</td>
<td>Breeding or holding where NO research is conducted</td>
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<tr>
<td>□ C</td>
<td>Procedure involving momentary or no pain or distress</td>
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<tr>
<td>✗ D</td>
<td>Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)</td>
</tr>
<tr>
<td>□ E</td>
<td>Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation</td>
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</table>

USDA PAIN CATEGORY: (Note change of categories from previous form)

Official Use Only

IACUC Approval Signature: [Signature]

Date of Approval: 12/1/14
**Principal Investigator Assurance**

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.

2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (ULAW).

3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.

4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.

5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.

6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.

7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.

8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.

9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.

10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.

11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*

12. I understand that by signing, I agree to these assurances.

---

Signature of Principal Investigator: [Signature]

Date: 10/10/14
NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
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<tbody>
<tr>
<td>1. Liyun Wang</td>
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<tr>
<td>2. Xiaohan Lai</td>
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</tr>
<tr>
<td>3. Shaopeng Pei</td>
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<td>4.</td>
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The Animal Use Protocol form has been developed to facilitate review of requests for specific research, teaching, or biological testing projects. The review process has been designed to communicate methods and materials for using animals through administrative officials and attending veterinarians to the Institutional Animal Care and Use Committee (IACUC). This process will help assure that provisions are made for compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Please read this form carefully and fill out all sections. Failure to do so may delay the review of this application. Sections that do not apply to your research must be marked “NA” for “Not Applicable.”

This application form must be used for all NEW or THREE-YEAR RENEWAL protocols.

All answers are to be completed using Arial 12 size font.

All questions must be answered in their respective boxes and NOT as attachments at the end of this form.

Please complete any relevant addenda:
- Hybridoma/Monoclonal Antibodies (“B”)
- Polyclonal Antibodies (“C”)
- Survival Surgery (“D”)
- Non-Survival Surgery (“E”)
- Wildlife Research (“F”)

If help is needed with these forms, contact the IACUC Coordinator at extension 2616, the Facility Manager at extension 2400 or the Attending Veterinarian at extension 2980.
1. Principal Investigator Information:

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</tr>
<tr>
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<td>UD</td>
</tr>
<tr>
<td>c. Department:</td>
<td>Mech Eng</td>
</tr>
<tr>
<td>d. Building/Room:</td>
<td>SPL204</td>
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<td>e. Office Phone:</td>
<td>2659</td>
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<td>f. Lab Phone(s):</td>
<td>2056</td>
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<td>g. Home Phone:</td>
<td>3024538372</td>
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<tr>
<td>h. Mobile Phone:</td>
<td>7323063026</td>
</tr>
<tr>
<td>i. E-Mail Address:</td>
<td><a href="mailto:lywang@udel.edu">lywang@udel.edu</a></td>
</tr>
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2. Protocol Status:

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<tr>
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| a. ☐ New Protocol OR ☒ Re-submission due to three (3) completed years.  
If re-submission, enter Protocol Number: 1149 |
| b. ☒ Research OR ☐ Teaching |
| c. ☒ Laboratory Animals OR ☐ Wildlife  
If “Wildlife” please complete Addendum “F” |
| d. Proposed Start Date: | 12/1/2014 |
| e. Proposed Completion Date: | 11/30/2017 |
| f. Funding Source: | UD |
| g. Award Number: Wang Startup and NIH R01AR054385 |

3. Personnel involved in Protocol (Include Principal Investigator):

Status: Indicate Prof, Post-Doc, Grad Student, Lab Manager, Research Assistant, Technician, etc.
**Qualifications:** Include procedures this person is proficient in performing on proposed species and the time they have been doing the procedure.  
**Be specific** (e.g. sub-mandibular bleeding on mice-2yrs, performing castrations on mice and rats-1yr, tail-vein injections on mice-2yrs, etc.) *(If no experience, list who will train.)*  

**Responsibilities:** Include all responsibilities this person will have with live animals on this protocol, including euthanizing animals.

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<th>Home/Cell Phone Number</th>
<th>Received Animal Facility Training</th>
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<td>a. Liyun Wang</td>
<td><a href="mailto:lywang@udel.edu">lywang@udel.edu</a></td>
<td>2659</td>
<td>7323063026</td>
<td>x [Click here to enter text]</td>
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</tbody>
</table>

**Status:** Associate Professor of Mechanical Engineering

**Qualifications:** Wang has experience with mice, including live animal loading-9yrs, tail-vein injection-15yrs, IP and SC injections-15yrs, anesthesia-15yrs, euthanasia-15yrs, and tissue harvesting-15yrs, and microsurgery on knee-7yrs

**Responsibilities:** Design & oversee the experiments; train and supervise lab members; coordinate with OLAM to perform all animal related procedures. In specific, she will oversee two new techniques that will be the focus of the renewal: dual-color Fluorescence Recovery After Photobleaching (FRAP) velocimetry to measure pericellular matrix in various clinical conditions; and ex vivo calcium imaging of cellular responses to mechanical loading.

<table>
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<tr>
<td>b. Xiaohan Lai</td>
<td><a href="mailto:laixhau@udel.edu">laixhau@udel.edu</a></td>
<td>6704</td>
<td>3024197960</td>
<td>x [Click here to enter text]</td>
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</tbody>
</table>

**Status:** Graduate student 5th year

**Qualifications:** Xiaohan had been working with mice for the past three years. She has experience with tail-vein injection-3yrs, anesthesia-3yrs, euthanasia-3yrs, tissue harvesting-3yrs, and FRAP experiments-3yrs.

**Responsibilities:** Xiaohan will perform dual-colored FRAP studies and ex vivo calcium imaging as well as other tasks required for the studies.
<table>
<thead>
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<th>Training</th>
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<tr>
<td>c. Shaopeng Pei</td>
<td><a href="mailto:shaopeng@udel.edu">shaopeng@udel.edu</a></td>
<td>6704</td>
<td>7162625186</td>
<td>x</td>
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</tbody>
</table>

**Status:** First year graduate student

**Qualifications:** Shaopeng has no previous animal experience. He will receive training from Dr. Wang and Xiaohan. He will get the training from Mr. Warren soon.

**Responsibilities:** Shaopeng will help Xiaohan in all her studies.

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**Qualifications:** Click here to enter text.

**Responsibilities:** Click here to enter text.

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</table>
4. Non-Scientific Summary: In language understandable to a high-school senior, very briefly describe the goals and significance of this study.

a. Specific Scientific Goals: Osteocytes are very important cells in bone. They serve as sensors to detect external forces and damages in the bone matrix, so that the bone can adapt to its mechanical usage and undergo self-repair. Because these cells are buried in the concrete-like mineralized matrix, they need obtain nutrients from and dispose of waste to blood vessels through a set of interconnected pores termed "osteocyte lacunar-canalicular system (LCS). In addition for the nutrition need, the fluid flow inside the LCS, termed in a mechanically loaded bone also provides physical signals that allow the osteocytes to detect the level of mechanical loading that bone experiences during daily life. The way that osteocytes detect mechanical loading is similar to that trees detect the direction and strength of blowing winds by sensing deformations in their branches. In bone LCS, a pericellular matrix (PCM) consisting of proteoglycan-like fibers are shown to fill the annular space. These PCM serve not only as a molecular sieve to filter passing solutes but also as a sensing apparatus (like branches for a tree) for osteocytes to detect the fluid flow (mechanical loading). Therefore, solute (and fluid) transport through PCM along LCS is critical for the survival of osteocytes and their vital function of detecting mechanical loading. In the past studies, we have successfully detected altered PCM in a transgenic fragile bone model where perilcan expression is lowered. For the next three years, we will test a better method in detecting the alterations of the PCM and to correlate the alterations of PCM with cell's intracellular calcium response to mechanical loading.

b. Significance of this Research (including the possible benefits to human and/or animal health, the advancement of scientific knowledge, or the betterment of society): The research will help gain fundamental understanding of how osteocytes detect and respond to mechanical signal. This knowledge will help us design patient-specific training program to increase bone strength.

5. Experimental Design: Explain the experimental design. This description should allow the IACUC to understand fully the experimental course of an animal or group of animals from its entry into the experiment to the endpoint of the study.

The inclusion of flow charts, diagrams, and/or tables are greatly encouraged to explain experimental design or sequential events.

Be sure to include all animal events and related details, i.e.,

- **All Procedures**- bleedings, injections, identification methods, genotyping methods, surgical procedures, euthanasia, etc.
- **Procedural details**- number of animals involved in procedure, approximate animal weight, if relevant (for injections, bleeding, etc.), route, frequency, volume, etc.
- **Pharmaceutical-grade and non-pharmaceutical grade compounds** – Identify any drugs, biologics, or reagents that will be administered to animals. If these agents are not human or veterinary pharmaceutical-grade substances, provide a scientific justification for their use and describe methods that will be used to ensure appropriate preparation.
- **Names of surgical procedures** (but reserve the surgical details for the proper Surgical Addenda)
• Monitoring—observations, measurements (animal weight, tumor size, etc.)
• Monitoring details—criteria, frequency, names of personnel monitoring, conditions for removing
  an animal from the study, etc.
• Endpoints—include endpoints for the animals/study and how will they be determined.

(Describe): Animals: perlecan-deficient homozygous mice (termed Hypo) are currently
maintained in UD animal facility by Dr. Liyun Wang and Dr. Catherine Kim-Safran. The
animals will be ear punched and genotyped using tails after weaning. All the procedures
except imaging will be done in OLAM and no live mice will be transferred to Spencer Lab.
We will use adult Hypo mice (males and females) at 2, 4, 6, 10 months to perform two
lines of studies: (1): Measuring PCM using Dual-color FRAP; and (2): Study how altered
PCM impacts the intracellular calcium elevation, one of the first cellular responses under
mechanical stimulation. Procedures of Study 1: Anesthesia: Although anesthesia is not
mandatory for tail vein injection, we have found that isoflurane anesthesia greatly
improved the successful rate of injection at the first shots, avoiding repeated piercing of
the tails and excess physical constrains on the animals, and thus reducing the animals’
stress. Mice will be anesthetized using isoflurane. Initial 4-5% will be used in the
induction phase and then remain at 1.5-3.5% later on. The duration of the anesthesia will
be approximately 5-10 min for one tail vein injection. Tail injection of dual tracers: To
measure the PCM density, one small tracer and one large tracer will be injected into the
same mouse. The small tracer is for tracking how fast fluid moves in the PCM. Due to the
hindrance of the PCM on larger tracer, larger tracer molecules will move slower than the
smaller tracer. By comparing the velocity difference between the small and large tracers,
we can obtain the information of how dense the PCM fibers are spaced near the
osteocytes. We call this new method “dual-color FRAP”, because the velocity of tracer
will be measured using FRAP technique that we pioneered (Price 2011; Wang 2014). A
bolus of lactalbumin or other proteins of similar molecular weight (10-20kDa)
fluorescently labeled with cy5 or other red dyes will be used as large tracer and injected
first to the mice. From our experience, this sized tracer will be reach equilibrium in 3-4
hours (Li 2009). 30 min prior to the equilibrium of the large tracer, a bolus of sodium
fluorescein (0.2mL, 2mg) will be injected at tail vein. Both the large red tracer and small
green tracer will reach equilibrium at the same time. The animals are allowed to come out
of anesthesia between the two injections and locomotion in fact promotes tracer
perfusion in bone. All the tracers and fluorophores can be purchased sterile from
Invitrogen or Sigma and dissolved in sterile PBS prior to injection. Sacrifice and FRAP
imaging: After the required circulation time, the mouse will be sacrificed with CO2 and
cervical dislocation and tibiae will be harvested for FRAP imaging under confocal
microscope and mechanical loading, following our previous publication (Wang 2014).
Since this is mainly an ex vivo study, only tail-vein injection and tissue harvesting
are involved in this animal protocol. The outcomes of study 1 are the PCM density
data at various age and gender compared with wild-type (WT) controls. The endpoints of
the procedures will be freshly sacrificed bone tissues perfused with two types of tracers,
which will be used for advanced imaging to probe the structure of the PCM. Procedures
of Study 2: To test whether altered PCM is associated with altered cell response to
mechanical loading, we will measure the intracellular calcium signaling in Hypo and WT
mice. Sacrifice and tissue harvest: Mice will be sacrificed with deep anesthesia and
cervical dislocation. Both tibiae will be promptly harvested in a sterile condition under a
biosafety hood. Bones will be cultured in an incubator for 2-4 hours. Ex vivo intracellular
calcium imaging, we will follow our collaborator Dr. Ed Guo’s protocol for imaging the intracellular calcium responses of osteocytes to mechanical loading using confocal microscopy (Jing 2014). Since this is an ex vivo study, only tissue harvesting is involved in this animal protocol. The outcomes of study 2 are the responding rates (percentage of cells showing elevated intracellular calcium within a certain time period (15 min) under physiological loading) for osteocytes in Hypo and WT bones. The endpoints of the procedures will be freshly sacrificed bone tissues with live and functional osteocytes, which will be used for advanced imaging of intracellular calcium responses to mechanical loading. Additional studies using live perfusion: To correlate the measured PCM density in the above experiments with TEM morphology, live perfusion procedure will be used. The principal reason of using live perfusion is that the bone PCM is very fragile and lacks intrinsic electron contrast. Live perfusion in deep anesthetized animals will allow fixatives to be carried by circulation into bone and preserve the PCM before autolysis and other forms of degradation occur. In addition, anions will be delivered into bone to bind to the negatively charged PCM for a better contrast for EM imaging as shown in our previous study (Thompson 2011). We will also use live perfusion techniques to explore the changes of vasculature in Hypo mice. Because perlecann is an important component of vascular base membrane, we need to make sure that our observed changes in osteocyte intracellular calcium responses are not due to changes in vasculature. We will perfuse a lead chromate-loaded silicon contrast agent (Microfil MV-117, Flow Tech Inc) at a rate of 1ml/min for ~15 min. Details seen in Terminal Surgery E form. References: Li (2009). “The dependency of solute diffusion on molecular weight and shape in intact bone.” Bone 45(5): 1017-23. Price (2011). “Real-time measurement of solute transport within the lacunar-canalicular system of mechanically loaded bone: direct evidence for load-induced fluid flow.” J Bone Miner Res 26(2): 277-85. Wang (2014). Perlecann-containing pericellular matrix regulates solute transport and mechanosensing within the osteocyte lacunar-canalicular system. J. Bone Miner Res. 29:878-91. Jing (2014): In situ intracellular calcium oscillations in osteocytes in intact mouse long bones under dynamic mechanical loading. FASEB J, 28(4):1582-92. Thompson (2011). Perlecann/4Hspg2 deficiency alters the pericellular space of the lacunoconnectal system surrounding osteocytic processes in cortical bone. J Bone Miner Res. 2011 Mar;26(3):618-29. doi: 10.1002/jbmr.236.

REFINEMENT, REDUCTION & REPLACEMENT

When using animals for research, it is important to consider the three Rs: reduction, refinement, and replacement to reduce both animal distress and the number of animals used in the laboratory.

Reduction: Minimizing the number of animals used
Refinement: Using techniques and procedures to reduce pain and distress
Replacement: Using non-animal methods or lower phylogenetic organisms

6. Justification for the Use of Animals (instead of in vitro methods)

(Check all that apply and explain):

a. ☒ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems: (Explain): The osteocyte lacunar-canalicular system and the pericellular matrix cannot be created in vitro. Animals have to be used.

Rev 10/2013 10
b. ☐ There is not enough information known about the processes being studied to design non-living models: *(Explain):* Click here to enter text.

c. ☐ Other: *(Explain):* Click here to enter text.

### 7. Justification for Species Appropriateness:
*(Check all that apply and explain):*

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>a. ☐ A large database exists, allowing comparisons with previous data: <em>(Explain):</em> Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>b. ☐ The anatomy or physiology is uniquely suited to the study proposed: <em>(Explain):</em> Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>c. ☒ This is the lowest species on the phylogenetic scale suitable to the proposed study: <em>(Explain):</em> Mouse is the lowest species with skeletal biology similar to human.</td>
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<tr>
<td>d. ☐ Other: <em>(Explain):</em> Click here to enter text.</td>
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</table>

### 8. Justification for Number of Animals Requested: *(Note: numbers should include animals used for breeding and all animals born)*

<p>| | |</p>
<table>
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<tbody>
<tr>
<td>a. ☐ Pilot study or preliminary project where group variances are unknown at the present time. Describe the information used to estimate how many animals will be needed: (Only a limited number of animals will be permitted.) <em>(Explain):</em></td>
<td></td>
</tr>
</tbody>
</table>

Rev 10/2013  11  #1149-2015-0
b. Group sizes are determined statistically. Describe the statistical analysis used to estimate the number (N) of animals needed: N may be estimated from a power analysis for the most important measurement in the study, usually based on the expected size of the treatment effect, the standard error associated with the measurement, and the desired statistical power (e.g., \( P < 0.05 \)). Data analysis methods should not be submitted unless directly applicable to the estimate of N.

An online calculator may be found at: http://www.math.iu.edu/~rlenth/Power/
or a stand-alone calculator that can be downloaded from http://wwwpsycho.uni-duesseldorf.de/abteilungen/aap/gpower3

(Explain): Although our previous study (Wang 2014) identified that the PCM structure changed in Hypo compared with WT, the variances for the two groups are currently unknown due to the technical limitations. The dual-color FRAP method will allow us to measure the PCM density in each mouse. We will begin with a sample size of n=10 per group per gender at the age of 2 months. This sample size can provide 61% power to detect a 30% difference between the two means with an effect size (mean difference/\( \text{std} \)) of 1. We have had used this sample size and found it adequate to detect bone responses to mechanical loading in our live animal loading study (Wang 2014). Similarly, we will begin a sample size of n=20 per group per gender to test the difference of intracellular calcium signaling between the two genotypes. This larger sample size is justified based on Dr. Guo’s experience and is necessary because we have to optimize the complicated calcium imaging protocol (Jing 2014). We also request a sample size of 10 per gender per genotype for live perfusion study of PCM and vasculature morphology and 5 per gender per genotype for breeding. After these initial studies on 2-month-old mice are completed in year 1, we will be in a better position to estimate how many animals are needed for other age groups and we will submit an amendment in year 2.

c. Group sizes are based on the quantity of harvested cells or the amount of tissue required for \textit{in vitro} studies. Explain how much tissue is needed based on the number of experiments to be conducted and the amount of tissue you expect to obtain from each animal (e.g., 10g of tissues are needed: Each animal can provide 2g. 10g/2g per animal = 5 animals needed.) (Explain): Click here to enter text.

d. Teaching protocol. Specify the number of students in the class, the student to animal ratio and how that ratio was determined: Animal numbers should be minimized to the fullest extent possible without compromising the quality of the hands-on teaching experience for students or the health and welfare of the animals. (Explain): Click here to enter text.

e. Study involving feral or wild animals. Animals will be captured and released in an attempt to maximize the sample size within logistical constraints. Describe the process by which you estimate these numbers and estimate the precision needed: (Explain): Click here to enter text.

f. Observational, non-manipulative study. Animals will not be captured, their behavior will not be
interfered with, and exact animal numbers cannot be predicted: (Explain): Click here to enter text.

g. □ Product testing. The number of animals needed is based on FDA guidelines. Provide the citation from the regulations, the IND tracking number, or relevant FDA correspondence: (Explain): Click here to enter text.

h. □ Other. Elaborate, indicating the method used to determine the group size. (Explain): Click here to enter text.

9. Animals Requested:

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Genus and Species</th>
<th>Total Number of Animals for Three Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. mouse</td>
<td>Mus musculus</td>
<td>200</td>
</tr>
<tr>
<td>2.</td>
<td>Click here to enter text.</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>3.</td>
<td>Click here to enter text.</td>
<td>Click here to enter text.</td>
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<tr>
<td>4.</td>
<td>Click here to enter text.</td>
<td>Click here to enter text.</td>
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<tr>
<td>5.</td>
<td>Click here to enter text.</td>
<td>Click here to enter text.</td>
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</tbody>
</table>

10. Where will animals be housed (or captured for wildlife)? UD OLAM

11. Where will the experiments take place?
If animals must be removed from the vivarium, please describe how they will be transported (such as hand carried in covered microisolator cages). Spencer 202 or OLAM

12. Will any animals be humanely killed, without treatment or manipulations, to be used to obtain tissue, cells, etc.? □ Yes □ No
If Yes, list types of tissue, etc: Bone
<table>
<thead>
<tr>
<th>Question</th>
<th></th>
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<tbody>
<tr>
<td><strong>Physiological Measurements</strong></td>
<td>☐ Yes ☐ No</td>
<td></td>
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<tr>
<td>If Yes, list and explain: Click here to enter text.</td>
<td></td>
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<tr>
<td><strong>Dietary Manipulations</strong></td>
<td>☐ Yes ☐ No</td>
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<tr>
<td>If Yes, list and explain (Note: if food or fluid will be restricted, describe method for assessing the health and wellbeing of the animals. Body weights must be recorded at least weekly. Amount earned (if animals work for food or fluid) during testing and amount freely given must be recorded. A scientific justification must be provided for departures from the recommendations of the Guide.) Click here to enter text.</td>
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<tr>
<td><strong>Environmental Stress (e.g., cold, restraint, forced exercise)</strong></td>
<td>☐ Yes ☐ No</td>
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<tr>
<td>If Yes, list and explain: Click here to enter text.</td>
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<tr>
<td><strong>Trauma or Burn Injury</strong></td>
<td>☐ Yes ☐ No</td>
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<tr>
<td>If Yes, list and explain: Click here to enter text.</td>
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<tr>
<td><strong>Production of Hybridoma/Monoclonal Antibodies</strong></td>
<td>☐ Yes ☐ No</td>
<td></td>
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<tr>
<td>If Yes, please complete Addendum “B”.</td>
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<tr>
<td><strong>Production of Polyclonal Antibodies</strong></td>
<td>☐ Yes ☐ No</td>
<td></td>
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<tr>
<td>If Yes, please complete Addendum “C”.</td>
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<tr>
<td><strong>Administration of Hazardous Chemicals, Drugs, Toxins, or Nanoparticles</strong></td>
<td>☐ Yes CAS#_____ ☐ No</td>
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</tr>
<tr>
<td>If Yes, describe hazards posed to personnel: Click here to enter text.</td>
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<td></td>
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<tr>
<td>Methods to control exposure: Click here to enter text.</td>
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<tr>
<td>Methods of Disposal of Animals and Bedding: Click here to enter text.</td>
<td></td>
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<tr>
<td><strong>Administration of radioactive materials</strong></td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>a. Type to be used. Include radioisotope(s) and chemical form(s): Click here to enter text.</td>
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</tbody>
</table>

Rev 10/2013

176
b. Describe the practices and procedures to be followed for minimization of radiation exposure to workers and for the handling and disposal of contaminated materials associated with this study: *(Include the methods for management of radioactive wastes and monitoring facility for radioactive contamination, if applicable.)*

Click here to enter text.

c. Who will be responsible for the daily care of animals containing radioactive materials?

Click here to enter text.

d. Approval received from UD- Environmental Health and Safety? ☐ Yes ☐ No ☐ Pending

Click here to enter text.

Please attach a copy of any approvals or provide the approval number.

Click here to enter text.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>a. Make, model, and location of irradiator to be used:</td>
<td>Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>b. Approval received from UD- Environmental Health and Safety?</td>
<td>☐ Yes ☐ No ☐ Pending</td>
<td></td>
</tr>
</tbody>
</table>

Please attach a copy of any approvals or provide the approval number. Click here to enter text.

<table>
<thead>
<tr>
<th>22. Administration of Biological Agents (eg microorganisms, recombinant DNA, HUMAN serum, tissue, cell lines, etc.)</th>
<th>☐ Yes</th>
<th>☐ No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal Biosafety Level</td>
<td>☐ 1</td>
<td>☐ 2</td>
</tr>
</tbody>
</table>

Describe hazards posed to personnel: Click here to enter text.

Methods to control exposure: Click here to enter text.

Methods of Disposal of Animals and Bedding: Click here to enter text.

Approval received from UD- Institutional Biosafety Committee? ☐ Yes ☐ No ☐ Pending

Please attach a copy of any approvals or provide the approval number. Click here to enter text.

| 23. Will tumor cells, tissue, sera, viral vectors or other biologies of RODENT origin – other than those isolated from rodents already housed in the facility – be administered to animals? | ☐ Yes | ☐ No |
24. Use of Genetically Engineered Animals

☐ Yes  ☐ No

If Yes, please describe any anticipated phenotypes that may cause pain or distress and any special care or monitoring that the animals will require.

Our Hypo mice harbor a mutant gene resulting in reduced expression of perlecain, a large heparan sulfate proteoglycan. The mice showed bone and cartilage abnormality. We have male formation of their teeth that may need special care (trimming). Our facility staff has been doing that.

Does the proposed work involve creating new genetically modified animals, or involve crossing two genetically modified animals to produce offspring with a new genotype.

☐ Yes  ☐ No

Approval received from UD- Institutional Biosafety Committee?

☐ Yes  ☐ No  ☐ Pending  ☐ Exempt (breeding of two lines of genetically-modified rodents is exempt if 1) both parents can be housed under BL1 containment and 2) neither parent strain incorporates more than one half of the genome of an exogenous eukaryotic virus or incorporates a transgene under the control of a gammaretroviral long terminal repeat and 3) the rodent that results from the breeding is not expected to contain more than one half of an exogenous viral genome)

Please attach a copy of any approvals or provide the approval number. Click here to enter text.

25. Special Study Requirements: Please describe any special study requirements such as a requirement for single housing of the animals, exemption from environmental enrichment, or special caging: Click here to enter text.

26. Any other procedures? ☒ Yes  ☐ No

If Yes, explain: We will run live perfusion procedures for a subset of mice to study the morphology of PCM and vasculature in lower limbs. Details will be attached in Terminal Surgery E. see below

27. Will this study involve surgery? ☒ Yes  ☐ No

If Yes, and it is “Survival Surgery,” please complete Addendum “D”.

If Yes, and it is “Terminal Surgery,” please complete Addendum “E”.
28. Will any animal undergo anesthesia for any reason other than surgery? Yes □ No □
If Yes,
  a. List Procedures and Reason(s) for using anesthesia: Although anesthesia is not mandatory for
tail vein injection, we have found from the past years that isoflurane anesthesia greatly
improved the successful rate of injection at the first shots, avoiding repeated piercing of the
tails and excess physical constrains on the animals, and thus reducing the animals’ stress. We
will inject one bolus of fluorescent tracer solution. The injection will take 1-5 min and the
animal will wake up in 5 min after being removed from the anesthesia machine. In this study,
we will inject two tracers in 2.5 hours apart.
  b. Check the type of anesthesia to be used.
    ☑ Isoflurane

☐ Injectable (For injectable, complete the following):
  Drug: Click here to enter text.
  Dose: Click here to enter text.
  Route: Click here to enter text.

29. Animal Use and Pain Distress. If you have indicated that animals in your study will experience pain
or distress, even if it will be fully alleviated, please mark the appropriate check boxes below and fill in
the requested information for each item marked.

You must conduct at least two (2) searches.

I have considered alternatives to the use of animals in my study. Alternatives refer to methods or
approaches which result in refinement of procedures which lessen pain and/or distress; reduction in
numbers of animals required; or replacement of animals with non-whole-animal systems or
replacement of one animal species with another, particularly if the substituted species is non-
mammalian or invertebrate. I have used the following methods and sources to search for alternatives:

- ☑ Medline
- ☑ Toxline
- ☑ Biosis
- ☐ Agricola
- ☐ CAB Abstracts
- ☐ Other (Specify): Click here to enter text.

Date of Search: Oct. 10, 2014
Years Covered: all years covered in Pubmed (1966-present)
Keywords Used (must include the word alternative): osteocyte, intracellular calcium, alternative
Number of Papers Found: 1

**Database Used:**
- Medline
- Toxline
- Biosis

**Date of Search:** Oct. 10, 2014
**Years Covered:** 1990-2014
**Keywords Used (must include the word *alternative*):** osteocyte, intracellular calcium, alternative
**Number of Papers Found:** none

Discussion of the Relevancy of the Papers Found: Click here to enter text.

---

30. **Unnecessary Duplication of Work.** Activities involving animals must not unnecessarily duplicate previous experiments performed by you or others. Provide a written narrative that assures that the activities of this project comply with this requirement and support this assurance by performing a literature search.

The search should return, at minimum, the related previous work from your laboratory.

You must conduct at least two (2) searches.

*(NOT REQUIRED FOR TEACHING PROTOCOLS)*

**Note:** You may need to do more than one search per database to look for duplication of work, especially if you are doing more than one experiment.

**Database Used:**
- Medline
- Toxline
- Biosis

**Date of Search:** Oct. 10, 2014
**Years Covered:** 1990-2014
**Keywords Used:** osteocyte, pericellular matrix, perlecan deficient
**Number of Papers Found:** 1

Discussion of the Relevancy of the Papers Found: This is the paper that Drs. Cindy Farach-Carson, Korn-Safran and I published together showing the TEM evidence of altered PCM in perlecan deficient mice. Only one age group (12 month) was examined in the study. We propose to look at younger mice.
Database Used:
- Medline
- Toxline
- Biosis
- Other (Specify): Click here to enter text.

Date of Search: Oct. 10
Years Covered: all years
Keywords Used: osteocyte, pericellular matrix, perlecan deficient
Number of Papers Found: 2
Discussion of the Relevancy of the Papers Found: Both papers are from our groups. One is on TEM study of PCM and the other is on tracer velocimetry measurement of PCM density. Both studies only examined one age group (12 month).

31. What is the expected disposition of animals at the end of the experiments?
(Check all that apply):
- Euthanized
- Maintained
- Released (Wildlife Only)
- Other (Specify): Click here to enter text.

32. Euthanasia*
Select methods that will be used in case of emergency and/or at the end of the procedure/experiment.
*NOTE:
- Methods must be approved by the AVMA or must be scientifically justified.
- A “Primary” and “Secondary” method must be selected (UD Double Kill Policy).
- If different methods will be used for different groups of animals, indicate the group after the procedure (e.g., write “Neonates” after Decapitation, “Adults” after CO2, “Terminal Surgery Animals” after Isoflurane Anesthesia Overdose, etc.).

- Animals will NOT be under anesthesia when euthanasia is performed.—For Dual-color FRAP study
- Animals will be under anesthesia when euthanasia is performed. (Check drug used below): For ex vivo calcium imaging study and live perfusion studies
- Isoflurane
- Injectable (Complete the following):
  - Drug: Click here to enter text.
  - Dose: Click here to enter text.
<table>
<thead>
<tr>
<th>PRIMARY method(s) of euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>☒ CO₂ by compressed gas cylinder <em>(Not for animals already under anesthesia or neonates)</em>. — For Dual-color FRAP study</td>
</tr>
<tr>
<td>☐ Barbiturate Euthanasia Solution - Injectable ≥150mg/kg <em>(Check route below)</em>:</td>
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<tr>
<td>□ IV □ IP □ IC</td>
</tr>
<tr>
<td>☐ Isoflurane Anesthesia Overdose - Inhalant</td>
</tr>
<tr>
<td>☒ Cervical Dislocation <em>(only under anesthesia)</em> For ex vivo calcium imaging study and live perfusion studies, where we want to maintain viability of the tissues and cells as long as possible under anesthesia without exposure to CO₂.</td>
</tr>
<tr>
<td>☐ Decapitation <em>(only under anesthesia or neonates)</em></td>
</tr>
<tr>
<td>☐ Exsanguination or Perfusion <em>(only under anesthesia)</em></td>
</tr>
<tr>
<td>☐ Incision of Chest Cavity – Bilateral Pneumothorax <em>(only under anesthesia)</em></td>
</tr>
<tr>
<td>☐ Pithing – <em>(only under anesthesia)</em> <em>(amphibians, reptiles only)</em></td>
</tr>
<tr>
<td>☐ Removal of Vital Organ(s) <em>(only under anesthesia)</em> <em>(Check all that apply)</em>:</td>
</tr>
<tr>
<td>□ Brain □ Kidneys</td>
</tr>
<tr>
<td>□ Heart □ GI Tract</td>
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<tr>
<td>□ Liver □ Lungs</td>
</tr>
<tr>
<td>□ Other Vital Organ(s) – <em>(Specify)</em>: Click here to enter text.</td>
</tr>
<tr>
<td>☐ Other Method of Euthanasia <em>(Describe and Scientifically Justify)</em>:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SECONDARY method(s) of euthanasia that will be used to ensure that the animal does not survive:</th>
</tr>
</thead>
<tbody>
<tr>
<td>☒ Cervical Dislocation — For Dual-color FRAP study</td>
</tr>
<tr>
<td>☐ Decapitation</td>
</tr>
</tbody>
</table>
- Exsanguination or Perfusion

- Incision of Chest Cavity – Bilateral Pneumothorax
  For ex vivo calcium imaging study and live perfusion studies

- Barbiturate Euthanasia Solution - Injectable ≥150mg/kg *(Check route below)*:
  - □ IV
  - □ IP
  - □ IC

- Pithing – Double pithing required *(fish, amphibians, reptiles only)*

- Removal of Vital Organ(s): *(Check all that apply)*:
  - □ Brain
  - □ Kidneys
  - □ Heart
  - □ GI Tract
  - □ Liver
  - □ Lungs
  - □ Other Vital Organ(s) – *(Specify)*: **Click here to enter text.**

- Other Method of Euthanasia: *(Describe and Scientifically Justify)*: **Click here to enter text.**
1. Complete:

<table>
<thead>
<tr>
<th>Species</th>
<th>Procedure</th>
<th>Number of Animals</th>
<th>Justification</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) mus musculus</td>
<td>Live perfusion</td>
<td>40</td>
<td>10 males and 10 females of either perlecan deficient Hippo mice or W1 mice will be perfused with either fixative for TEM imaging of PCM or a contrast agent for microCT imaging of vasculature trees in lower limbs</td>
</tr>
</tbody>
</table>

2. Medications:

   a. Pre-operative medications [drug; dose (mg/kg); route; frequency]:
      NONE

   b. Anesthesia [drug; dose (mg/kg); route; frequency]:
      Inhalant isoflurane, initial 4-5% induction, 1.5-3.5% maintenance of anesthesia plane

3. Surgical Procedure: Non-survival perfusion
a. Briefly describe your non-survival surgical procedure:

For TEM imaging of PCM:

1. Mice will be under an adequate level of anesthesia, confirming with lack of pedal reflex
2. Open chest, insert pump needle (inlet for fixatives) into left ventricle and cut open right auricle (outlet for drainage);
3. Pump fixatives of approximate 100 ml that contains blood substitutes (Perfluoro-compound FC-75), glutaraldehydes and/or paraformaldehyde, and anionic electron contrast ruthenium or terbium into heart for 30 min. Mice will be dead with fixative perfusion. The total time for the procedure including the preparation time will be approximately 45 min to 1 hr;
4. In addition to the fixative perfusion, cervical dislocation will be performed as secondary euthanasia;
5. Bone tissues (tibiae, femurs) will be harvested for EM

For Vasculature Perfusion:

The procedure is similar to the above perfusion fixative, except for replacing the fixatives with contrast agent. We will use a lead chromate-loaded silicon contrast agent (Microfil MV-117, Flow Tech Inc) at a rate of 1ml/min for ~15 min.
Appendix D

PUBLICATION INFORMATION

Chapter 2

Paper Title:
Perlecan-Containing Pericellular Matrix Regulates Solute Transport and Mechanosensing within the Osteocyte Lacunar-Canicular System

Paper Authors
Xiaohan Lai,1* Bin Wang, 1,2* Christopher Price,1 William R Thompson,3 Wen Li,1 Tonima R Quabili,1 Wei-Ju Tseng,4 Xiaowei Sherry Liu,4 Hong Zhang,5 Jun Pan,2 Catherine B Kirn-Safran,6 Mary C Farach-Carson,7 and Liyun Wang1

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1 This paper was published in JBMR, 2014, 29: 878-891
Chapter 3

Paper Title:
The Dependences of Osteocyte Network on Bone Compartment, Age, and Disease

Paper Authors:
Xiaohan Lai, Christopher Price, Shannon Modla, William R. Thompson, Jeffrey Caplan, Catherine B Kirn-Safran, and Liyun Wang

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2 This paper was published in Bone Research (2015) 3, 15009; doi:10.1038